

CELLULAR ORIGIN, LIFE IN
EXTREME HABITATS AND ASTROBIOLOGY

Algae and Cyanobacteria in Extreme Environments

Edited by
J. Seckbach



Springer

ALGAE AND CYANOBACTERIA IN EXTREME ENVIRONMENTS

Cellular Origin, Life in Extreme Habitats and Astrobiology

Volume 11

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Joseph Seckbach

The Hebrew University of Jerusalem, Israel

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Algae and Cyanobacteria in Extreme Environments

Edited by

J. Seckbach

*The Hebrew University of Jerusalem,
Israel*



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LEGENDS FOR THE COVER PICTURE

An arrangement of 44 diatoms surrounded by images of cyanobacteria and chlorophyte algae. The diatoms include species from the genera *Actinoptychus*, *Amphitetras*, *Aulacodiscus*, *Caloneis*, *Cocconeis*, *Cymatopleura*, *Cymbella*, *Didymosphenia*, *Diploneis*, *Gyrosigma*, *Hemiaulus*, *Mastogloia*, *Melosira*, *Navicula*, *Neidium*, *Paralia*, *Pleurosigma*, *Rhabdonema*, *Stauroneis*, *Stictodiscus*, *Surirella*, *Synedra*, *Tetracyclus*, *Triceratium*, and *Trinacria*. The algae bordering the diatoms include (from top left corner): the desmid *Staurastrum artiscon*, the cyanobacterium *Anabaena*, the desmid *Micrasterias hardyi*, the coiled filaments of *Spirogyra*, the tapered cork screw-like filaments of the cyanobacterium *Spirulina*, and the coiled filaments of *Zygema*. Border algae images are contributed by **Dr. Gordon W. Beakes**, Newcastle University, UK. The diatom arrangement and photomicrograph, and the composite image of all algae, are by **Dr. Stephen S. Nagy** (MD), Montana Diatoms, USA.

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FOREWORD

This volume encompasses a great diversity of subjects as well as authors, most of whom are well recognized and established in their fields and others who are younger, upcoming scientists of whom we will hear more in the future. The subject is extremophiles, and is almost entirely focused on photosynthetic microbes. It is a complementary and updated version of a volume of similar subject matter published in 2001 (*Algae and Extreme Environments: Ecology and Physiology*, eds. J. Elster, J. Seckbach, W.F. Vincent and O. Lhotsky, Nova Hedwigia, Beiheft 123: 1–602. Schweizerbart’sche Verlagsbuchhandlung, D-70176 Stuttgart. <http://www.schweizerbart.de/pubs/books/bo/novahedwig-051012300-desc.ht>, Berlin/Stuttgart, 602 pp.). Few of the authors of the present volume were involved in the earlier publication, and many of the subjects are quite different.

The studies of extremophiles have increased almost exponentially in the last several years, mainly because of the interest in early life on this planet and possible life (past or present) on others. This has necessitated the creation of a new and well-received journal, *Extremophiles* (Volume 1, 1997 to present). Unfortunately, few of the papers published in this journal to date have focused on phototrophs. Papers on tolerance to environmental extremes by phototrophic microorganisms are also frequently found in a number of journals, including *Journal of Phycology*, *Applied and Environmental Microbiology*, *Environmental Microbiology*, *Microbial Ecology*, *Archives of Microbiology*, and several others, and, of course, in the series of volumes entitled “*Cellular Origin, Life in Extreme Habitats and Astrobiology*” (www.springer.com/series/5775) edited by Joseph Seckbach.

The current volume is divided into nine subject areas in addition to an opening section: General introductory chapter (1), (2) Phototrophs at high and low light, (3) Phototrophs in the marine environment, (4) Phototrophs in cold environments, (5) Phototrophs in hot alkaline and acidic environments and non-thermal acidic habitats, (6) Phototrophs under water stress: dry and hyper-saline environments, (7) Adaptability to changing environments, (8) Other microorganisms and extreme habitats, and (9) Outlook.

We need to remember that phototrophs, except for a few, do not generally extend as far into some extreme environments as do the non-photosynthetic Bacteria and Archaea. With respect to upper temperature limits, a few Cyanobacteria and Chloroflexi extend to the lower 70°C which marks the upper boundary for a few unique organisms of each of these phyla, and consequently the upper temperature limit for chlorophyll- or bacteriochlorophyll-based photosynthesis (a limit known and not disputed for many decades). With respect to upper salinity limits, there too the non-photosynthetic Bacteria and Archaea dominate the picture, but there are exceptions. Some phototrophic purple

bacteria (e.g., *Halorhodospira* spp.) nearly equal the upper salinity limits of some of the non-phototrophs, and a few cyanobacteria (e.g., *Halothece* spp.) and diatoms come close, as does the green alga, *Dunaliella salina*. The non-phototrophs do not have the monopoly on cold or freezing tolerance, although they may have with respect to true psychrophily, since most of the cyanobacteria isolated from cold habitats are psychrotolerant rather than psychophilic. Cyanobacteria appear to have an edge over eukaryotic microalgae with ability to withstand freezing in nature.

Acidophily or acidotolerance are well represented by the non-phototrophic Bacteria and Archaea. The Cyanobacteria and anoxygenic phototrophic Bacteria are almost excluded from acidic environments below pH 4–4.5 [with the exception of *Acidophilium* spp. (α -Proteobacteria) that use Zn in the center of the porphyrin ring of bacteriochlorophyll instead of Mg].

Periodic and long-term desiccation is another extreme that is well represented by phototrophs, particularly cyanobacteria – these to a greater extent than microalgae. It is not clear that non-phototrophic Bacteria or Archaea have the edge with desiccation as the stressor.

The chapters vary in subject matter from algae and cyanobacteria in sub-aerial urban environments, in environments that are exposed to freeze–thaw cycles and permanent coldness, as well as speculations as to the versatility and evolution of cyanobacteria in extremes and the role of reduced iron as a reductant in early photosynthesis. Some papers are more focused on individual species or natural groups of species and their responses to various extremes. Many habitats or natural groups of organisms included here have barely been described or discussed before in the context of extreme environments. There is extensive speculation in some of the chapters, particularly those that constitute brief reviews of the subjects. From a scan of abstracts and concluding remarks, I also believe that portions or conclusions of some of the chapters may be somewhat or highly controversial, and therefore should stimulate discussion.

With respect to the Eukarya (algae, except for a couple of chapters on “other” protists or fungi), there is a chapter that emphasizes the uniqueness of the dinoflagellates as a distinct group, and another, their probable propensity to acquire endosymbionts in crowded benthic environments. Diatoms are also discussed, particularly with respect to the mysteries of their intricate and rigidly controlled wall patterns, and in another chapter, their remarkable ability to thrive in cold seawater and even to endure freeze–thaw episodes.

Acidic environments are involved in a few chapters. The acidic Rio Tinto of southwestern Spain drains ancient mine pits. There are descriptions of other acid mine drainways that are also very rich in heavy metals that would be toxic for most algae, but these habitats nevertheless sustain a mixed assemblage of a few specialized species of algae, including diatoms, euglenoids, and green algae. The biominerall deposits of the extreme Rio Tinto are suggested as possible analogs in the search for remnants of possible microbial life in ancient deposits on Mars. Primitive photosynthetic, unicellular red algae of the order Cyanidiales inhabit

acid environments from pH 0.0 to 4.0 with temperatures from about 40 to 56°C. A few species of acidophilic eukaryotic algae also inhabit waters of this pH range, but at lower temperature. Thermoacidic environments such as the sulfurous vents near Naples, Italy, are discussed with respect to the Cyanidiales that reside and thrive there. Another chapter delves into understanding the biology of these algae based on knowledge of their genomes.

Other chapters describe the distribution of various algae and cyanobacteria in varied habitats (e.g., under UV stress, in dim light, on man-made substrates, high diversity in urban environments, soils, and in other periodically xeric or aero-terrestrial environments).

About half of the chapters focus on prokaryotes (namely cyanobacteria or mixed communities). Some are reviews, such as that on the diversity, versatility, and specialization of cyanobacteria that may help to explain their extraordinary ability to inhabit various extreme environments. Three other chapters examine the tolerances of cyanobacteria in polar and other cold environments. Some authors have focused on specific cyanobacteria, such as *Acaryochloris*, the only cyanobacterium utilizing chlorophyll *d* instead of chlorophyll *a*, the extreme desiccation-tolerant cyanobacterium, *Chroococcidiopsis*, that may represent a type close to a form that may have inhabited a terrain like that of Mars in happier times past, and *Mastigocladus* cf. *laminosus*, a common global, thermophilic cyanobacterium that exhibits somewhat dissimilar genotypes in different portions of the thermal gradient of the same alkaline, geothermal stream as well as in near and distant geographic locations.

There are, of course, other topics that I have not mentioned. However, this compilation has such a remarkable variety of subject matter that I believe that it should not be treated simply as a reference volume for specialists who would concentrate on one habitat or on a few species, but should be read in full by every biologist interested in extreme or unusual environments and their organisms.

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Richard W. Castenholz

Biodata of **Richard Castenholz**, author of the *Foreword*.

Dr. Richard W. Castenholz is professor emeritus in the Center for Ecology and Evolutionary Biology at the University of Oregon. He received his B.S. in Botany at the University of Michigan (1952) and his Ph.D. in Botany at Washington State University (1957). He has been a faculty member at the University of Oregon since 1957. His early research was focused on the ecology of both freshwater and marine epilithic diatoms, but in the late 1960s he switched almost entirely to the study of cyanobacteria and anoxygenic phototrophic bacteria of hot spring ecosystems. During this period, the anoxygenic phototrophs, *Chloroflexus* and *Heliothrix*, were first described by Pierson and Castenholz (1974) and Pierson et al. (1985), respectively. These unique new genera (with the later addition of others) have formed the base of a new phylum, the Chloroflexi. Later, this expanded into ecological and physiological studies of phototrophic prokaryotes from microbial mats in nonthermal freshwater, marine, and hypersaline habitats, including mats of Antarctic melt ponds dominated by cyanobacteria. The research questions that were asked include: how do various microorganisms adapt to environmental extremes (e.g., high and low temperatures, low pH, high salinity, desiccation, normally toxic sulfide concentrations, and high solar irradiance, especially UV radiation)?

In these studies, he and his students have characterized scytonemin, a UV-shielding compound in the extracellular sheaths of many highly exposed cyanobacteria, and have demonstrated its role in increasing fitness under UV exposure, even when cells are metabolically inactive. We have also shown that some motile cyanobacteria have a lifesaving escape response to UV exposure by moving downward into soft mats or sediments in geothermal, temperate, and polar environments. Currently, his main interest is in the ecology and diversity of the unicellular algae of the rhodophytan order Cyanidiales, a group of a few genera and species that inhabit hot (40–56°C) acid waters (pH 0.5–4.0) in Yellowstone Park and other volcanic regions of the earth.

Throughout the last 25 years, Castenholz has also been trying (with others) to unravel the extremely disordered and complex taxonomy of cyanobacteria and was heavily involved in writing and editing the cyanobacterial sections in two editions of *Bergey's Manual of Systematic Bacteriology* (1989 and 2001).

He has also been honored several times (e.g., J.S. Guggenheim Fellow, 1970–71; Fulbright Scholar, 1977–78; Fellow, AAAS; Trustee, Bergey's Manual Trust, 1991–2001; Fellow, American Academy of Microbiology, 1996; Bergey Medal for Distinguished Achievement in Bacterial Taxonomy, 2005).

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PREFACE

This volume, number 11 in the series of “COLE” (*Cellular Origins, Life in Extreme Habitats and Astrobiology*, see: www.springer.com/series/5775), deals with algae growing at the edge of life under extreme conditions. It contains over 40 chapters contributed by 50 authors from 17 countries.

Photosynthetic microorganisms such algae (including cyanobacteria) occupy most environments on Earth that are illuminated with visible light. Among these habitats are several places which are, from the anthropocentric view, inhospitable and different from the “normal” places. The microbes occurring in those environments are referred to as “extremophiles.” We could assume that those extremophiles regard their harsh habitats as an oasis or a paradise. Not only bacteria and Archaea, which are known to be flexible and may occupy almost every niche and cope with severe habitats, but also algae are among the extremophiles. Extremophiles are classified into various categories according to the “extreme” character of their environments, such as very high or very low temperatures limit, pH values, salinity, dryness, high concentration of heavy metals, very high or low levels of radiation, especially ultraviolet radiation, and to a certain extent anaerobic environments.

Thermophiles, organisms that love elevated temperature levels, grow in hot environments such as hot springs. The highest temperature for algae is in the upper 50°C and for the cyanobacteria close to 70°C. At the lower temperature scale are the cold lovers (psychrophiles) growing in geographical regions such as in the Arctic, Antarctica, and the permafrost of Siberia. They tolerate low temperatures as long as the internal cytosol is not damaged from freezing ice inside the cells. Their cellular membranes protect their internal content by selective permeability, and in some cases the cells produce compounds to provide an “antifreeze” effect. Among the eukaryotic algae are the ice *Chlorophytes* (green algae) which “paint” ice in various colors.

Algae also grow in alkaline or acidic media. The alkaliophiles occur at higher ranges of pH as in the soda lake in Africa, while the acidophiles thrive in acidic media at the lower ranges of pH scale. They occur, for example, in acidic hot springs and abandoned coal mines. Among the acidophiles are eukaryotic algae such as *Cyanidium caldarium*, *Galdiera sulfuraria*, and certain diatoms. Other algae, the halophiles, may be found in high salinity, which include the eukaryotic alga *Dunaliella salina*, some diatoms, and to a certain extent also *Galdiera sulfuraria* could be regarded as halotolerant. Extensive research has been carried out with the halophiles, growing in media of high salt solution (up to saturation). Some halophilic algae synthesize and accumulate organic compounds in their cytosol, such as glycerol. These compounds balance internal–external osmotic

pressure and prevent plasmolysis from occurring. Fewer algae have been observed growing at depths where hydrostatic pressure could be a factor, but where there is still minimal light availability.

Some environments are characterized by more than one extreme factor. The microbes dwelling in those habitats are referred to as poly-extremophiles. Higher temperature may go together with acidic media (which is the habitat of thermoacidophilic microorganisms); hot or cold environments are often exposed to UV irradiation (occurring in such places are xerophilic algae and cyanobacteria; observed in the deserts or near the polar regions), likewise, high temperatures and high pH levels occur in salinity areas.

These extremophiles can provide important answers to the ecology and biochemistry and lead to biotechnological applications and industrial aspects. Furthermore, understanding of the diversity of algal life in various environments is vital also for the study of the origin and evolution of life on Earth. They thrived in conditions which are probably similar to the current extreme habitats, and it is well possible that similar phototrophs also live elsewhere in the universe.

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Biodata of **Joseph Seckbach**, author of *Preface*, “Oxygenic Photosynthetic Microorganisms in Extreme Environments: Possibilities and Limitations” (with co-author Aharon Oren), and “Algae and Cyanobacteria under Environmental Extremes: Final Comments” (with co-authors David J. Chapman, David J. Garbary, Aharon Oren, and Werner Reisser).

Professor Joseph Seckbach is the initiator and chief editor of *Cellular Origins, Life in Extreme Habitats and Astrobiology* (COLE) book series (see www.springer.com/sereis/5775). He is the author of several chapters in this series. Dr. Seckbach earned his Ph.D. from the University of Chicago, Chicago, IL (1965) and spent his postdoctoral years in the Division of Biology at Caltech (Pasadena, CA). Then he headed a team for searching for extraterrestrial life at the University of California at Los Angeles (UCLA). He has been appointed to the faculty of the Hebrew University (Jerusalem, Israel), where he performs algal research and teaches biological courses. He spent his sabbatical periods in Tübingen (Germany), UCLA, and Harvard University, and served at Louisiana State University (LSU) (1997/1998) as the first selected occupant of the John P. Laborde endowed Chair for the Louisiana Sea Grant and Technology transfer, and as a visiting Professor in the Department of Life Sciences at LSU (Baton Rouge, LA). Recently, he spent 3 months in Ludwig Maximilians University in Munich with a DAAD fellowship from the German service of exchange academicians, where several forward steps of this volume have been performed.

Among his publications are books, scientific articles concerning plant ferritin (phyt ferritin), cellular evolution, acidothermophilic algae, and life in extreme environments. He also edited and translated several popular books. Dr. Seckbach is the co-author (with R. Ikan) of the *Chemistry Lexicon* (1991, 1999) and other volumes, such as the Proceeding of *Endocytobiology VII Conference* (Freiburg, Germany, 1998) and the Proceedings of *Algae and Extreme Environments* meeting (Trebon, Czech Republic, 2000) (see <http://www.schweizerbart.de/pubs/books/bo/novahedwig-051012300-desc.htm>). His recent interest is in the field of enigmatic microorganisms and life in extreme environments.

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INTRODUCTION TO THE ALGAL WORLD

If you leave a glass of water near the window, after a while you may notice changes in color and clarity of the water. If you then examine a sample of the water under a light microscope you will see a beautiful, marvelous microscopic world unfold. Microalgae are everywhere! They occupy virtually every habitat including those with extreme conditions. Many microalgae live in extremely hot environments ($>70^{\circ}\text{C}$) such as deserts and the outflow of geothermal springs. They can live in the Dead Sea, where salt concentrations are seven times that of the oceans, and in highly acidic or extremely alkaline conditions. They can even live in snow!

Such extreme environments may be either their optimum niche, or be “limiting” to their growth. A sudden change in environment parameters generally induces two kinds of response in microalgae: changes in their metabolism and biochemical composition, and/or changes in their morphological structure. In addition, some taxa may change the stage in their life cycle (a common seasonal variation for many phytoplankton species). For instance, the alga *Haematococcus* forms green vegetative cells with two flagella that grow autotrophically in the light and heterotrophically in the dark. Environmental stressors, including high light, increased salinity, or shortage of nutrients such as nitrogen, induce encystment. The cells change their morphology and life stage. They lose their flagella and become spherical. Their protoplast is enveloped within a closely adherent palmlellar membrane; cells increase their volume and start to produce the secondary carotenoid astaxanthin. This is a naturally occurring carotenoid, which provides a wide range of antioxidant benefits protecting cell membranes and other sensitive structures against free radical attack.

Microalgae can produce more than 100 different carotenoids, with comparable structural diversity. Primary carotenoids are generally synthesized under optimal growth conditions whilst the production of secondary (keto) carotenoids, located outside the chloroplasts, is often enhanced under stress conditions. The composition and content of ketocarotenoids vary depending on the alga.

Haematococcus and *Dunaliella* are the two main organisms employed in the commercial manufacture of algal carotenoids, producing astaxanthin and β -carotene, respectively. Like *Haematococcus*, *Dunaliella* produces carotenoids when subjected to stress conditions. Under elevated salt concentrations, cells lose their flagella, become surrounded by mucus, and form resistant cysts. *Dunaliella* is the most halotolerant eukaryotic organism, and is capable of living under salt concentrations ranging from 0.1 to 4 M NaCl. To cope with this wide range of osmotic potentials it employs a unique osmoregulatory mechanism changing its cellular glycerol concentration. It naturally grows in hypersaline lakes or lagoons

with low nitrogen concentration exposed to high solar irradiation. In these stressful conditions, *Dunaliella* may produce more than 12% of its dry weight as β-carotene. β-Carotene is a pro-vitamin A carotenoid and a natural antioxidant, which has been used as food and animal feed additive and cosmetic ingredient.

In addition, microalgae are also a source of a wide range of fats, oils, hydrocarbons, and sterols. Many of these metabolites have the potential to be used either for biodiesel production or pharmaceutical applications. The lipid synthesis pathways in algae are similar to those found in higher plants. However, there are some differences. Thus, exposure to stress conditions causes variations in the fatty-acid composition of the oils in algae, and lipid synthesis by algae continues, in spite of the reduction in photosynthetic activity observed under stress conditions. Nitrogen limitation stress has the greatest influence on lipid storage of algae. Limitation of other key nutrients, for instance silica starvation in diatoms, may also result in increased lipid content. In contrast, some microalgae such as *Dunaliella* and *Tetraselmis* respond to stress conditions by decreasing their lipid content and producing carbohydrates. Thus, as for commercial carotenoid production, knowledge on the fundamental responses of individual taxa to extreme stresses is the key to the success of any future commercial production processes.

Many cyanobacteria have the ability to fix atmospheric nitrogen. Nitrogen fixation of cyanobacteria is catalyzed by the enzyme nitrogenase, which is sensitive to oxygen and is irreversibly inactivated in the presence of free oxygen. Some taxa have evolved heterocysts (special thick-walled cells that protect nitrogenase against damage from oxygen); however, there appears to be no universal system to protect the enzyme complex from both atmospheric and intracellular sources of oxygen in non-heterocystous cyanobacteria. During cyanobacterial evolution, it seems reasonable to assume that nitrogen fixation preceded oxygenic photosynthesis. After the evolution of this event, in the Precambrian period, the atmospheric oxygen level started to rise. It is clear that environmental perturbations and their associated stress induction have acted as a major stimulus to the evolution of stress.

This book highlights a number of examples of microalgae surviving, or even thriving, in extreme niches, and the mechanisms they have evolved that allow them to cope with the stresses to which they are subjected.

To paraphrase William Shakespeare:
To survive, or not to survive
To evolve, or not to evolve
To be, or not to be: These are the questions . . .

I congratulate the editor, Prof. Joseph Seckbach, and the authors of “*Algae and Cyanobacteria in Extreme Environments*.” I’m sure this book will be a guide source in this exciting field.

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Biodata of **Meltem Conk Dalay**, author of the chapter “*Introduction to the Algal World.*”

Dr. Meltem Conk Dalay is an Associate Professor of Limnologic Sciences and Algal Biotechnology at Ege University, Engineering Faculty, Bioengineering Department, Izmir, Turkey. She obtained Ph.D. from the Faculty of Aquatic products at Ege University (1997). Dr. Conk Dalay’s research over the past 15 years has emphasized the isolation, cultivation, and valuable chemical ingredients of algae, especially the effects of culture conditions on growth and biochemical composition of algal biomass. In addition, she has been working on photobioreactors and commercially important microalgae production facilities.

She coordinated the first commercial microalgae (*Spirulina*) production in Turkey with a private sector and her University cooperation project (2000) and she established the first microalgae culture collection (Ege-MACC) in Turkey. Dr. Conk Dalay has been coordinating many national and international scientific and industrial projects related to her scientific interests. Among her publications are over 30 scientific articles and the book entitled *Aquatic Plants*, published by Ege University press (2001). She has also organized several educational meetings and symposia and has been awarded two prizes by the Ebiltem (Ege University Science and Technology Centre).

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OXYGENIC PHOTOSYNTHETIC MICROORGANISMS IN EXTREME ENVIRONMENTS: *Possibilities and Limitations*

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1. Introduction

Oxygenic phototrophic microorganisms are abundantly found in environmental extremes of temperature, pH, salt concentration, and radiation. These extremophilic phototrophs include both prokaryotes (cyanobacteria) and eukaryotes (different types of algae).

The prokaryotic cyanobacteria, belonging to the eubacterial domain, do not possess a defined nucleus and have no intracellular membrane-surrounded organelles, while the eukaryotic algae have nucleated, more complex and larger cells that contain chloroplasts and other organelles. It is nowadays well accepted that the prokaryotic anucleated cells have evolved into more developed eukaryotic organisms in a process termed eukaryogenesis.

In this chapter we present a survey of the occurrence of oxygenic phototrophs in extreme environments, exploring the existence of phototrophic thermophiles (lovers of high temperature), psychrophiles (cold-loving organisms), halophiles (high salt-loving organisms), acidophiles (cells thriving at low pH), alkaliphiles (cells living at high pH), and radiation-resistant phototrophs. We then compare the performance of the prokaryotic and the eukaryotic phototrophs in each of the environmental extremes, and discuss the findings in the light of the evolutionary ideas relating to the formation of the eukaryotic cell.

2. Eukaryogenesis

Prokaryotic cells such as cyanobacteria have been documented to have inhabited Earth for 3.5 billion years at least. For eons, the cyanobacteria were the sole photosynthesizers and they contributed oxygen to the ancient reducing and anaerobic atmosphere. The first unicellular eukaryotic microbes appeared only 1.5–2.2 billion years ago. It may be assumed that the first evolving eukaryotic cells, whose ancestors had been living under anaerobic conditions, were poisoned

upon exposure to oxygen produced by the cyanobacteria. Only those early eukaryotic cells that could cope with the presence of oxygen survived. Oxygen requirement developed only after mitochondria were acquired, enabling the use of aerobic respiration for ATP production. When chloroplasts were acquired, the photosynthetic way of life was open to the eukaryotes as well.

The origin of the eukaryotes and the nature of the transition from the prokaryotic phase to eukaryotic status is still a biological and evolutionary mystery. The principles of the Darwinian theory of natural selection can be applied to the evolution that led to eukaryogenesis (Seckbach et al., 1998). There are two main schools of thought attempting to explain the evolution of simple prokaryotic cells into more advanced nucleated organisms (Seckbach, 1994b). The “classical hypothesis” advocates an autogenic slow process of compartmentalization (or “direct filiation”), leading to the establishment of the pro-eukaryotic cell. The second eukaryogenetic concept is that of endosymbiosis. The proponents for each of these hypotheses try to “prove” and justify their approach by applying various tools derived from comparative morphology, biochemistry, molecular biology, and genome analysis. Currently, the endosymbiosis model is the more popular of the two theories.

The concept of the endosymbiosis theory can be traced to the beginning of the twentieth century (Mereschkovsky, 1909; see also Margulis, 1981), but only since the 1970s has the model gathered momentum (Klein and Cronquist, 1967; Margulis, 1981). In the 1960s, both chloroplasts and mitochondria were found to contain specific DNAs and ribosomes, different from those in the nucleus and the cytosol of the host eukaryote. These discoveries led to the concept that free-living prokaryotic microorganisms, like cyanobacteria or aerobic bacteria (or in some cases even whole eukaryotic cells), penetrated, or were incorporated, into a host cell (of archaeal or eukaryotic type) by phagocytosis. Following some degree of genetic exchange with the host, the new lodgers turned into organelles. Incorporation of cyanobacteria led to the development of the chloroplast, and aerobic symbiotic bacteria turned into the respiratory organelle – the mitochondrion. In some algal groups, such as in the dinoflagellates, cryptophytes, and euglenoids, the chloroplast is believed to have originated from an engulfed eukaryotic alga. Such chloroplasts are surrounded by an envelope composed of three or four membranes. Such a structure can be explained as originating as a result of a secondary endosymbiosis. Recently, Lee and Kugrens (2000) proposed that the evolution of algal secondary endosymbioses was directly correlated to the decrease in atmospheric CO₂ during the late Paleozoic. Such secondary endosymbioses probably arose continuously from the time of evolution of the chloroplast, which took place about 2 billion years ago. The proponents of the endosymbiosis theory have suggested that many other organelles, such as the flagellum, golgi apparatus, lysosomes, and microbodies, have originated from symbionts. Seckbach (1996) termed the symbiotic association as “One and One = One,” and reported a personal communication of L. Bogorad stating that there are still many unsolved questions around the endosymbiosis theory.

Cells proposed as possible candidates for the first host cells include organisms similar to the archaeon *Thermoplasma acidophilum*, which possesses some proto-eukaryotic characteristics. The first eukaryotes could have been nucleated cells lacking some organelles common to most present-day eukaryotes, such as (chloroplasts and) mitochondria, but similar to the protozoan *Giardia lamblia*. Likewise, the large ameba *Pelomyxa palustris* is a primitive eukaryote lacking mitochondria, Golgi bodies, and a typical endoplasmatic reticulum; aerobic endosymbiotic bacteria function within this host as mitochondria; *Pelomyxa* is therefore considered to be a contemporary proto-eukaryotic cell (Ebringer and Krajčovič, 1994; Seckbach, 1994b). Other primitive eukaryotic microbes include the photosynthetic unicellular flagellate *Cyanophora paradoxa* (Glaucophyta), possessing cyanelles (Seckbach, 1994b), or the thermoacidophilic eukaryote *Cyanidioschyzon* (Cyanidiophyceae). *Cyanidioschyzon merolae* has been considered a primitive eukaryote, proposed as the most primitive among the Cyanidian members which compose the “algal bridge” linking between the cyanobacteria and the primitive Rhodophyta (Seckbach et al., 1983; Seckbach, 1987, 1994a, 1996). Seckbach and coworkers proposed an evolutionary line within the Cyanidiaceae from *C. merolae* (the most primitive eukaryote with ultra-small cells which still possess pro-eukaryotic features) through *Cyanidium caldarium*, a more complex alga, to *Galdieria sulphuraria*, the most advanced cell in this family, resembling the lower red algal division (Seckbach et al., 1983; Seckbach, 1992, 1994a, b, 1999).

These symbiotic proposals based on new-old evidence shook the proponents of the compartmentalization model (Klein and Cronquist, 1967). At present, there are only few who advocate the nonsymbiotic concept, among them are Jensen (1994, 1999) and Nakamura (1999) (see also Seckbach, 1994b). The basic question is whether the eukaryotic cell developed from a single prokaryotic evolving cell (the autogenous pathway) or from a few bacterial precursors (the symbiotic concept), so that the eukaryotic cell can be considered as a multiple complex of prokaryotic “cells.” For further data on eukaryogenesis, see Seckbach (1981, 1987, 1994b), Ebringer and Krajčovič (1994), Nakamura (1999), and Lee and Kugrens (2000).

3. Photosynthetic Microorganisms in Extreme Environments

Recently, much attention has been drawn to the various forms of life existing at the edge of biological limits under extreme physiological conditions. Extremophiles can be defined as organisms thriving in uncommon habitats. Kristjansson and Hreggvidsson (1995) defined an extremophile as one whose optimal growth conditions are found beyond their “normal” environments, “normal” meaning those that have a temperature between 4 and 40°C, a pH between 5 and 8.5, and salinity between that of freshwater and that of seawater (see also Walsh and Seckbach, 1999). The extremophilic inhabitants consider their hostile

habitats as an oasis or even as “Garden of Eden.” All three domains of life (Archaea, Eubacteria, and Eukaryotes) are represented in extreme environments. Some extremophilic microorganisms may resemble the early cells adapted to life in primordial environments exposed to the primitive atmosphere. In addition, they may serve as analogues for possible extraterrestrial life (Seckbach and Oren, 2001).

Many types of oxygenic phototrophic microorganisms can be found in extreme environments, both prokaryotic (cyanobacteria) and eukaryotic. They occur both at extremely high (hot springs) and extremely low temperatures (in severely cold zones, such as the permafrost areas in Siberia and Antarctica). Some phototrophs have colonized hypersaline waters or strongly acidic or alkaline habitats. Some extremophilic phototrophs are highly radiation resistant.

In Section 4, we discuss the oxygenic photosynthetic extremophiles that live in harsh conditions of temperature, pH, hypersalinity, and harmful radiation. For additional sources of data on extremophiles, see Brock (1969), Seckbach et al. (1970), Hausmann and Kremer (1995), Seckbach (1997, 2000b, c), Horikoshi and Grant (1998), Davies (1999), Roberts (1999), Madigan (2000), Seckbach and Oren (2000, 2001), and Gerday and Glansdorff (2007).

4. Thermophilic Oxygenic Phototrophs

Charles Darwin already proposed that life emerged from “a little warm pond” (see Davies, 1999). It is now generally believed that the first prokaryotic cells originated in warm environments (see Seckbach, 1994b, 1996). Photosynthesis does not appear to be compatible with very high temperatures: while archaeal life exists at as high as 114°C and possibly even higher, and certain heterotrophic eubacteria can grow up to 95°C, the most thermophilic cyanobacteria grow up to about 74°C only. Such cyanobacteria abound in hot springs worldwide (Brock, 1978; Ward and Castenholz, 2000). Photosynthetic eukaryotes are even less thermotolerant. The highest temperature supporting eukaryotic photosynthesis is 57°C, being in the same range as the maximum temperature enabling growth of protozoa and fungi.

4.1. PROKARYOTIC THERMOPHILIC PHOTOTROPHS

The existence of thermophilic cyanobacteria has been extensively documented in the course of the microbiological characterization of hot springs, first in Yellowstone National Park, Wyoming, USA (Brock, 1969, 1978) and later in other geothermal areas all over the world. Many geothermal springs emit water near the boiling point. The gradual cooling of the water in the outflow channels provides a stable temperature gradient in which populations of microorganisms position themselves according to their temperature requirements. Thus it was

established that 73–74°C is the maximum temperature enabling development of cyanobacteria. Different types of unicellular cyanobacteria, classified in the genus *Synechococcus* (*Thermosynechococcus*), are the most thermophilic. There are distinct differences in the communities found in hot springs in different parts of the world: thermophilic *Synechococcus* (*Thermosynechococcus*) species, abundant in Yellowstone, are absent from similar hot springs in Iceland (Ward and Castenholz, 2000). Filamentous cyanobacteria are less thermotolerant: *Mastigocladus laminosus*, *Phormidium* spp., and different thermophilic *Oscillatoria* species have their upper temperature limit for growth between 55 and 62°C. Some thermophilic cyanobacteria such as *M. laminosus* (upper temperature limit 58°C) and a strain designated “*Chlorogloeopsis* HTF” (upper temperature limit 64°C) are nitrogen fixers. An in-depth overview of the ecology, taxonomy, and physiology of thermophilic cyanobacteria was given by Ward and Castenholz (2000).

4.2. EUKARYOTIC THERMOPHILIC PHOTOTROPHS

C. caldarium (Cyanidiophyceae) and its relatives are the most thermophilic eukaryotic algae known. They live at temperatures between 45 and 57°C. These unicellular Rhodophyta not only tolerate elevated temperature levels, but are also able to thrive in very acidic media (pH 2–4), and even tolerate an atmosphere of pure CO₂ (Seckbach et al., 1970).

In the older literature it was claimed that *C. caldarium* has an upper temperature range of 75–80°C. Only after Doemel and Brock conducted ¹⁴CO₂ incorporation measurements in natural communities of the alga and in laboratory cultures was the upper temperature determined to be 57°C (Brock, 1978). Earlier reports of higher temperature limits of this alga were based on the observation of (dead) cells in hotter sites, where they had been carried from the cooler areas in which they had grown. Further detailed information on the Cyanidiaceae can be found in the treatise by Seckbach (1994a).

5. Psychrophilic Oxygenic Phototrophs

In the Arctic and Antarctic zones the surface layers of the sea are close to freezing. In spite of the low water temperature, phytoplankton may develop to high densities, and primary production may be quite high. Active photosynthetic communities also are found on the Antarctic continent, both in and on ice, in freshwater and saline lakes and streams, and below and within rocks. Moreover, cold environments are found at high altitudes in temperate zones.

The diversity of photosynthetic microorganisms that live in cold environments is surprisingly great. Low temperatures appear to cause no special problems for the functioning of oxygenic photosynthesis. Photosynthetic microorganisms,

prokaryotic as well as eukaryotic, are able to regulate the lipid composition of their membranes to adjust the membrane fluidity to the ambient temperature, and they can function as long as the cytoplasmic water is unfrozen.

5.1. PROKARYOTIC PSYCHROPHILIC PHOTOTROPHS

There exists an extensive literature on cyanobacteria found at low temperatures. Much information on the life of cyanobacteria near the freezing point of water was collected in the Antarctic (Vincent, 1988, 2000). A wide range of species have been found, unicellular as well as filamentous. Extensive areas of the McMurdo ice shelf are covered with mats of *Oscillatoria* species, accompanied by *Nostoc*. Benthic mats lining the bottom of ice-bound pools in different areas in Antarctica are composed of *Oscillatoria*, *Lyngbya*, *Phormidium*, and *Microcoleus*. Cyanobacteria are found in all freshwater environments of Antarctica. *Phormidium* and *Synechococcus* abound in Lake Vanda. *Phormidium frigidum* develops in lakes of the Dry Valleys, sometimes together with *Lyngbya mertensiana*. The nitrogen-fixing species *Nostoc commune* is abundant in Antarctic soils. Below rocks and in cracks within Antarctic rocks, *Chroococcidiopsis* can often be found (Vincent, 1988, 2000; Grilli-Cailola and Billi, 2007).

The Antarctic cyanobacteria are no true psychrophiles, as most types found grow optimally at temperatures far above those of the environment, typically in the range of 15–35°C. Their growth rate in the cold polar regions is therefore very low. Their survival is to a large extent based on their tolerance to desiccation and freezing, adaptation to low nutrient levels and to often high light and UV radiation levels (see Section 9.1), and the lack of significant levels of predation. In the marine environment of the Arctic and the Antarctic, cyanobacteria are rare or virtually absent (Vincent, 2000).

5.2. EUKARYOTIC PSYCHROPHILIC PHOTOTROPHS

The occurrence of red, pink, green, or yellow patches of algae growing on melting snow shows that certain eukaryotic algae are adapted to growth at low temperatures (Hoham, 1975). The majority of the snow algae belong to the Chlamydomonadaceae (Chlorophyceae). The best known species of snow algae is *Chlamydomonas nivalis*. Active growth of the alga occurs mainly in the spring and the summer when the snow melts, nutrients are available, and light penetrates through the snow pack. The vegetative cells are pigmented green, but its spores are bright red. The phenomenon, sometimes described under the name “water-melon snow,” is known from all continents with the exception of Africa. Other algae growing on and within snow belong to genera such as *Chloromonas*, *Ankistrodesmus*, *Raphidonema*, *Mycanthococcus*, and others. Snow algae have complex life cycles in which vegetative cells, sexual stages, and spores alternate (Hoham and Ling, 2000).

Eukaryotic microalgae are also abundantly found in the cold seas of the Arctic and the Antarctic. Diatoms are especially well adapted to life at low temperatures. The Antarctic ice-shelf diatom communities consist of *Nitzschia westii*, *Pinnularia cymatopleura*, *Navicula* sp., *Melosira* sp., and others. The sea ice is populated by *Pleurosigma*, the colonial *Nitzschia stellata*, and the chain-forming *Amphiprora*. *Nitzschia* species abound in the marginal ice zone of Antarctica. In the open ocean in the Antarctic, pinnate and centric diatoms, such as *Chaetoceros*, *Corethron*, *Nitzschia*, *Thalassiosira*, *Fragillariopsis*, and others, dominate. In addition to diatoms, other classes of algae (green algae, dinoflagellates) occur in the Antarctic as well (Vincent, 1988).

6. Halophilic Oxygenic Phototrophs

Presence of high concentrations of salts does not preclude the occurrence of oxygenic photosynthesis, and some phototrophs may thrive at NaCl concentrations up to saturation. Truly halophilic and/or highly halotolerant microorganisms can be found both in the bacterial and the eukaryal domains, and halophilic cyanobacteria as well as halophilic eukaryotic algae contribute to the primary production in salt lakes (Borowitzka, 1981; Javor, 1989).

6.1. PROKARYOTIC HALOPHILIC PHOTOTROPHS

Cyanobacteria feature prominently among the phototrophic biota found in hypersaline environments such as salt lakes, hypersaline lagoons, and solar salterns. Many highly salt-requiring and salt-tolerant species, unicellular as well as filamentous, have been described from such environments (Golubic, 1980; Javor, 1989). A general review on the occurrence and properties of halophilic cyanobacteria was recently presented by Oren (2000).

One of the most widely occurring filamentous species is *Microcoleus chthonoplastes*, a benthic mat-building species, found worldwide up to salinities of 200 g/l and higher (Javor, 1989). Its trichomes are generally encased in multiple-filament sheaths. An in-depth taxonomic study of *Microcoleus* specimens collected from all over the world has shown that there is a single, cosmopolitan species with a great ability to adapt to a wide range of salinities (Garcia-Pichel et al., 1996). Another type of filamentous cyanobacterium widely encountered in high-salt environments is the coiled *Halospirulina tapetiformis* (Nübel et al., 2000). The most widespread and best-known unicellular halophilic cyanobacterium is *Aphanthece halophytica* (also known under a range of other names, including *Coccochloris elabensis*, *Cyanothece*, *Halothecace*, and others).

Attempts to review the occurrence of cyanobacteria in hypersaline environments are hampered by the present confusing state of cyanobacterial nomenclature (Oren, 2000). Recent attempts toward the application of modern

approaches in the study of taxonomy, including molecular methods, have brought some clarity to the previous confusion, especially in the characterization of *M. chthonoplastes* (Garcia-Pichel et al., 1996), the *Aphanothece* (*Cyanothece*, *Halothece*) group (Garcia-Pichel et al., 1998), and the coiled *Halospirulina* (Nübel et al., 2000).

In the Great Salt Lake (Utah, USA) cyanobacteria are a characteristic component of the lake's biota. *A. halophytica* is found up to the highest salinities. In addition, filamentous species such as *Phormidium* or *Oscillatoria*, as well as *Microcoleus*, *Spirulina*, and *Nodularia*, were found in the shallow sediments of the lake (Post, 1977). In the Dead Sea (Israel), however, cyanobacteria do not form an important component of the biota. A varied community of cyanobacteria, unicellular as well as filamentous, was described from the hypersaline Solar Lake (Sinai, Egypt) (salinity 80–180 g/l), both in the water column and in the benthic microbial mats (Cohen et al., 1977). Solar salterns are also a rich source of halophilic cyanobacteria (Javor, 1989). The main component of the community is generally *M. chthonoplastes*, which forms coherent, highly productive mats. At higher salinities *Phormidium*, *Spirulina*, *Aphanothece*, and *Synechococcus* become dominant (Golubic, 1980; Javor, 1989). Of special interest are the layered cyanobacterial communities within the deposits of gypsum found in saltern ponds of intermediate salinity (Caumette et al., 1994; Oren et al., 1995b; Sørensen et al., 2004). The upper layer contains *Aphanothece* (*Cyanothece*), embedded in mucoid substance, imparting a yellowish-brown color to the bottom of the ponds. Below a green layer is found, mainly inhabited by *Phormidium*-like filamentous cyanobacteria.

Halophilic and halotolerant cyanobacteria maintain their intracellular ionic concentrations at relatively low levels, although ions such as K⁺ and Cl⁻ can transiently enter the cells following increases in medium salinity. To provide osmotic equilibrium with the environment, organic solutes are accumulated. The less salt-tolerant types generally use disaccharides such as sucrose and trehalose as osmotic solutes (Mackay et al., 1984; Reed et al., 1986). Many marine and moderately halophilic species, including the abundant *Microcoleus*, produce the heteroside glucosylglycerol (2-O- α -D-glucopyranosyl-(1 \rightarrow 2)-glycerol) as osmotic stabilizer. In recent years, the biosynthesis of glucosylglycerol and its regulation at the molecular biological level has been studied in depth in *Synechocystis* PCC 6803. Glucosylglycerol is produced in a two-step reaction from ADP-glucose and glycerol-3-phosphate with glucosylglycerol phosphate as an intermediate. The glucosylglycerol-forming enzyme system requires activation by salt and/or hypertonic conditions (Hagemann et al., 1999). Those cyanobacteria adapted to life at the highest salt concentrations (*A. halophytica*, *Halospirulina*) produce glycine betaine as their osmotic solute. Additional solutes such as L-glutamate betaine have been found as well (Mackay et al., 1984; Reed et al., 1986).

6.2. EUKARYOTIC HALOPHILIC PHOTOTROPHS

Green algae of the genus *Dunaliella* (Chlorophyceae, Volvocales) are found worldwide in hypersaline environments at salt concentrations up to NaCl saturation. The genus *Dunaliella* contains a number of species, including large-celled types ($12\text{--}16 \times 6\text{--}9 \mu\text{m}$ and more), such as *Dunaliella salina* and *Dunaliella bardawil*, and smaller species (about $12 \times 8 \mu\text{m}$), such as *Dunaliella parva*, *Dunaliella viridis*, and *Dunaliella tertiolecta* (Javor, 1989). Some species have complex life cycles in which both motile vegetative cells, nonmotile cells, haploid asexual resting cysts, and encysted zygotes may occur. *Dunaliella* may be defined as a genus of moderately halophilic algae with a wide salt tolerance. *D. viridis* was reported to grow optimally at around 60 g/l NaCl and to tolerate up to about 230 g/l; *D. salina* has its salt optimum at about 120 g/l and grows up to NaCl saturation. *D. viridis* has a minimum NaCl requirement of about 9 g/l, and is thereby obligatory halophilic to a larger extent than *D. salina*, which can even grow at 0.2% NaCl (Borowitzka, 1981).

Dunaliella is the main or only primary producer in hypersaline lakes with pH values close to neutrality. The Dead Sea surface waters are at times densely populated with small green *Dunaliella* cells. Numbers of up to 40,000 cells/ml were counted in 1964. A systematic survey from 1980 onwards showed that the development of a *Dunaliella* community in the Dead Sea is possible only when the upper water layers become diluted to a significant extent (10% at least) by flood waters; *Dunaliella* does not grow in undiluted Dead Sea water (presently more than 340 g/l total salts, with about 1.9 M Mg²⁺ and 0.4 M Ca²⁺, in addition to 1.6 M Na⁺ and 0.2 M K⁺). Following the rainy winter of 1979–1980, a bloom of up to 8,800 *Dunaliella* cells/ml developed, and following the even larger dilution (down to 70% of the original salinity) in the beginning of 1992, algal cell numbers even reached 15,000/ml (Oren, 1988; Oren et al., 1995a). Also in the Great Salt Lake *Dunaliella* is the main primary producer. In the 1970s, the north arm had about 330 g/l total salts, and was populated by *D. salina* (typically 200–1000 cells/ml, with maxima of more than 30,000 up to 100,000 cells/ml). The less-saline south arm (120 g/l salts) contained up to 2×10^5 *D. viridis* cells in the late spring (Post, 1977). In recent years drastic changes in the salinity of the Great Salt Lake have occurred, and no systematic studies have been made of the effect of these changes on the behavior of the *Dunaliella* communities. *Dunaliella* is also found worldwide in solar saltern ponds (Borowitzka, 1981; Javor, 1989).

Dunaliella cells maintain intracellular salt concentrations much below those of the outside medium. Measurements of intracellular ionic concentrations showed that *D. salina* cells contain only 20–100 mM Na⁺ when grown over the range of 1–4 M NaCl, showing that Na⁺ is effectively excluded from the cells. K⁺ is accumulated, with intracellular concentrations being in the range of 150–250 mM (Pick et al., 1986). To provide osmotic balance with the high salt concentrations in the medium, glycerol is accumulated as osmotic solute. Cells grown

at 1.5 M salt will contain about 2.1 M glycerol, and in cells grown at 5 M salt, glycerol concentrations of up to 7 M have been measured. As *Dunaliella* lacks a rigid cell wall, cells rapidly swell and shrink in reaction to changes in external salinity. Subsequently, they produce additional glycerol or convert excess glycerol into osmotically inactive starch to compensate for the changed osmotic pressure of the medium, and within 1.5–2 h the original cell volume is restored (Ben-Amotz, 1975). Glycerol is produced by reduction of dihydroxyacetone-phosphate to glycerol-3-phosphate, which is then dephosphorylated to glycerol. Excess glycerol is removed by the oxidation to dihydroxyacetone, followed by phosphorylation to form dihydroxyacetone phosphate (Ben-Amotz, 1975). The NAD-dependent glycerol-3-phosphate dehydrogenase is located in the chloroplast, while the NADP-specific glycerol dehydrogenase is found in the cytosol. The affinity of the glycerol dehydrogenase for glycerol is extremely low (K_m of 1.5 M), agreeing well with its use at extremely high glycerol concentrations. In contrast to most biological membranes, the plasma membrane of *Dunaliella* is very little permeable to glycerol, and only at supraoptimal temperatures are significant amounts of glycerol released to the environment (Wegmann et al., 1980). Increase in medium salinity causes the induction of at least two proteins in the plasma membrane of *D. salina*, one being a salt-resistant carbonic anhydrase (size 60 kDa), postulated to optimize CO₂ uptake in hypersaline media, the second being an internally triplicated transferrin-like protein of 150 kDa size, which is probably involved in iron uptake (Gokhman et al., 1999).

Dunaliella is the most widespread and the best-known halophilic eukaryotic alga, but it is by no means the only one. *Asteromonas gracilis* (Prasinophyceae) grows at NaCl concentrations between below 30 g/l and 260 g/l, and similar to *Dunaliella* accumulates glycerol, with intracellular concentrations as high as 5.5 M being reported (Wegmann et al., 1980). Diatoms may be found in solar salterns and hypersaline lakes up to about 210 g/l salt (Javor, 1989). They have also been found in the Great Salt Lake. Even in the alkaline (pH 9.8) saline (up to 90 g/l salts) Mono Lake, CA, several genera such as *Navicula*, *Amphora*, and *Nitzschia* have been observed.

7. Acidophilic Oxygenic Phototrophs

Eukaryotic algae, and to a lesser extent cyanobacteria, grow in various acidic environments scattered over the globe. Habitats for acidophilic algae are found in places such as the sulfataric fields of Pozzuoli (near Naples, Italy), Iceland, and Yellowstone National Park (Wyoming, USA). Microorganisms dwelling in acidic environments have to protect their internal constituents, such as chlorophylls, DNA, and ATP, which are unstable at low pH. Acidophilic microorganisms maintain their intracellular medium at a pH close to neutral (Beardall and Entwistle, 1984). Their surface barrier is extremely impermeable to protons, and selective mechanisms prevent H⁺ ions from entering the cell. Acidophiles use

proton pumps as a defense mechanism to maintain their intracellular pH at the desired, near-neutral values.

7.1. PROKARYOTIC ACIDOPHILIC PHOTOTROPHS

Cyanobacteria generally grow in environments of neutral and alkaline pH, and are rarely found at low pH. Brock (1973) stated in his survey that benthic and planktonic cyanobacteria were never found below pH 4–5, while eukaryotic algae proliferate even at pH levels below 3.0. However, more recently Steinberg et al. (1998) demonstrated that acid-tolerant cyanobacteria do exist. Populations of two filamentous cyanobacteria resembling *Oscillatoria Limnothrix* and *Spirulina* sp. were found in acidic Bavarian lakes, one of which had a pH of 2.9. Interestingly, eukaryotic phytoplankton was almost absent in that lake. Moreover, a survey of hundreds of lakes in Sweden and Canada showed that cyanobacteria are always present even in the most acidic lakes, down to a pH of about 3.7. Cyanobacteria such as *Aphanocapsa* sp. and several *Chroococcus* spp. have been found to dominate acidified Canadian lakes (Steinberg et al., 1998).

7.2. EUKARYOTIC ACIDOPHILIC PHOTOTROPHS

C. caldarium is an extreme thermoacidophilic rhodophytan (Seckbach 1994a). This microalga and its relatives are the sole photosynthesizers in warm acidic environments, where they have no competitors. This cosmopolitan red thermophilic alga has been shown to tolerate 1 N of sulfuric acid (Allen, 1959), while it grows optimally between pH values of 0 and 4, at a maximal temperature of 57°C. The chlorophytan *Dunaliella acidophila* also grows at very acidic conditions (Pick, 1999). Other green algae such as *Chlamydomonas acidophila* were observed to develop at pH values below 1–2 in Czechoslovakia and in Japanese volcanic waters, while *Euglena mutabilis* is able to grow between pH 1 and 5 (Gessner, 1959). In addition, there are acidophilic diatoms such as *Pinnularia braunii* (Pinnulariaceae), occurring in the pH range 0–4. Populations of unicellular rhodophytes, diatoms, and chlorophytes represent the main photosynthetic biomass in acidic habitats. A new species of *Pinnularia* has been observed in iron-rich, highly acidic (pH ranges from 2.7 to 3.2) strip-mined coal pits of south-central Iowa (Czarnecki and Cawley, 1997). *Pinnularia* is the only diatom occurring in thermal and nonthermal sulfuric acid habitats in Italy (pH 1.0–3.0) (Czarnecki and Cawley, 1997).

To protect the acid-sensitive cell components, the intracellular pH of acidophilic eukaryotic algae is maintained at near-neutral values, as shown in the examples presented in Table 1. Interestingly, four species of (nonacidophilic) brown algae (Dictyotales, Phaeophyceae) have been reported to be highly acidic as they accumulate sulfuric acid in their vacuoles, to yield pH values as low as

Table 1. Intracellular pH values measured in *Cyanidium caldarium* and in acidophilic chlorophytes. Data were derived from Seckbach (2000a), Beardall and Entwistle (1984), and Pick (1999)

Alga	External pH	Internal pH
<i>Cyanidium caldarium</i>	2.1	6.6
<i>Chlorella saccharophila</i>	4.0	7.1
<i>Chlorella vulgaris</i> Beij	5.3	6.6
<i>Chlorella pyrenoidosa</i> Chick	3.1	6.6–7.4
<i>Chara corallina</i> Klein ex Wild	4.5	7.3
<i>Scenedesmus quadricauda</i> (Turp.) Breb	3.1	6.8–7.0
<i>Euglena mutabilis</i> Schmitz	2.8	5.0–6.4
<i>Dunaliella acidophila</i>	0.5–3.0	6.2–7.2

0.5–0.9 (Sasaki et al., 1999). The cytoplasmic contents are protected against damage by the sulfuric acid by the properties of the vacuolar membrane.

8. Alkaliphilic Oxygenic Phototrophs

Photosynthetic CO₂ consumption leads to an increase in pH, and as a result, communities of cyanobacteria and eukaryotic algae may be exposed to elevated pH values during daytime. Most phototrophic microorganisms function well up to pH 9–10. Higher, stable pH values are encountered in alkaline lakes, and these also harbor oxygenic phototrophs, some of which may be obligate alkaliphiles. In contrast to the wealth of information available on halophilic phototrophic microorganisms, only little basic research has been performed on the adaptation of phototrophs to life at high pH.

8.1. PROKARYOTIC ALKALIPHILIC PHOTOTROPHS

The soda lakes of East Africa provide ample documentation for the existence of cyanobacteria adapted to life in highly alkaline environments. In these lakes, *Spirulina platensis* may reach very high community densities and high primary productivity at pH values of 11 and above (Grant and Tindall, 1986). Other species, such as the heterocystous *Anabaenopsis* (*Cyanospira*) and unicellular types such as *Synechococcus* and *Gloeocapsa*, may be present as well (Boussiba et al., 2000). *Gloethece linearis* and *Microcystis aeruginosa* have their optimum near 10, and growth of *Plectonema nostocorum* was reported up to pH 13, the highest pH at which life has been recorded.

S. platensis is an obligate alkaliphile, which grows best at pH 9–10, and still grows at 80% of its maximum growth rate at pH 11.5. *Spirulina* is the major

source of food for the dense communities of flamingos that feed on the African soda lakes. It is being exploited as food supplement for humans and animal, and has also found its way to the health food market (Cifferi, 1983). In the highest pH range, the organism shows an elevated requirement for Na^+ ions: at pH 9–10, 250 mM Na^+ is needed for optimal growth, and no growth occurs below 50 mM Na^+ . In the absence of Na^+ , cells die and lyse. It has been established that Na^+ is involved in pH homeostasis through the activity of Na^+/H^+ antiporters (Boussiba et al., 2000).

Of special interest are such environments that combine high pH values with the presence of high salt concentrations. One such environment is Mono Lake, CA. Here eukaryotic algae dominate (see later), but cyanobacteria have also been found in this lake (Javor, 1989). In the alkaline (pH 10.8–11.2) saline lakes of the Wadi Natrun, Egypt, *Spirulina* is found up to 92 g/l salt (Lake Gabara). Lake Muluk (159 g/l salt) has mats of *Phormidium* and *Synechococcus*. Other even more saline hypersaline alkaline lakes in the Wadi Natrun area, such as lakes Hamra, Gaar, Rizunia, and Zugm (240, 374, 389, and 394 g/l salts, respectively), contain cyanobacteria as well (Javor, 1989; Oren, 2000).

8.2. EUKARYOTIC ALKALIPHILIC PHOTOTROPHS

Diatoms are a prominent component in the biota of many alkaline lakes. A study of the diatom community in the East African soda lakes (pH from 8.3 up to 10.6) showed *Cyclotella meneghiniana* to be dominant at the lowest alkalinities, *Nitzschia frustulum* at the highest values, with *Coscinodiscus rudolfi* and *Navicula elkab* being occasionally found at intermediate alkalinites (Hecky and Kilham, 1973). In the highly alkaline and saline Mono Lake, CA, the green alga *Nannochloris* dominates, found together with *Chlamydomonas* (or *Dunaliella*?), *Ctenocladus circinnatus*, and diatoms such as *Nitzschia communis* and *Amphora coffeaeformis* (Javor, 1989). To our knowledge, hardly any basic research has been performed to elucidate the specific mechanisms enabling these eukaryotic algae to thrive in environments of extremely high pH.

9. Radiation-Resistant Oxygenic Phototrophs

Photosynthetic microorganisms cannot function naturally in the absence of light. Often they are exposed to full sunlight, including harmful ultraviolet radiation. Radiation damage can be caused by reactive oxygen species formed at high light intensities, such as singlet oxygen. The shorter the wavelength, the more potentially damaging the radiation is. Ultraviolet radiation (UV-A: 320–400 nm; UV-B: 290–320 nm; UV-C: 200–290 nm) is most harmful, and results in the hydroxylation

of cytosine and uracil, formation of pyrimidine dimers in DNA, interstrand cross-linking of DNA, and chain breaking and denaturation of DNA (Rothschild, 1999).

Microorganisms protect themselves both actively and passively against radiation damage. Active mechanisms include moving away from environments with too high light intensities and different mechanisms to repair damaged cell components. Passive protection involves the accumulation of protecting substances (“sunscreen compounds”) that prevent the harmful radiation from reaching the sensitive targets within the cell. One class of compounds that provides protection in many groups of microorganisms is the carotenoids. Carotenoid pigments quench excited state singlet oxygen. The quenching reaction involves direct transfer of energy between singlet oxygen and the carotenoid to yield the excited triplet state carotenoid and ground-state oxygen. Carotenoids absorb in the visible range above 400 nm. Their efficacy as UV screen is minimal, but they almost completely screen off the violet/blue part of the visible spectrum. Their function in providing protection against UV damage has never established, and if such a function does exist it is indirect only, by their activity as quenchers of singlet oxygen and inhibitors of free radical reactions (Castenholz and Garcia-Pichel, 2000). Another class of pigments, present in many groups of microalgae, is the mycosporine-like amino acids (MAAs). These are derivatives of cyclohexenone with amino acid or imino alcohol residues. Their biosynthesis derives from the shikimate pathway. Monosubstituted MAAs absorb around 310 nm, while bisubstituted derivatives have their absorbance maximum between 320 and 360 nm (Jeffrey et al., 1999; Castenholz and Garcia-Pichel, 2000).

9.1. RADIATION RESISTANCE IN PROKARYOTIC PHOTOTROPHS

Cyanobacteria are often found in environments exposed to high light intensities, including high levels of UV radiation. They often grow on walls and pavements, exposed to full sunlight. Some of the most extreme levels of radiation that cyanobacteria encounter are found in Antarctica, where degradation of the ozone layer has brought about an increase in the amount of solar ultraviolet radiation that reaches the surface, and cyanobacteria are still very common (Vincent, 2000, see also Section 5.1).

Different pigments provide passive protection of cyanobacteria against excessive levels of radiation. In the visible light range, communities exposed to high intensities have often high levels of carotenoid pigments (see e.g., Oren et al., 1995b). In polar areas, cyanobacteria often contain high levels of canthaxanthin, myoxanthophyll, and other carotenoids, the ratio carotenoids/chlorophyll *a* being maximal under low temperatures, high light regimes, and moderate UV radiation (Vincent, 2000).

To provide protection against UV radiation many cyanobacteria contain MAAs, often in large concentration within their cytoplasm (Castenholz and

Garcia-Pichel, 2000). A special case is the glycosylated MAA of *N. commune*, which is excreted and accumulated extracellularly to provide UV protection. An additional sunscreen pigment often encountered is scytonemin, a unique dimeric indole alkaloid located in the sheath surrounding the cells of many species, coloring the cells yellow-brown, and providing light absorption around 390 nm (Castenholz and Garcia-Pichel, 2000). Moreover, cyanobacteria may possess effective mechanisms to repair UV-induced damage (Rothschild, 1999).

An extreme case of radiation resistance in cyanobacteria was provided by a *Synechococcus* isolate from an intertidal evaporitic gypsum crust from Baja California, Mexico. When cells were exposed to the high radiation (10^4 kJ/m² between 200 and 400 nm) and vacuum in outer space during a 2-week space flight in June 1994, only a slight reduction in viability and activity occurred (Mancinelli et al., 1998).

9.2. RADIATION RESISTANCE IN EUKARYOTIC PHOTOTROPHS

High levels of carotenoids are also accumulated by many eukaryotic algae exposed to high light intensities. The halophilic green algae *D. salina* and *D. bardawil* produce β-carotene up to 8–12% of their dry weight, mainly as a mixture of the all-trans and the 9-cis isomers. The β-carotene is found as globules within the interthylacoidal space of the chloroplast, and makes the cells resistant to photoinhibition (Ben-Amotz and Avron, 1983; Ben-Amotz et al., 1989). The β-carotene content of the cells is enhanced when grown at high light, high salinity, low nutrient concentrations, and low temperatures (Ben-Amotz, 1999). β-Carotene is valuable as a food-coloring agent, source of provitamin A in food and animal feed, and as a health food antioxidant. *Dunaliella* is presently grown in several countries for β-carotene production in large nutrient-enriched lagoons or in highly intensive ponds in which all parameters of growth are strictly controlled (Ben-Amotz and Avron, 1983; Ben-Amotz, 1999).

The snow algae present another well-known case of carotenoid pigments providing protection against damaging high light intensities (see Section 5.2). Snow is highly reflective to visible radiation, and its high light-scattering properties may create very high photon fluence rates. At high elevations, the fluence rate of UV-B is also high. The spores of *C. nivalis* contain large amounts of astaxanthin esterified with fatty acids which accumulates in extrachloroplastic lipid globules (Bidigare et al., 1993). Aplanospores of snow algae exposed to UV-A (365 nm) and UV-C (254 nm) also produce flavonoids as antioxidant compounds, further reducing the level of free radicals that may damage chlorophyll in the chloroplast thylacoids (Duval et al., 2000). Thanks to these protection mechanisms, the red spores of *C. nivalis* resist the high UV irradiation level to which they are exposed in their natural environment (Hoham and Ling, 2000).

MAA sunscreen compounds are also widely found in eukaryotic microalgae, as shown by a recent survey of 152 species of marine microalgae from 12 classes

(including cyanobacteria, see Section 9.1). Very high ratios of UV/visible light absorbance (between 2.4 and 6.75) were reported in surface bloom-forming dinoflagellates, cryptomonads, prymnesiophytes, and raphidophytes. Intermediate values (0.9–1.4) were observed in chrysophytes, some prasinophytes, and other prymnesiophytes. Many others, including diatoms, chlorophytes, eustigmatophytes, rhodophytes, dinoflagellates, and prymnesiophytes, contained lower concentrations of UV-absorbing compounds, probably MAAs (Jeffrey et al., 1999).

10. Comparison of the Performance of Prokaryotic and Eukaryotic Photosynthetic Microorganisms in Extreme Environments – Conclusions

The outline presented earlier shows that both prokaryotic and eukaryotic microorganisms are abundantly found in environmental extremes of temperature, pH, salt concentration, and radiation. It has often been stated that in general the prokaryotes are much better adapted to environmental extremes than eukaryotes (see e.g., Elster, 1999). Such a statement is surely true in the case of adaptation to high temperatures: here the prokaryotes – and especially the Archaea – greatly outperform the eukaryotes. However, a comparison of the performance of the photosynthetic prokaryotes and eukaryotes in extreme environments (Table 2) shows that in some cases the more complex eukaryotic cell is more resistant to the environmental extremes than its prokaryotic counterparts. One example is the growth of *Dunaliella* at saturating salt concentrations. While many cyanobacteria can grow up to 200–250 g/l salt (Oren, 2000), they are rarely found in high numbers at the highest salinities. The success of *Dunaliella* may possibly be attributed to the use of glycerol as osmotic solute, a molecule that can be produced cheaply, and can be accumulated in sufficiently high concentrations. Prokaryote membranes are permeable to glycerol, and so are the membranes of most eukaryotes. The special properties of the *Dunaliella* cell membrane, possibly related to its high sterol content, ensure the retention of the glycerol inside the cell. The most halotolerant cyanobacteria use glycine betaine as osmotic solute, a compound less soluble and energetically more expensive to produce than glycerol (for a discussion see Oren, 1999). Another case in which photosynthetic eukaryotes have a clear advantage over the prokaryotes is in low pH environments. Cyanobacteria perform poorly in acidic environments, while organisms such as *Cyanidium* and *D. acidophila* function optimally. It may be speculated that the compartmentation of the eukaryotic cell allows for a more effective regulation of the pH to which the photosynthetically active membranes are exposed.

It thus appears that prokaryotic and eukaryotic photosynthetic microorganisms complement each other in their abilities to colonize extreme environments. While the prokaryotes may be intrinsically more adaptable to the most extreme of conditions, the formation of the eukaryotic cell has opened up a number of new possibilities, enabling the colonization of extreme ecological niches not or hardly occupied by the prokaryotes.

Table 2. Oxygenic photosynthesis in extreme environments: comparison of the abilities to prokaryotic and eukaryotic microorganisms to live under different environmental extremes. For details see Sections 4–9

Environmental parameter	Potential of photosynthetic microorganisms	
	Prokaryotes	Eukaryotes
High temperature	Unicellular cyanobacteria (<i>Synechococcus</i> [<i>Thermosynechococcus</i>] sp.). photosynthesize up to 73–74°C, the highest temperatures enabling photosynthesis	The most thermotolerant eukaryotic alga (<i>Cyanidium</i>) is capable of photosynthesis up to 57°C
Low temperature	Cyanobacteria are abundantly found in the Arctic and Antarctic, and grow slowly at near-freezing temperatures. They are psychrotolerant rather than truly psychrophilic	Eukaryotic algae, especially diatoms and green algae, grow in the cold ocean in or around sea ice; snow algae develop in melting snow, and may be true psychrophiles
High salt concentration	Cyanobacteria are abundantly found at high salt concentrations, but seldom develop massively at salt concentrations above 250 g l ⁻¹	Unicellular green algae of the genus <i>Dunaliella</i> are found worldwide at salt concentrations up to NaCl saturation
Low pH	Cyanobacteria are seldom, if at all, found in acidic environments	Specialized acidophilic photosynthetic eukaryotes (<i>Cyanidium</i> , <i>D.acidophila</i>) grow at pH values as low as 0–1
High pH	Cyanobacteria, especially <i>Spirulina</i> , occur massively in alkaline lakes, some of them are obligate alkaliophiles	Many eukaryotic algae grow in high pH environments
High radiation levels	Cyanobacteria are often found in high radiation environments and tolerate high levels of visible and ultraviolet radiation	Some carotenoid-rich eukaryotic microalgae grow in high light environments

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PART 2: PHOTOTROPHS AT HIGH AND LOW LIGHT

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EFFECTS OF ULTRAVIOLET RADIATION ON CYANOBACTERIA AND THEIR PROTECTIVE MECHANISMS

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1. Introduction

Enhanced solar ultraviolet radiation (UVR) due to stratospheric ozone depletion is a major stress factor for many phototrophic organisms in aquatic and terrestrial ecosystems (Franklin and Forster, 1997). UVR includes the wavelengths below those visible to the human eye. According to the CIE (Commission Internationale de l'Eclairage), the spectral range is divided into three wavebands: 315–400 nm UVA, 280–315 nm UVB and 190–280 nm UVC. UVA is not attenuated by ozone, and hence its fluence rate will be unaffected by any ozone layer reduction reaching aquatic and terrestrial organisms. Increases in UVB have been particularly reported in Antarctica (McKenzie et al., 2003) and the adjacent geographic regions (southern parts of South America and Australia) (Buchdahl, 2002; Deschamps et al., 2004), as well as in more recent years in the Arctic region (Knudsen et al., 2005). UVB exposure is potentially harmful to all living organisms, but especially to photosynthetic organisms due to their requirement for light. UVB represents less than 1% of the total solar radiation reaching the earth's surface, because it is absorbed partly by the ozone layer. It is particularly this waveband, which is influenced by changing stratospheric ozone concentrations caused by anthropogenic emissions of greenhouse gases, such as chlorinated fluorocarbons (Fraser et al., 1992). From recent five (1998–2003) years of study on continuous solar-irradiation measurements in Valdivia, Chile (39° S) it was reported that daily maximum dose rates (clear days) averaged in winter-summer: UVB as high as 0.3–3.7 Wm⁻² and UVA as high as 20.2–60.5 Wm⁻² respectively (Huovinen et al., 2006). In contrast, UVC is strongly mutagenic and lethal to most organisms. However, due to its complete absorption by the atmospheric ozone layer it does not reach the biosphere.

Almost everywhere on earth, especially in extreme environments, such as Antarctic ice shelves, deserts and hot springs, cyanobacteria are an important component of microbial communities (Potts and Friedmann, 1981; Ward et al., 1987). Cyanobacteria are evolutionarily the oldest oxygen-producing photosynthetic prokaryotes. In their natural habitats, these organisms are often exposed to

high solar radiation including UVR. The effects of UVR on cyanobacterial populations have been studied with increasing intensity in the recent years. Many effects reported in the literature indicate species-specific responses. UVB exposure causes physiological changes including inhibition or damage of photosynthetic processes and loss of enzymatic activity inhibiting growth and is sometimes accompanied by a high mortality (Kumar et al., 1996; Wu et al., 2005). UVB also affects membrane permeability, pigment stability, nutrient uptake mechanisms and signal transduction through phytochrome or specific UVB photoreceptors (Vincent and Roy, 1993; Portwich and Garcia-Pichel, 2000; Kumar et al., 2003; Cadoret et al., 2005). Lipids, proteins and DNA are also major targets of UVB directly or indirectly by oxidative damage from reactive oxygen species (ROS) induced by UVB (He and Häder, 2002a).

As cyanobacteria developed early during the Precambrian era (between 2.8 and 3.5×10^9 years), that is before the existence of the present ozone shield, it is presumed that these organisms faced more intense solar UVR than other phylogenetically much younger phototrophs (Castenholz and Garcia-Pichel, 2000). Therefore, most probably cyanobacteria possess efficient mechanisms to prevent or counteract the harmful effects of present (increasing) solar UVR. This review will summarize the effects of UVR on cyanobacteria and their protective mechanisms listed in Fig. 1.

2. Avoidance

2.1. MAT OR CRUST FORMATION

Cyanobacteria are the most successful crust-forming organisms. They are closely associated with the substrate and produce mat or crust-like structures, which can range from a thickness of few micrometers to decimeters. Mats are often composed of a varying number of different cyanobacterial taxa (10–40 species) (Büdel, 1999). They occur all over the world in rather extreme habitats, such as alkaline

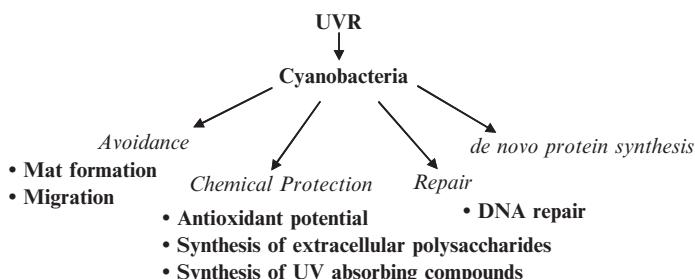


Figure 1. Cyanobacterial protective mechanism under UVR.

hot springs (Miller et al., 1998), Arctic fresh waters (Quesada et al., 1999), marine and hypersaline environments like the intertidal zone or evaporates (sediments left after evaporation of sea water) (Sørensen et al., 2005), rice fields (Adhikary and Sahu, 2000), hot arid areas (Scherer et al., 1984) and terrestrial rock surfaces (Pattanaik and Adhikary, 2002) (Fig. 2a). At all these places, the cyanobacteria have to tolerate great changes and frequent fluctuations of extreme environmental conditions. The composition of cyanobacterial mats varies according to different environmental conditions and substrata as well as the colonizing species abilities and properties. Generally, large filamentous cyanobacteria colonize first, most probably because of their possession of thick extracellular sheaths or mucus layers (Fig. 2b), which improve the water retention properties (Tomaselli and Giovannetti, 1993; Kovacik, 2000).

Since mat systems are considered as joint ventures of different taxa, these organisms are beneficial to each other. In marine habitats, such as the hypersaline Laguna Guerrero Negro, Mexico, the extensive cyanobacterial mats are dominated by the filamentous *Lyngbya aestuarii* and *Calothrix* sp. (Javor and Castenholz, 1984). Two distinct cyanobacterial layers were also reported in a mat associated with a subtropical mangrove system growing fully exposed to solar radiation (Karsten et al., 1998). While the 0.5-mm thick top layer was dominated by *L. aestuarii*, which is characterized by having a sheath with the UVA-absorbing yellowish-brown pigment scytonemin, the lower layer was mainly formed by the bundle-forming *Microcoleus chthonoplastes*, a species unable to produce this particular UV-sunscreen. Therefore, scytonemin produced by *Lyngbya* served as a photoprotective mechanism ("umbrella") for *M. chthonoplastes* too (Karsten et al., 1998).



Figure 2. (a), Circle indicating the mats on the rock surface of a mountain in Orissa, India; (b), Microscopic photograph of filamentous cyanobacteria (*Lyngbya* sp.) in the mat possessing extracellular sheath.

Gypsum crust formations were reported from the ponds of commercial salterns in Eilat, Israel (Oren et al., 1995; Canfield et al., 2004), and on ice-free rocks at Alexander Island, Antarctic Peninsula (Hughes and Lawley, 2003). The gypsum crystals provide protection from desiccation, rapid temperature variation and UVR. Both systems showed two distinct horizontal oxygenic phototrophic communities inside the crusts, which experienced considerably different irradiation levels. The upper carotenoid rich brown cyanobacterial (*Halothecce*-like) layer was exposed to highest radiation intensity and produce most of the oxygen in the crust. The rate of oxygen production of this layer responded strongly to radiation intensities. However, the lower green cyanobacterial layer (*Phormidium*-type) experienced much less light intensities (less than 0.1% of the radiation) and the rate of oxygen production did not apparently respond to changes in the intensity of incident solar radiation due to photosaturation indicating their adaptation to extremely low light intensities.

In contrast to all these aquatic mat systems, terrestrial habitats are generally much more extreme because of periodic long-term desiccation and higher UVR (Potts, 1994). Terrestrial substrates are often covered by black- or dark-coloured cyanobacterial crusts due to the presence of scytonemin or “gloeocapsin” (Büdel et al., 1997). Many of the warm and cold deserts are dominated by cyanobacterial crusts which often represent the most important primary producers (Garcia-Pichel and Belnap, 1996; Mazor et al., 1996). In these habitats, the organisms have extremely thick outer sheath layers to better withstand under high solar radiation and desiccated conditions particularly during summer. *Nostoc*, *Scytonema*, *Calothrix* and *Tolyphothrix* sp. are the most dominant aeroterrestrial cyanobacterial taxa (Tripathy et al., 1999).

Under different extreme environmental conditions the dominant cyanobacterial species in the mat or crust exhibit some protective characteristics against high solar UVR and desiccation, and thereby protect other associated taxa without these capabilities (Quesada and Vincent, 1995; Karsten et al., 1998).

2.2. MIGRATION

In planktonic cyanobacteria, sinking and floating regulated by gas vacuoles are also protective strategies against UVR (Reynolds et al., 1987). To escape from high solar radiation, motile cyanobacteria in mats often migrate up- and downwards depending on the spectral waveband (Bebout and Garcia-Pichel, 1995). Ramsing and Prufert-Bebout (1994) reported a downward movement of motile Oscillatoriales from microbial mat surfaces into the mat matrix or into soft sediments during periods of high insolation. In the mat of McMurdo Ice Shelf, Antarctica, two closely related cyanobacterial species *Phormidium murrayi* and *Oscillatoria priestleyi* showed substantial differences concerning their ability to escape damaging UVR effects. In response to changes in ambient light, the motile trichomes of *O. priestleyi* remained well below the microbial mat surface

consisting of the non-motile filaments of *P. murrayi*, which has the greater ability to tolerate UVA and UVB exposure. And when the mat was shaded for several hours, *O. priestleyi* migrated to the surface (Vincent and Quesada, 1994; Quesada and Vincent, 1995).

UVR is a primary cue for the vertical movement of cyanobacteria (Garcia-Pichel and Castenholz, 1994). Daily vertical migration to avoid periods of incident high solar irradiance has been reported for *Oscillatoria* sp. and *Spirulina* cf. *subsalsa* (Garcia-Pichel et al., 1994). In the hypersaline mats of Guerrero Negro, motile *Spirulina* and *Oscillatoria* showed the upward movements under low PAR (20–90 Wm⁻²) or in complete darkness. Downward movements of cyanobacteria were reported in response to PAR over 400 Wm⁻² and UVA irradiance above 10 Wm⁻² (Kruschell and Castenholz, 1998). *Microcoleus chthonoplastes* in the microbial mats of Solar Lake, Sinai, Egypt showed highest incidence of migration in response to UVB compared to UVA and PAR, indicating UVB was the most effective waveband (Bebout and Garcia-Pichel, 1995). The populations of these organisms were able to sense UVB directly, most probably due to the presence of a UVB-specific (280–315 nm) photoreceptor. While UVR-induced inhibition of physiological activity may occur within very short time intervals, natural daily vertical movements usually occur in response to gradual increases and decreases of solar irradiance (Donkor et al., 1993). Although sudden changes in natural insolation may occur especially during summer at cloudy days the motile cyanobacteria are usually well photoprotected underneath the mat surface within time scales that permit survival. Migration and vertical movement in the mats or sediments definitely appears to be an efficient strategy of cyanobacteria to avoid long-term exposure to high UVR and PAR and hence to save energy for photo-protective acclimation (Castenholz, 1997).

3. Chemical Protection

3.1. ANTIOXIDANT POTENTIAL

Cyanobacteria perform oxygenic photosynthesis using water as an electron donor. Thereby, they release molecular oxygen into the environment, which can be accumulated and converted into potentially harmful ROS. The interaction between UVR, oxygen and certain organic compounds can produce toxic intermediates such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroperoxy radicals (HO_2^-) and hydroxyl radicals (OH^-). These ROS can cause extensive damage to proteins, nucleic acids and other biological structures (Cadenas, 1989). Cyanobacteria have evolved a complex defense system against ROS including non-enzymatic antioxidants like carotenoids, tocopherols (vitamin E), ascorbic acid (vitamin C) and reduced glutathione. Enzymatic antioxidants are superoxide dismutase (SOD), catalase and glutathione peroxidase as well as the enzymes involved in the ascorbate-glutathione cycle, such as ascorbate peroxidase (APX),

mono-dehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase.

Carotenoids are well known for their antioxidant activity. In cyanobacteria, carotenoids occur in the outer cellular membrane as well as in thylakoids. During long-term exposure to high natural or artificial radiation very high ratios of carotenoids to chlorophyll *a* occur. These high ratios are the prerequisite for a broader tolerance against excessive irradiation, particularly at suboptimal growth temperature (Castenholz, 1972), and in response to UVR (Garcia-Pichel and Castenholz, 1991; Ehling-Schulz and Scherer, 1999). Carotenoids exhibit multiple physiological roles, that is as light-harvesting pigments in photosynthesis, as a general radical trapping antioxidant to prevent lipid peroxidation and by quenching triplet state of chlorophyll *a* to dissipate excess photochemical energy (Burton and Ingold, 1984; Edge et al., 1997; Kerfeld, 2004). The accumulation of high carotenoid (mostly myoxanthophylls and canthaxanthin) concentrations was visible in some Antarctic cyanobacterial mats dominated by *Nostoc commune* due to their bright orange or pink colouration (Vincent and Quesada, 1994). In the tropical Guyana-shield region of Venezuela, maximum canthaxanthin/β-carotene concentrations (Lakatos et al., 2001) and an increase in carotenoid/chlorophyll *a* ratios in response to UVA and UVB radiation (Ehling-Schulz et al., 1997) were detected in terrestrial cyanobacteria supporting a photo- and UV-protective function of these pigments. To counteract UVB-induced ROS formation, an increase in the synthesis of carotenoids in *Microcystis aeruginosa* 854 was observed (Jiang and Qiu, 2005). Carotenoids exerted their protective function as antioxidants in *Synechococcus* PCC7942 by inactivating UVB-induced radicals in the photosynthetic membrane (Goetz et al., 1999). It is also reported that outer membrane bound carotenoids provided a fast active SOS response to counteract acute cell damage (Ehling-Schulz et al., 1997).

Tocopherols are the second major class of lipid-soluble antioxidants in photosynthetic membranes. The primary function of tocopherols is to protect cells from lipid peroxidation as documented in *Synechocystis* PCC6803 (Steiger et al., 1999; Maeda et al., 2005). Ascorbic acid also significantly acts against lipid peroxidation and DNA damage. In *Anabaena* sp., ascorbic acid and *N*-acetyl-L-cysteine reversed the UVB-induced oxidative stress and protected against chlorophyll bleaching and damage of the photosynthetic apparatus (He and Häder, 2002b).

However, the non-enzymatic antioxidants are not considered as the efficient detoxifying agents (Wolfe-Simon et al., 2005). The most important enzymes, which detoxify superoxide radicals are the SOD family eliminating the noxious superoxide radical anions. Different metalloforms of SOD (Fe, Mn, CuZn and Ni) protect different cellular proteins and provide an in vivo tool to study cellular responses to oxidative stress (Lesser and Stochaj, 1990). Under desiccation and rehydration during UVA and UVB irradiation, an accumulation of active FeSOD was detected in *N. commune* (Shirkly et al., 2000; Ehling-Schulz et al., 2002). APX appears to be another important key enzyme in a cascade of reactions generating antioxidants. High activities of APX and catalase were reported in *Nostoc*

muscorum 7119 and *Synechococcus* sp. 6311 (Tel-or et al., 1986; Miyake et al., 1991). APX effectively removes low concentrations of peroxides whereas catalase eliminates H₂O₂ produced under photooxidative conditions. However, the importance of antioxidative enzymes under elevated UVR in cyanobacteria deserves further investigation.

3.2. SYNTHESIS OF EXTRACELLULAR POLYSACCHARIDES

Synthesis of extracellular polymeric substances (EPS) in cyanobacteria leads to a better survival capacity compared to many other phototrophic microorganisms under UVR (Helm et al., 2000). The EPS-containing sheath of cyanobacteria forms a buffer zone between the cell and the physico-chemical environment. The composition and structure of EPS vary widely among different cyanobacterial taxa and is responding to environmental conditions (Stal and Krumbein, 1985). Even one single strain may produce more than one type of EPS at different stages of growth (Christensen et al., 1985; Schüßler et al., 1997). The principal components of the EPS in vivo are glycan, UVA/B-absorbing pigments and water-stress proteins (WspA) (Tamaru et al., 2005). In *Nostoc commune*, cells were embedded and immobilized in a water-absorbing sheath composed of glycan, which played a crucial role in the stress tolerance during desiccation, freezing and thawing by physically replacing water and maintaining membrane integrity (Hill et al., 1997; Tamaru et al., 2005). In addition, the production of extracellular glycan and WspA in *N. commune* was stimulated threefold and fourfold respectively under UVB, indicating that EPS provides protection against this waveband (Ehling-Schulz et al., 1997; Ehling-Schulz et al., 2002; Wright et al., 2005).

3.3. SYNTHESIS OF UV-ABSORBING COMPOUNDS

To prevent photodamage, cyanobacteria acquired another important mechanism by synthesizing molecules that absorb a substantial portion of harmful UVR which consequently cannot interact with potential cellular targets anymore.

Mycosporine-like amino acids (MAAs) are colourless, water-soluble, low molecular weight compounds, which are accumulated in large quantities in cyanobacterial cells (Garcia-Pichel and Castenholz, 1993; Karsten and Garcia-Pichel, 1996). Structurally, these are cyclohexenone or cyclohexenimine chromophores conjugated with the nitrogen substituent of an amino acid or its imino alcohol, having absorption maxima ranging from 310 to 360 nm and an average molecular weight of around 300 (Castenholz, 1997; Dunlap and Shick, 1998; Cockell and Knowland, 1999). Their biosynthesis probably originates from the first part of the shikimate pathway via 3-dehydroquinic acids and 4-deoxygadusol (4-DG) (Favre-Bonvin et al., 1987). About 20 MAAs have been reported from various cyanobacterial species growing in different habitats. The most common

MAAs are shinorine, asterina-330, porphyra-334 and mycosporine-glycine. Several new compounds have also been characterized in recent years such as the novel compound 2-(E)-3-(E)-2, 3-dihydroxyprop-1-enylimino-mycosporine-alanine in the unicellular cyanobacterium *Euhalothece* sp. strain LK-1, isolated from a gypsum crust of a hypersaline saltern pond in Eilat, Israel (Volkmann et al., 2006). Although some cyanobacterial MAAs have been identified, most still await their chemical characterization.

Several studies provide evidence that MAAs protect cyanobacteria by absorbing harmful UVR (Scherer et al., 1988; Ehling-Schulz et al., 1997; Ehling-Schulz and Scherer, 1999). The specific content of MAAs can be significantly induced by exposure to UVA/UVB radiation with maximum effect at 320 nm (Garcia-Pichel and Castenholz, 1993), however, its synthesis is mostly induced by UVB. The induction of an eightfold MAA accumulation was also reported in the benthic cyanobacterium *Microcoleus chthonoplastes* after UVB treatment indicating the involvement of these compounds in photoprotection against UVR (Karsten, 2002). Cells with high concentrations of MAAs are approximately 25% more resistant to UVB than those with no or low concentrations of MAAs (Garcia-Pichel and Castenholz, 1993).

The protection against UV damage by MAAs depends on the species and the location of the sunscreen. In *N. commune* MAAs are thought to play an important role in photoprotection as they are located in the extracellular glycan layer covalently linked to oligosaccharides (Böhm et al., 1995; Ehling-Schulz et al., 1997). In most cyanobacteria, however, MAAs are located in the cytoplasm, where only 10–26% of harmful radiation is absorbed by this compound (Garcia-Pichel and Castenholz, 1993). Besides acting as sunscreens, MAAs may provide additional protection as antioxidants (Dunlap and Yamamoto, 1995). The MAA precursor 4-deoxygadusol was found to be a strong antioxidant with an activity comparable to that of ascorbic acid (Masaki et al., 1996). Oren (1997) has suggested that MAAs may also function as osmolytes because high concentrations of MAAs can be found in natural microbial populations living in hypersaline environments, but Karsten (2002) questioned this hypothesis by undertaking salt treatment experiments on *Microcoleus chthonoplastes*, which did not indicate any major involvement of these compounds in the process of osmotic acclimation.

Another UV-absorbing component “scytonemin” is also known for UV-screening properties in cyanobacteria. It is an extracellular, yellowish-brown, lipid-soluble dimeric pigment with a molecular weight of 544 Da and a structure based on indolic and phenolic subunits. Scytonemin is formed by condensation of tryptophan and phenyl-propanoid derived subunits (Proteau et al., 1993). The biochemical pathways that are involved in the biosynthesis are still unknown. However, scytonemin synthesis is strongly induced by UVA and due to its high extinction coefficient it may prevent 90% of incident UVA entering the cell, thus, efficiently functioning as a UVA-sunscreen (Garcia-Pichel et al., 1992; Brenowitz and Castenholz, 1997). After its synthesis, scytonemin remains chemically stable

and thereby persists for a long time under natural conditions which supports the screening activity. Photodegradation of this pigment does not occur, which is evident from its long persistence in terrestrial cyanobacterial crusts or dried mats (Brenowitz and Castenholz, 1997). Scytonemin effectively reduces inhibition of photosynthesis and photobleaching of chlorophyll *a* by UVA in *Chlorogloepsis* sp. (Garcia-Pichel et al., 1992). Increase in both, temperature and photooxidative conditions, in conjunction with UVA caused a synergistic increase in the scytonemin production in *Chroococcidiopsis* sp. (Dillon et al., 2002). In *Tolypothrix arenophila* and *Nostoc microscopicum*, the scytonemin pigment remained almost unaffected after 24 h of UVB. Increased ratio of chlorophyll *a*: scytonemin in the irradiated cells indicating an enhanced stability and protective role against UVB (Pattanaik and Adhikary, 2004).

4. Repair

Reparation (rebuilding by synthetic processes) is the last alternative mechanism in cyanobacteria to persist in nature under moderate to high UV irradiance (Castenholz, 1997). The repair mechanism involves active processes, in which UV-damaged molecules can be replaced by increased synthesis of the target or by repair of damaged targets without *de novo* synthesis (i.e. in DNA repair).

DNA-repair mechanisms are universal for all types of organisms. Exposure of DNA to UVR causes several types of DNA lesions, which are mainly repaired by photoreactivation (light-dependent) and excision repair (light-independent) mechanism. Photoreactivation occurs with the help of the photolyase enzyme that specifically binds to cyclobutane-pyrimidine dimers (CPDs) or 6–4 photolyase (6–4PPs) and reverses the damage after absorption of light energy at 400 nm (Sinha and Häder, 2002). The major photoreactivating factor *phrA* in the cyanobacterium *Synechocystis* sp. PCC6803 codes for a cyclobutane-pyrimidine dimer-specific DNA photolyase (Ng et al., 2000). In the excision repair process, various enzymes (e.g. glycosylases or polymerases) are involved. First, the damaged DNA is nicked and then the short single strand segments are removed and the gaps are filled by nucleotide re-synthesis. Both photoreactivation and excision repair activity have been found in cyanobacteria (Levine and Thiel, 1987). DNA damage occurs easily under *in vitro* conditions, but *in vivo* DNA damage is often not found, because repair mechanisms are very efficient (Rozema et al., 2002).

5. De Novo Protein Synthesis

Increased synthesis of any UVR-damaged target protein or of UV-resistant iso-forms may contribute to counteract UV damage. Most cyanobacteria have efficient biosynthetic capacities in response to UV stress especially for the

de novo formation of the D1 and D2 proteins, the key proteins in photosystem II (PSII) (Sass et al., 1997; Vass et al., 2000). Increased turnover of D1 and D2 proteins in *Synechocystis* sp. PCC6803 (Sass et al., 1997) and complete replacement of the D1:1 protein with D1:2 in *Synechococcus* sp. PCC7942 (Campbell et al., 1998) are considered as an important defense mechanism against detrimental UVB effects. A key step in this repair process is the differential transcription of the *psbA2*, *psbA3* and *psbD1*, *psbD2* genes encoding identical D1 and D2 proteins, respectively (Máté et al., 1998; Viczián et al., 2000). UVB irradiation increases the level of *psbA2* mRNA 2- to 3-fold, *psbA3* mRNA 20- to 30-fold and increases the accumulation of *psbD1* mRNA 1.5- to 2-fold, *psbD2* mRNA 5- to 7-fold in *Synechocystis* sp. PCC6803 indicating their defense response against UVB stress. A specific UVB related signal transduction pathway is involved in the induction of *psbA3*. The primarily expressed *psbA3* and *psbD2* serve as UV-stress gene and this effect is regulated, at least partially, at the level of transcription (Viczián et al., 2000).

6. Conclusion

UVR has various detrimental effects on cyanobacteria and many facts are already known about their different protective strategies to survive under such environmental stress. However, many open questions still have to be answered.

In crusts, the migration ability in response to UVB and the related physiological mechanisms are still obscure and need to be investigated. The role of radical scavenging and detoxifying molecules and enzymes as well as the respective signalling pathways are not fully understood yet. Concerning UV-absorbing substances, the contribution of extracellular polymers is still unclear. Although MAAs are induced under UV in many cyanobacteria and some of them are identified, their chemical diversity is not clarified as well as other potential functions related to nutrient status and osmotic stress. In addition, MAA biosynthesis per se, its molecular basis and regulation are unstudied. There are only few other photoprotective compounds, like scytonemin, which are undoubtedly connected to UVR.

Especially in terrestrial habitats, cyanobacteria are exposed to multiple environmental stress factors. Therefore, protective strategies that defend the cells against multiple stress seem beneficial and totally plausible. However, uni- and multifactorial experiments must be designed to address the most important regulating factors, reception molecules, signal pathways especially concerning synergistic effects and defense mechanisms. Not only because cyanobacteria contribute significantly to microbial primary production, but primarily because they must have developed sophisticated and efficient UV protection strategies early during their evolution as the earth still lacked any ozone layer, the investigation of the molecular basis of these abilities is almost unstudied in cyanobacteria.

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THE HIDDEN LIFE OF ALGAE UNDERGROUND

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1. Introduction

The term “algae” designates a most diverse and ancient group of organisms that is polyphyletic by evolution and artificial by taxonomy. Its only common feature is the ability to perform aerobic photosynthesis. Algae range by size from tiny cyanobacterial cells of the picoplankton to the giant kelps dominating rocky coastlines. They settle most diverse aquatic habitats such as hot springs and Arctic ice, live on and in rocks and various organisms, travel by air currents for thousands of miles and can be found in groundwater. Algae have taken an important part in the evolution of Gaia and gave rise to embryophytes (plants: bryophytes and spermatophytes).

In most textbooks, it is common wisdom that the success of algae is dependent on sufficient light available for net photosynthesis. However, there exists another world, hidden and without light, offering a plethora of aphotic habitats. That is the world underground, which is not only settled by bacteria, fungi and the terrestrial fauna, but also by algae.

The following is meant to give an introduction into what we know and suggest on the life of algae underground, that is on algae not living, as crust forming algae do, on but in soil substrates. The leitmotiv shall be to look for lessons they can tell us about the tremendous potential of life to adapt to habitats, that – at first glance – appear to be rather hostile and strange. For practical reasons all kinds of both pro- and eukaryotic algae living on and within soil substrates will be designated as “soil algae.”

2. Numbers

Current knowledge on the numbers of algae living on and within soil bases on few simple experimental setups: an aliquot of soil matter is diluted with tap water and is observed either by a microscope under UV-light in order to count particles showing the typical red fluorescence of chlorophyll: each dot represents one algal cell. Or particles with a diameter above a certain size are eliminated by filtration and the rest is spread on an inorganic agar-medium enriched with mineral salts and kept under conditions that favour autotrophic growth of algae. After a couple of days, colonies of algae are formed and can be counted easily. That method

allows an estimation of the amount of viable algal cells in a sample whereas the observation of chlorophyll fluorescence gives a total of all algal cells present. Data derived from those type of countings tell us that algae are present on and in nearly all types of soil. What is the more amazing is the fact that all counts represent minimum numbers of viable algae due to the method applied. Real number of algae might be even higher since usually a selective (inorganic) agar medium is used.

In general, highest numbers of algae are observed at the surface of soil and these numbers usually depend on season and extent of covering by plants (Shimmel and Darley, 1985), but also by other factors such as acid rain (Lukesová and Tajovsky, 1999). Accordingly, most algal cells are counted at the surface of farmland: Hunt et al. (1979) report on 3.3×10^7 algal cells/g topsoil whereas in the upper layer of forest soils they counted up to 1.6×10^6 algal cells. However, under special environmental conditions as are reported for Antarctic habitats most algal cells live below the soil surface that probably shields them from UV and desiccation (Davey and Clark, 1991).

Oesterreicher (1990) calculates the algal biomass for farmland as up to 1,500 kg/ha with mean values between 500 and 200 kg/ha, and for forest soils up to 20 kg/ha. Thus the biomass of soil algae may represent about 5–10% of the total microbial biomass in soil (Ramsay and Ball, 1983; Oesterreicher, 1990).

Data on the amount of algae below the surface and in deeper (aphotic) zones are less well corroborated. In general, number of algae decreases with depth (Table 1). At a depth of 1 m viable algae are still present their number amounting to about 10% of the population size at the surface. Other measurements report viable algae at a depth of about 2.7 m (Stina, 1969). There are also records for viable algae in groundwater (Wöhler et al., 1999). Data shown in Table 2 (Reisser, unpublished), correspond to algal numbers of about 6.5×10^4 /g dw of a forest soil at the surface (0 cm in Table 2) and to about 2.5×10^3 /g dw at a depth of 5 cm.

Those observations set the frame for a rather fascinating scenario of an algal world existing underground that yet shows only vague outlines and more questions

Table 1. Number of algal cells in different types of soil and depths.*

Type of soil	Depth (cm)	Number of algal cells ($\times 10^3$ /g soil)
Lawn	0	110
	20	80
	60	30
	100	20
Farmland	0	100
	20	100
	60	25
	100	10

*Data from Stina (1969).

Table 2. Distribution of algal taxa in different depths of a forest soil.*

Taxon	Isolation from depth of (cm)		
	0	1	5
<i>Bracteacoccus</i> sp.	4	12	11
<i>Chlamydomonas</i> sp.	7	5	7
<i>Chlorella</i> sp.	2	15	12
<i>Chlorococcum</i> sp.	11	6	7
<i>Eustigmatos vischeri</i>	7	5	10
<i>Klebsormidium sterile</i>	36	12	4
<i>Pseudochlorella pyrenoidosa</i>	7	5	4
<i>Spongiocyclotis</i> sp.	2	12	11
<i>Stichococcus bacillaris</i>	2	8	7
<i>Stichococcus</i> sp.	9	4	9
<i>Tetracystis</i> sp.	11	8	11
unidentified chlorophycean alga	2	8	7

*Number of cells is given as % of total number at each depth.

than answers. Only the most basic questions can be discussed here. They will deal with taxonomy, ecophysiology and the possible ecological impact of soil algae.

3. Taxonomy

Although taxonomy of algae living in soil habitats has been far less intensively studied than of species living in aquatic habitats, there is ample evidence that the overwhelming part of soil algae belongs to chlorophycean, xanthophycean, bacillariophycean and cyanobacterial taxa. For an overview, see Ettl and Gärtner (1995) and Metting (1981). Metting (1981) counted 185 genera of aeroterrestrial algae, 112 of them belonging to chlorophycean, 38 to cyanobacterial, 15 to bacillariophycean, 13 to xanthophycean, 3 to eustigmatophycean, 2 to rhodophycean and 2 to euglenophycean genera. Ettl and Gärtner (1995) report among eukaryote soil algae of 170 genera with about 1,000 species, 91 genera belonging to the chlorophycean, 34 to the xanthophycean, 29 to the bacillariophycean (64 species belonging to *Navicula* sp.), 5 to the euglenophycean, 4 to the rhodophycean, 3 to the chrysophycean, 3 to the dinophycean and 1 to the cryptophycean algae. According to Starks et al. (1981), more chlorophycean algae are found in acid soils than in neutral and alkaline soils where cyanobacteria are dominating.

It has repeatedly been asked whether there exist endemic taxa among soil algae, but the question is still open for discussion. Most probably soil algae represent a subgroup of the aeroterrestrial algae due to the fact that there is a constant influx of algae from the air and from rainwater and an efflux from soil back into the air by wind-driven erosion and into the groundwater (Broady, 1979; Wöhler et al., 1999). Observations suggest that a significant part of those algae

that come in by air currents are taken away again by wind-driven erosion and only part of incoming algae is able to settle either the soil surface permanently or to get into the soil. In general, algae are passively washed into the soil by rainwater, smaller cells getting into deeper layers faster than bigger cells, filamentous taxa or algae that form mucilaginous sheaths. Our observations show that in a given soil type a set of algal taxa is present, however, both absolute and relative cell numbers might differ with depth: As shown in Table 2 for a Middle European forest soil numbers of colony forming units (cfu) drop from 65,000 cfu/g dw soil at the surface to 5,500 cfu/g dw soil at 1 cm depth and to 2,500 cfu/g dw soil at 5 cm depth. Relative cell numbers, that is numbers of cfu per species, also may change with increasing depth: At the surface there is a rather inhomogenous distribution with one species (*Klebsormidium sterile*) dominating representing about 36% of the total cell number. With increasing depth this drops to about 4% whereas other species such as *Bracteacoccus* sp. increases its relative cell number from 4% at the surface to 11% at 5 cm depth. Cell numbers of other species such as *Tetracystis* sp. do not show a strict correlation with depth (11% at both surface and 5 cm depth). Data suggest that morphology of taxa may have some influence on the transport of algae into the soil: among taxa coming in by air filamentous specimen such as *K. sterile* tend to rest at the surface of the soil whereas coccoid taxa such as *Bracteacoccus* are washed into the soil more easily.

4. Physiology

Knowledge on the physiology of soil algae is rather limited and based rather on assumptions and deductions than on experimental data. This unusual situation reflects the fact that soil algae are not in the mainstream of current phycological research interests and is probably also due to the erroneous belief that algae per se have to perform photosynthesis and otherwise have no chance to survive. As to soil algae, a simple observation casts serious doubt on this assumption: all data of the amount of algal cells in soil are based on the count of cells exhibiting chlorophyll fluorescence or on cells that are able to form viable colony forming cells. This tells us that at a given moment a tremendous number of living algal cells is present in a permanently aphotic habitat. Experimental evidence scattered in literature backs this observation and suggests that the ability to use inorganic carbon as C-source does not principally exclude the use of organic carbon, neither in the light when photosynthesis is active, nor in the dark. Unfortunately, since the pioneering work of Bristol Roach (1926, 1927, 1928) the potential of soil algae to rely solely on organic carbon sources has not been examined systematically. Table 3 summarizes some data from literature.

Thus it is a reasonable working hypothesis that at least part of the soil algal population is actively growing heterotrophically when sufficient supply with soluble organic carbon and water is guaranteed. Unfortunately data on productivity of soil algae are rare and do not allow to differentiate between the activity of

Table 3. Soil algae growing in continuous darkness with organic carbon-sources.

Species	Source of Carbon	Reference
<i>Ankyra sp.</i>		4
<i>Bracteacoccus sp.</i>	Glu	5
<i>Characichloris sp.</i>		4
<i>Characiopsis sp.</i>		4
<i>Characium sp.</i>		4
<i>Chlamydomonas sp.</i>		4
<i>Chlorella sp.</i>	Fru, Gal, Glu, Mal, Man, Suc	2
<i>Chlorococcum sp.</i>	Fru, Gal, Glu, Mal, Man	2
<i>Chlorosarcina sp.</i>		4
<i>Chlorosarcinopsis sp.</i>		4
<i>Cyanidium sp.</i>		4
<i>Cystococcus sp.</i>	Fru, Gal, Glu, Mal, Man, Suc	2
<i>Dictyococcus sp.</i>		4
<i>Friedmannia sp.</i>		4
<i>Gloeotilopsis sp.</i>		4
<i>Heterochlamydomonas sp.</i>		4
<i>Klebsormidium sp.</i>		4
<i>Monodus sp.</i>		4
<i>Murielopsis sp.</i>		4
<i>Neochloris sp.</i>	Glu	5
<i>Neospongiococcum sp.</i>		4
<i>Oocystis sp.</i>		4
<i>Ourococcus sp.</i>		4
<i>Planktosphaeria sp.</i>		4
<i>Pleurastrum sp.</i>		4
<i>Pseudotrebouxia sp.</i>		4
<i>Radiosphaera sp.</i>		4
<i>Scenedesmus sp.</i>	Glu, Mal, Man, Suc	1, 2, 3
<i>Spongiochloris sp.</i>		4
<i>Tetracystis sp.</i>		4
<i>Tribonema sp.</i>		4

1: Bristol-Roach (1926), 2: Bristol Roach (1927), 3: Bristol Roach (1928), 4: Metting (1981), 5: Parker (1961), Fru: Fructose, Gal: Galactose, Glu: Glucose, Mal: Maltose, Man: Mannose, Suc: Sucrose.

algae at the (photic) soil surface and in (aphotic) deeper layers. Shimmel and Darley (1985) calculate the annual productivity of “soil algae” (0–2 cm) as 39 g C m⁻² a⁻¹ with maximum amounts in June (76 mg C m⁻² h⁻¹), that is about 5% of the activity of vascular plants at the same site. Stina (cited in Oesterreicher, 1990) estimates for farmland soils (0–10 cm) the production of about 500 kg of algal biomass (fresh weight) within a vegetation period of five months. This is in the same order of magnitude as data for fungi (2,000 kg h⁻¹, Oesterreicher, 1990) and bacteria (1,600 kg ha⁻¹, Oesterreicher, 1990). According to Ramsay and Ball (1983), the algal biomass in a pasture soil at a depth of 1–5 cm represents about 1.25 µg C g⁻¹, that is about 5% of the total microbial biomass.

A well known feature of some algae is the ability to form resting stages. This has been repeatedly studied in chlorophycean algae and can be triggered by, for example nitrogen deficiency and increasing light intensities. Usually resting stages are discernible from vegetative cells by a thickening of the cell wall, the formation of sporopollenin-like substances and secondary carotenoids that give cells a reddish-orange color. It is very interesting to note that those stages are rather seldom observed among soil algae *in situ*. However, there is indirect evidence that chlorophycean and cyanobacterial algae are able to survive in a desiccated status for an astonishingly long period of time: As has been shown for hot and dry desert soils algae are able to form cells that are identical to standard vegetative cells by morphology but have reduced their metabolic activity and thus can survive for 20 years and more (Trainor, 1970, 1985). Metting (1981) reports on algae that could be revived from 83 year old herbarium sheets. The more fascinating is the fact that after wetting of those cells and regardless to how long they have stayed in a dormant stage, it takes only few minutes until full metabolic activity is achieved and for example new flagella are formed (Trainor, 1985). Thus formation of resting stages may be a common strategy among algae to survive unfavourable conditions. In this context it should also be born in mind, that environmental conditions for soil algae may be rather harsh. Even if at least some of them are able to master aphotic conditions, stress could be built up by lack of water and a corresponding increase of salinity. Therefore, although only viable cells are counted by the agar plate method, this does not mean automatically that all those algal cells have also been active *in situ*.

As to the physiological prerequisites to withstand periods of osmotic stress and dryness, only general assumptions, mainly derived from the study of marine algae and phycobionts in lichens, are available. Thus various osmolytes such as monosaccharides, sugar alcohols and glycerol (Kirst, 1989) have been reported to be accumulated in marine algae but adequate information on soil algae is lacking. Some authors speculate on a water-storing capacity of exopolysaccharides formed as slimes or capsules by some soil algae in order to prolong the period of metabolic activity, but this needs confirmation by experimental data.

In recent time a further facet of survival strategies in soil algae was discovered by the revival of algal cells from up to 10×10^6 year old permafrost soils of North-Eastern Siberia and Antarctica. In those places viable coccoid chlorophycean algae and cyanobacteria were isolated from deep subsurface layers of about 14 m depth where they had persisted at temperatures of about -23°C at cell numbers of about 10^4 cells/g of sediment (Vishnivetskaya et al., 2003). As astonishing as those observations for eukaryotic cells are they are in agreement with a growing amount of data and experience gained from modern cryopreservation techniques used for long time conservation of cell cultures in for example algal collections.

Soil algae may be adapted not only to low temperatures but can stand also elevated temperatures up to 40°C in a dry soil for months (Trainor 1983; Buzer et al., 1985). Thus soil algae are able to persist in a wide temperature range.

Another kind of survival strategies is shown by cryptoendolithic algae. Those are mostly cyanobacteria and chlorophycean algae that penetrate rocks by small fissures and by the excretion of organic acids form small caverns under the rock surface where they perform photosynthesis while being protected from rapid temperature variations, desiccation and UV radiation (Friedmann, 1980; Bell et al., 1986).

5. Ecology

Information on the ecological impact of soil algae is as rudimentary and speculative as on their physiology. However, some assumptions can be taken for granted: soil algae in aphotic layers contribute directly (by respiration and/or fermentation) or indirectly (as a source of food for other soil organisms) to the CO₂-release of terrestrial ecosystems. They thus represent a deposit of carbon and thus a source of CO₂ in terrestrial ecosystems that has been widely neglected until now and should be taken into account for the discussion of scenarios following an increase in soil temperature in general (Davidson and Janssens, 2006) and in melting of permafrost soils that represent about 20–25% of the world's land surface. Recent estimates (Knorr et al., 2005) suggest that there is in soil much more carbon stored (1,500 GT) than in the surface vegetation (600 GT) and in the atmosphere (720 GT). Unfortunately, in a soil algae population the proportion of resting stages with a reduced metabolism is unknown and will widely depend on soil type and the properties of microhabitats that are offered to algae. What is more, even in the same soil type conditions may vary according to depth, rainfall, temperature, availability of free water in soil pores, etc.

Soil algae may interfere in many ways with soil structure and soil fertility. They can release substances such as exoenzymes, organic acids and exopolysaccharides (Paulsen et al., 1992; de Caire et al., 1997) that have an impact on soil structure and soil formation in general. Experiments with farmland soils show that exopolysaccharides that are excreted by some soil algae, aggregate soil particles, may help to keep moisture in the soil and thus to minimize wind-driven erosion (Bailey et al., 1973; Hu et al., 2002). Soil algae also have been reported to be part of the diet of earthworms and other members of the terrestrial fauna.

Besides being part of the terrestrial food chain (Lukesová and Tajovsky, 1999), soil algae may interfere with other organisms in soil by producing substances such as exopolysaccharides and antibiotic compounds that attract, for example bacteria and fungi or repel them. It is conceivable that there exist associations between algae and bacteria that attach to or live within the mucus- or sheath-like material surrounding cell walls of some algae.

Experiments with soil algae and Gram-positive and Gram-negative bacteria indicate that algae can produce antibiotic substances that stop the growth of bacteria (Tables 3 and 4, after Safonova and Reisser, 2005).

Table 4. Growth of *Escherichia coli* in co-culture with *Chroococcus turgidus* and *Tetra*cystis sp.

	Increase (+ %) or decrease (- %) of bacterial cell number at start (cfu $\times 10^4 \times ml^{-1}$) days after start				
	1	2	3	4	7
<i>Chroococcus turgidus</i>					
<i>E. coli</i> without alga (control)		+1300		+1700	+1575
<i>E. coli</i> with alga		-17		-37	-80
<i>Tetra</i> cystis sp.					
<i>E. coli</i> without alga (control)	+110	+255	+577	+682	
<i>E. coli</i> with alga	-32	-97	-96	-77	

Table 5. Growth of *Micrococcus luteus* in co-culture with *Chroococcus turgidus* and *Xanthonema Debile*.

	Increase (+ %) or decrease (- %) of bacterial cell number at start (cfu $\times 10^4 \times ml^{-1}$) days after start				
	1	2	3	4	7
<i>Chroococcus turgidus</i>					
<i>M. luteus</i> without alga (control)		+19		+17	+37
<i>M. luteus</i> with alga		-45		-57	-98
<i>Xanthonema debile</i>					
<i>M. luteus</i> without alga (control)		-5		-23	-10
<i>M. luteus</i> with alga		-27		-86	-10

Table 6. Attachment of soil algae to the surface of monocot (*Triticum* sp.) and dicot (*Pisum* sp.) roots.

Alga	Chlorophyll a bound to root surface (ug $\times g^{-1}$) of	
	<i>Triticum</i> sp.	<i>Pisum</i> sp.
<i>Chlorella</i> sp.	32	50
<i>Chlorococcum</i> sp.	38	12
<i>Tetra</i> cystis sp.	65	2

Plants were cultivated in liquid medium (BBM: Bold, 1942). Algae were incubated by gentle shaking with plant roots for seven days in the dark. Plants then were taken from vessels and roots directly extracted for chlorophyll

In another set of experiments, the production of chitinases by soil algae was shown (Reisser, unpublished). Those data back the assumption that soil algae are able to grow heterotrophically and thus compete *in situ* with bacteria and fungi for organic C-resources (Table 5).

Soil algae may also play a role in and around the rhizosphere. Our observations show that some soil algae can attach specifically to roots of mono- and dicots, respectively (Table 6) and support their growth. In one case (*Tetracystis* sp.) the release of phytohormones with auxin-like activity was shown (Reisser, unpublished).

6. What Is the Message ?

- A considerable amount of algae, mainly belonging to chlorophycean, xanthophycean and cyanobacterial taxa, lives in soil under permanently aphotic conditions. They represent a biomass and physiological activity that is deeply involved in soil-bound food webs and should be taken into account when making up the C-balance of soil ecosystems.
- Algae living in soil interfere with other soil organisms and plants directly and indirectly either by forming a substrate for herbivores or by releasing substances that change soil structure such as organic acids and exopolysaccharides or that have antibiotic and phytohormone activity.
- By having adopted various kinds of survival strategies under extreme conditions such as are lack of water supply under both high and low temperature regimes, cyclic freezing and desiccation as well as exposure to high and zero light intensities soil algae may contribute to our knowledge on the potentials of the extraterrestrial existence of plant life as we know it.

7. Acknowledgements

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Biodata of **Klaveness**, author (with F. Løvhøiden) of “*Meromictic Lakes as Habitats for Protists: Life in the Chemocline and Below?*”

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MEROMICTIC LAKES AS HABITATS FOR PROTISTS: *Life in the Chemocline and Below?*

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1. Introduction

The meromictic lake is, in effect, two different habitats separated by a distinct vertical gradient (“the chemocline”, Hutchinson, 1937). By passing through the chemocline, the conditions with regard to dissolved salts and organic matter, particulates, gases and pH/eH may be altered significantly. Contrary to holomictic lakes where gradients may be established seasonally by temperature differences, the chemical gradient in truly meromictic lakes is sufficiently robust to withstand seasonal mixis. While the meromictic condition in temperate lakes may last for several to thousands or more years, some basins of marine origin may have conserved a gradient towards anoxia for millions of years, if unperturbed by glaciations. The origin, terminology, properties and significance of land-locked waters (fjords being isolated from the sea by postglacial isostatic equilibration) and various aspects of meromixis have repeatedly been discussed in papers and reviews (e.g., Strøm, 1936; Findenegg, 1937; Hutchinson, 1937; Kjensmo, 1967; Walker and Likens, 1975; Degens and Stoffers, 1976; Hakala, 2004) and textbooks (e.g., Ruttner, 1940; Hutchinson, 1957; Wetzel, 2001; Kalff, 2002). A review of biological implications at the chemocline level has been written by Tyler and Vyverman (1995). More information is hidden in the literature, and emerging from further studies of meromictic lakes of different origin (cf. Hakala, 2004). Here, information about protists inhabiting the chemocline and possibly the monimolimnion (or anoxic water of isolated fjords) are presented from unpublished observations and from literature. Since these authors’ experience is mainly from Norway and Sweden, examples will be chosen from the geo-diversity of fjords and lakes here. This presentation is a sequel and extension to the review by Tyler and Vyverman (1995) and the treatise on meromictic lakes in Finland by Hakala (2004).

In Fig. 1, two hypothetical meromictic basins are illustrated in a simplified manner, indicating a different position of the chemocline relative to the lake surface. The lake indicated in Fig. 1A has a chemocline located relatively close the surface, implying a large volume of the underlying monimolimnion, a large surface of the interface receiving sedimenting particles from the mixolimnion, and also the possibility of developing phototrophic communities in the chemocline gradient if light penetration through the mixolimnion is sufficient. Examples of

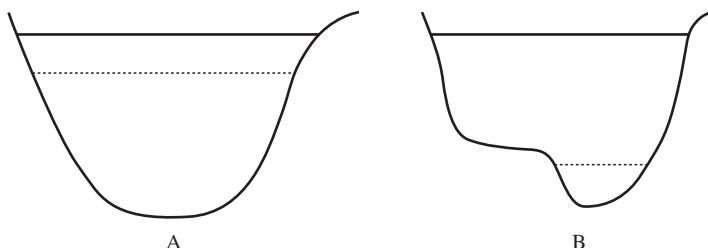


Figure 1. Diagrammatic representation of meromictic lakes where the chemocline (stippled) is located near the surface (A) and the monimolimnion fills a substantial part of the basin's volume, and (B) a lake where the chemocline is limited to a deep depression at the bottom of the lake and where the monimolimnion is a minimal part of the lake's volume. In case (A), the monimolimnion may serve as an efficient trap of sedimenting particles and prevent return of nutrients released during decomposition. Further, a clear-water mixolimnion may allow light penetration to the chemocline and stimulate photosynthesis by different organisms, types depending upon the local chemical environment. In case (B), the cleansing effect of the monimolimnion is slight, and light may not reach the chemocline. Then, the chemocline may mainly stimulate chemoheterotrophic processes by density-dependent accumulation of sedimenting particles and associated decomposition. There may be different variants of these types (see Table 1 and text for examples).

lakes of this kind are well represented in the literature, for example, lake Blankvann near Oslo in Norway (Strøm and Østtveit, 1948; Larsson, 1971); the “fjord lake” Framvaren in South Norway (Skei and Dyrssen, 1988); and lakes listed or discussed below as lake Vesle Bakketjern (Hongve, 1980, 2002) and lakes Pollen (Faafeng, 1976) and Hunnebotn (Braarud and Føyn, 1958; Strøm and Klaveness, 2003). The lake indicated in Fig. 1B has a chemocline located deep below the lake surface, here indicated within a local depression of the lake bottom. Light does not penetrate to the chemocline. The monimolimnion represents a small fraction of the entire lake water volume and is a minor recipient of sedimenting particles; its effect as a trap of sedimenting matter from the mixolimnion is therefore slight. Meromictic lakes of this kind may lack the cleansing effect of the “subsurface irreversible nutrient trap” sometimes seen in lakes of the type shown in Fig. 1A–B. Scientific treatises on this type of meromictic lakes include those of Strøm (1957, 1962) and his colleagues and students (e.g., Bøyum, 1973) on lakes with “fossil” seawater, Kjensmo (1967) on a number of “iron-meromictic” lakes, and a crenogenic lake (Bøyum and Kjensmo, 1970) mostly not including studies of the biota, and Hongve (1974, 1980, 1994, 1997, 1999, 2002) on lake Nordbytjern. Since the protist biota of meromictic basins to be discussed here may include those of fjords cut off from the sea and inland lakes stagnating through biological processes, the Venice convention terminology (Symposium on the Classification of Brackish Waters, 1959) will be used to indicate salinity differences between the mixolimnion (above the chemocline) and the monimolimnion (below the chemocline). Selected lake type examples, already visited and investigated by biologists or still wanting this research focus, are listed in

Table 1. The typology of meromixis follows the conventions of Walker and Likens (1975), although viewpoints of Hakala (2004) may well apply. The term protist is here applied in the modern sense (including eukaryote algae), since chloroplasts have originated as endosymbionts within protozoa from prokaryote or eukaryote “algal” ancestors. Therefore, the top-level classification and nomenclature follow Cavalier-Smith (2004), while lower-level nomenclature (here, at class or order level when applied) is based on the most recent textbooks (but see also Adl et al., 2005).

Table 1. Selected lake type examples with recorded chemocline or monimolimnion populations, or types lacking biological records (due to lack of sampling for this purpose). The records by microscopy is here emphasized, but molecular methods may indicate more to disclose (e.g., the chemocline of Framvaren fjord, see reference). The terminology of salinity levels refers to that introduced by the “Venice system” (Symposium on the Classification of Brackish Waters, 1959). In the right column, references are coded, where a star (*) indicates background (environmental, general) information, and numbers (1, 2) indicate organisms or populations discussed. Lakes with few data, included here, only indicate potential type habitats for interesting organisms under combinations of light, salinity and redox conditions, not yet investigated

Character of chemocline, level (), type (cf. Fig. 1).		Light conditions at chemocline level	Example lake	Chemocline populations (protists)	Monimolimnion populations	References
Mesohaline–polyhaline (18–20 m), type A. $Z_{\max} = 183$ m	PAR ca. 0.5% of surface level at 18 m	Framvaren fjord lake, located at sea level, restricted water exchange by channel and shallow sill 2–2.5 m	<i>Euglena proxima</i> + Ciliates ¹ , <i>Actuariola framvarensis</i> ² , more by molecular methods ³	Indicated by molecular methods ³	Skei and Dyrssen (1988), ¹ Klaveness (unpublished), ² Stoeck et al. (2005), ³ Behnke et al. (2006)	
Limnetic–polyhaline (7–9 m), type A $Z_{\max} = 18$ m	PAR ca. 1% of surface level at 7 m	Pollevann (Pollen), 1 m. a.s.l., outlet to sea by creek, 1.2 km.	Possible predators upon the bacterial plate ⁴ not recorded	No records	⁴ Faafeng (1976), ⁴ Stewart (1968)	
Limnetic–polyhaline (132–137 m), type B $Z_{\max} = 147$ m (405 m), type B $Z_{\max} = 464$ m	No light	Rørholtfjord lake (Tokke) Salsvatn	No records	No records	*Strøm (1957, 1962), *Barland (1991), *Bøyum (1973)	

(Continued)

Table 1. cont'd.

Character of chemocline, level (), type (cf. Fig. 1). Max. depth of lake (Z_{\max})	Light conditions at chemocline level	Example lake	Chemocline populations (protists)	Monimolimnion populations	References
Limnetic-limnetic (18–20 m), type A $Z_{\max} = 55$ m	PAR ca. 0.5% of surface level at 18 m	Blankvann	No records	<i>Astasia</i> spp., <i>Distigma curvatum</i> , <i>Gloeotila curta</i> ⁵	*Strøm and Østtvit (1948), *Larsson (1971), ⁵ Skuja (1956)
Limnetic-limnetic (0–3 m), type A–B. $Z_{\max} = 10$ m	PAR ca. 0.01% of surface level at 2 m, polyhumic	Vesle Bakketjern	<i>Chlamydomonas</i> sp., <i>Scourfieldia cordiformis</i> , a.o. ⁶	<i>Distigma striato-granulatum</i> ⁶	*Hongve (1980), ⁶ This paper, (Klaveness and Løvhoiden 2007)
Limnetic-limnetic (17–18 m), type B $Z_{\max} = 22$ m	No light at chemocline level. Seasonal redox-cline at 12 m indicated by (e.g., nitrite)	Nordbytjern	No records	No records	*Hongve (1974), *Klaveness (1977)

2. Meromictic Lakes and Protist Biota

Here, natural meromictic lakes at temperate to arctic latitudes are discussed. Meromixis due to tropical thermal conditions (see textbooks in limnology), and the situation in “heliothermic lakes” (Kirkland et al., 1983; Klaveness, 1990) are not included. For the very relevant conditions in the mainly anaerobic but holomictic lakes in Spain, see references in Tyler and Vyverman (1995). The successes of Fenchel, Finlay and co-workers in investigating protist communities in reducing environments have led to publications of potential relevance for meromictic systems also (e.g., Finlay et al., 1983, 1988; Fenchel and Finlay, 1990, 1991; Fenchel and Bernard, 1993, a.o.).

The spatial structure of the meromictic lake invites the establishment and maintenance of at least four different biological communities within the lake proper (here with specific reference to protists):

- The mixobionts: those living within the mixolimnion between the lake surface and the chemocline. The conditions for the mixobionts may in many respects be comparable to those of the epilimnion in holomictic lakes, with thermal stratification, periods of complete mixing, and inverse thermal stratification

beneath a covering of ice and snow. Parts or all of the mixolimnion may be within the euphotic zone, and availability of nutrients within different lakes may permit productivity ranges from ultraoligotrophy to eutrophy. Therefore, the protist plankton of the mixolimnion of meromictic lakes may be compared to and classified with those of the holomictic lakes (e.g., Hutchinson, 1967; Reynolds et al., 2002).

- The clinobionts: those living close to the chemocline, in a level within the decreasing gradient of oxygen towards the abrupt redox-cline, or within the anoxia below the redox-cline and exposed to dramatically different activities of metal ions, pH, and levels of dissolved gases – some of which may be toxic. Some clinobionts perform vertical excursions through the redox-cline, others possess organelles permitting life under permanent anoxia.
- The monimobionts: those living permanently below the chemocline, always exposed to anoxic conditions and well adapted to surviving osmotrophically, or grazing upon prey organisms or detritus, and reproducing under these conditions.
- The pelobionts: those living on or close to the surface of or within the sediment, at any level within the lake basin. Pelobiont communities are found in all lakes, and to what extent they differ in meromictic lakes may depend upon local conditions, even at small spatial scales (e.g., permanent or temporal/seasonal anoxia may just as well be present at or within the sediment in holomictic lakes – or variously distributed at micro-sedimentscape level). The concept here is taken to include the biofilm communities on rock and mud surfaces, as well as organisms always found close to or at the sediment surface, regardless of the oxygen tension.

2.1. CLINOBIONTS

Examples of clinobionts, from lakes of Table 1 or the literature, may include:

Actuariola framvarensis Stoeck et al. (2005) (Protozoa, phylum Euglenozoa). This newly described species is present in the chemocline of Framvaren fjord lake (Table 1). Formal diagnosis and pictures: Stoeck et al. (2005). The 18S rRNA sequence is deposited in GenBank with accession number AY963571.

Chlamydomonas sp. (Plantae, phylum Chlorophyta). A species of *Chlamydomonas* was found peaking at the chemocline level, but always extending into the anoxic zone below, in Vesle Bakketjern during the summer season (Fig. 2 E–H). It was closely associated with *Scourfieldia*, and took the largest share of the phytoplankton biomass in the summer with peaks of up to 10^8 cells/L in the chemocline. A similar occurrence of a *Chlamydomonas* sp. was found in lake Mekkojärvi (Finland, Arvola et al., 1992) where *Daphnia* was present (not recorded in Vesle Bakketjern).

Coleps hirtus Nitzsch (Protozoa, phylum Ciliophora). This species is a voracious predator commonly found where organic particulates, living and dead,

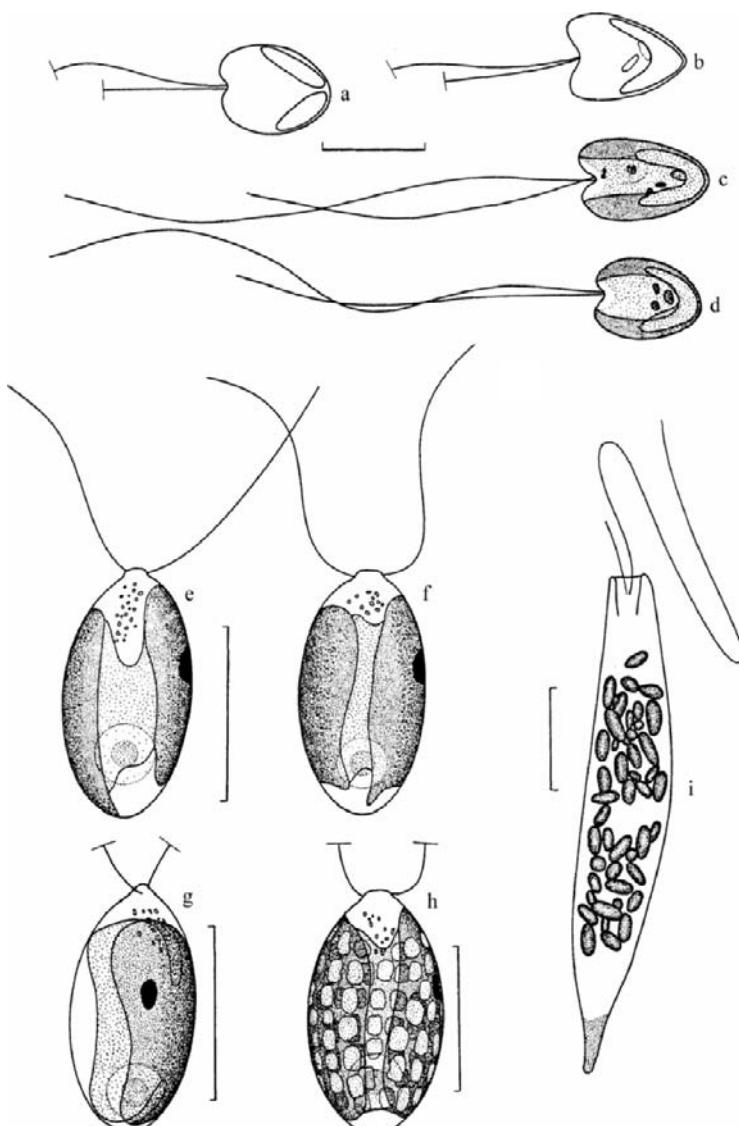


Figure 2. Figures are drawn from samples taken in Vesle Bakketjern. (A–D): *Scourfieldia cordiformis* Takeda, living cells with cordiform to horseshoe shapes. (A–B) with assimilation product indicated, (C–D) with the chloroplast and other cellular inclusions added [(C) with nucleus outlined]. (E–H): *Chlamydomonas* sp. 1, living cells showing cell shape and chloroplast with eyespot, nucleus and cellular inclusions indicated. (E–F) Dorso-ventral aspects. (G) Lateral aspect. (H) Dorso-ventral aspect of cell with accumulated assimilation product. (I): *Distigma striato-granulatum* Skuja, from material fixed by Lugol's solution. Scale bars = 5 µm. All drawings by Finn Løvhøiden.

accumulate as in pycnoclines of meromictic lakes of biogenic origin (limnetic-limnetic, cf. Table 1), and in summer chemoclines of stagnating holomictic lakes. It is, however, a very versatile predator of algae (Klaveness, 1984) and other protists, with the ability to cruise from mixolimnia to anoxic monimolimnia – the latter ability may be connected with its content of *Chlorella*-endosymbionts (cf. Stabell et al., 2002) – and it has a high survival ability under forced anoxic conditions (e.g., Lindeman, 1942). Its eurytopic nature is revealed whenever a sample of phytoplankton from almost any freshwater lake is left overnight – a steadily increasing number of *Coleps hirtus* is frequently found. GenBank: U97109 etc.

Cryptomonas rostriformis Skuja (1948) (as *C. rostrata*, nec *C. rostrata* Troitzkaja) = (?) *Campylomonas rostriformis* (Skuja) Kugrens and Clay (2003) (Chromista, phylum Cryptista). This is regarded as a thiophile species, occurring in water with detectable amounts of H_2S (Skuja, 1948; Huber-Pestalozzi, 1968). It is a clinobiont and possibly a facultative monimobiont in the limnetic environment (a pendant to *Euglena proxima* in the haline environment). It was frequently also recorded in the littoral of numerous Swedish lakes (as epi-pelobiont?), and is in “Schwefelgewässern Lettlands recht verbreitet” (Skuja, 1948). The thiophile limnetic community may have more members from the class Cryptophyceae, e.g., *Cryptomonas phaseolus* Skuja (1948) (see there, and also in Huber-Pestalozzi, 1968; Tyler and Vyverman, 1995; Gervais, 1998). For a recent review on taxonomy of and literature on the class Cryptophyceae, see Kugrens and Clay (2003). One rRNA sequence is deposited in GenBank: AB240953. More information about this strain (NIES 277) is available from the paper by Ishimitsu and Chihara (1984).

Euglena proxima Dangeard (Protozoa, phylum Euglenozoa). A species epithet given to a photoautotrophic *Euglena* frequently found under mesohaline conditions, inhabiting chemoclines under low-light conditions and very tolerant to H_2S (e.g., Fig. 3). *Euglena proxima* may also be found in ditches and lakes

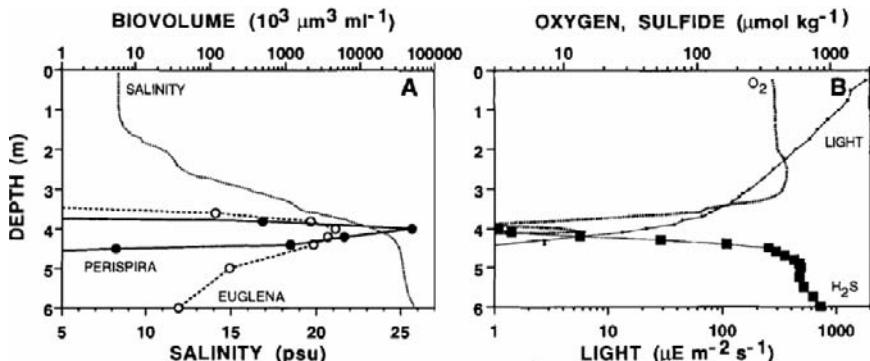


Figure 3. The clinobiont *Euglena proxima* and its predator, the ciliate *Perispira ovum*, in the chemocline of Pettaquamscutt River estuary, recorded on April 26, 1989 by Johnson et al. (1995). Reproduced with permission from Blackwell Publishing.

(Huber-Pestalozzi, 1955), and from there easily grown in biphasic (soil-water) cultures (Pringsheim, 1956), but not in pure cultures (Pringsheim, loc. cit.). It is, however, possible to keep pure cultures of at least one strain isolated from a mesohaline habitat (lake Hunnebotn, cf. Braarud and Føyn, 1958; Strøm and Klaveness, 2003, for environmental data) in glass-stoppered bottles under anaerobic conditions in algal medium with Na₂S added, when low light is provided (unpublished). Its apparent preference for these conditions in nature may well be due to the release and availability of certain nutrients under anoxic conditions, and the H₂S is tolerated as long as the intracellular oxygen generator is functioning. *Euglena proxima* is obviously a species quite well defined by morphological characters to critical microscopists, but more by habitat to ecologists – this interesting species complex is in need of supplementary studies by modern methods. Informative literature comprises Conrad and van Meel (1952), Huber-Pestalozzi (1955, limnetic habitats and records), Pringsheim (1956, species concept), Butcher (1961, marine records) – treated as freshwater species in John et al. (2002, cf. also Wehr and Sheath, 2003). GenBank deposit: AY626050 (chloroplast 16S rRNA), from SAG culture 1224–11b (freshwater), grown in biphasic (soil-water) medium NH₄E (see <http://www.epsag.uni-goettingen.de/html/sag.html> for details).

Loxodes spp. (Protozoa, phylum Ciliophora). This “primitive” ciliate is typical of many water bodies in which the bottom water periodically becomes anoxic (Finlay et al., 1983). There, bacterial nitrification and denitrification processes may be operative seasonally, and nitrate may serve as terminal electron acceptor in place of oxygen for *Loxodes* spp. (Finlay et al., 1983). It will be of interest to know whether *Loxodes* also may occur under permanent anoxic conditions as in the monimolimnion of meromictic lakes. In Vesle Bakketjern (Table 1), undetermined ciliates were recorded only in the very limited mixolimnion during the ice-free period. The chemical conditions in this particular lake favour the accumulation of reduced species of nitrogen compounds, and the availability of nitrate and formation of nitrite is very limited throughout the entire year. There is information about *Loxodes striatus* and *Loxodes magnus* in GenBank, e.g., U24248, L31519 etc.

Perispira ovum Stein (Protozoa, phylum Ciliophora). The ultrastructure and ecology of this sp. was recently treated in Johnson et al. (1995). Its large size and characteristic morphology, and its being a predator upon *Euglena* (Kahl, 1930; Johnson et al., 1995) should make it easy to recognize where *Euglena proxima* is present. *Perispira ovum* seems to be an euryhaline species, tolerating salinities from freshwater (e.g., Levander, 1894; Dewey and Kidder, 1940) to 2% (Kahl, 1930, p. 174) or higher (Johnson et al., 1995). It is therefore remarkable that it was not found within the abundance (14,000–50,000 cells/L) of *Euglena proxima* at the chemocline level in Framvaren fjord lake in June 1989, when a comprehensive plankton investigation was done there: the *Perispira ovum*–*Euglena proxima* – association may not be obligate. There is no information about the genus *Perispira* in GenBank.

Scourfieldia cordiformis Takeda (Plantae, phylum Chlorophyta, Prasinophyceae). This well-established sp. is frequently recorded beneath the ice

in winter and spring, frequently together with cryptomonads (Ettl, 1983). Here, in Vesle Bakketjern (Table 1, and Fig. 2 A–D) it was quantitatively most important during summer at the chemocline level and below under microaerobic and anaerobic conditions and extremely low light, even at mid-day. In this respect its occurrence is quite similar to that of the same species in lake Mekkojärvi, another polyhumic lake – with a similar community to that of Vesle Bakketjern (see also *Chlamydomonas* sp.). *Scourfieldia caeca* (Korschikoff) Belcher & Swale is considered a close relative by Ettl (1983), and this species occurs under similar conditions in some of the exogenous meromictic lakes of Tasmania (e.g., Croome and Tyler, 1985; Miracle et al., 1991). There is so far only fragmentary information about this genus in GenBank, L42854.

2.2. MONIMOBIONTS

These are protists preferring the monimolimnion as permanent habitat rather than performing diel excursions into the anoxic realm. Included here may be members of the *Metopetum* (cf. Foissner and Berger, 1996) comprising obligately anaerobic ciliates (lacking mitochondria but with hydrogenosomes) tolerating H₂S and feeding upon (sulphur) bacteria, members which also belong to the communities described by Lauterborn (1901, 1916). In the cases of true meromictic lakes, their presence will require further investigation. Among monimobionts may be recorded:

Astasia spp. (Protozoa, phylum Euglenozoa). Recorded by Skuja (in Skuja, 1956) in the upper monimolimnion (at 20–40 m) of lake Blankvann (cf. Table 1, on the 17th of October 1948). Probably a facultative monimobiont, maybe in line with several spp. of *Astasia* described from nature by Skuja (1939, 1948) like *A. pygmaea*, *A. hypolimnetica*, *A. kathermerios*, and further by Skuja (1956): *A. curta*, *A. elongata*, *A. robusta*, and others. They are found “zusammen mit zahlreichen anderen, teils thiophilen und saprophytischen Formen” (e.g., *A. pygmaea*, Skuja, 1956, p. 242) – an interesting community found in Swedish meromictic lakes like Hönsan, Storacksen and Lushavet (Skuja, 1956, p. 387). The ecology of *Astasia* spp. remains obscure pending more field-studies and experimental work (and a new tolerant and generous scientific climate) to rematerialize. There is genetic information about *Astasia* spp. in GenBank (AJ532394, AF112871 and more) and much can be learnt from papers by Christen (1958, 1959, 1963), Skuja (1939, 1948, 1956), Pringsheim (1942, 1963) and Leedale, in Leedale and Vickerman (2000).

Distigma curvatum Pringsheim (1936) (Protozoa, phylum Euglenozoa). This species was also recorded in some Latvian and Swedish lakes (Skuja, 1939, 1956) and was (reported in Skuja, 1956) recorded in the monimolimnion of the well-buffered lake Blankvatn (Norway, Table 1) under anoxic conditions, at a depth (20–40 m) where methane and some H₂S is found (Strøm and Østveit, 1948; Larsson, 1971). There is information in GenBank from several strains of this sp. isolated from continental Europe, for example, with accession numbers

AF099081, AF386642, AF386641, AF386640. Literature: Pringsheim (1942, 1963), Leedale, in Leedale and Vickerman (2000).

Distigma striato-granulatum Skuja (1948) (Protozoa, phylum Euglenozoa). This species was described (translated from German) as “slightly thiophilic; it occurs mostly littoral or above mud, particularly in water containing small amounts of H₂S, also singly in plankton of small lakes”. It was recorded in two Swedish lakes (Erken and Säbysjön), and previously known to that author from Latvia. *Distigma striato-granulatum* (Fig. 2I) was noted here as a regular component of the plankton in Vesle Bakketjern (cf. Table 1, and Fig. 2I), close to the redox-cline or in larger numbers up to the surface under anaerobic conditions beneath the ice (Fig. 4). It probably is a species preferring monimolimnion conditions in this lake, or a facultative clinobiont with a wide tolerance for the conditions in this lake. The species has possibly a wider distribution; so far there is no information about this one in GenBank. See Pringsheim (1942, 1963), Leedale, in Leedale and Vickerman (2000).

Euglena cf. hemichromata Skuja (1948) (Protozoa, phylum Euglenozoa). A common cline/monimobiont occurring in Vesle Bakketjern (Table 1), mainly during the summer, and accumulating under the ice under anoxic conditions during winter. Skuja (1964) recorded *Euglena hemichromata* “in moorigen Kleingewässern” in the mountain area of Abisko, there mostly scattered among other microorganisms. There is no information about this particular species in GenBank, but much information on other species of the genus *Euglena*.

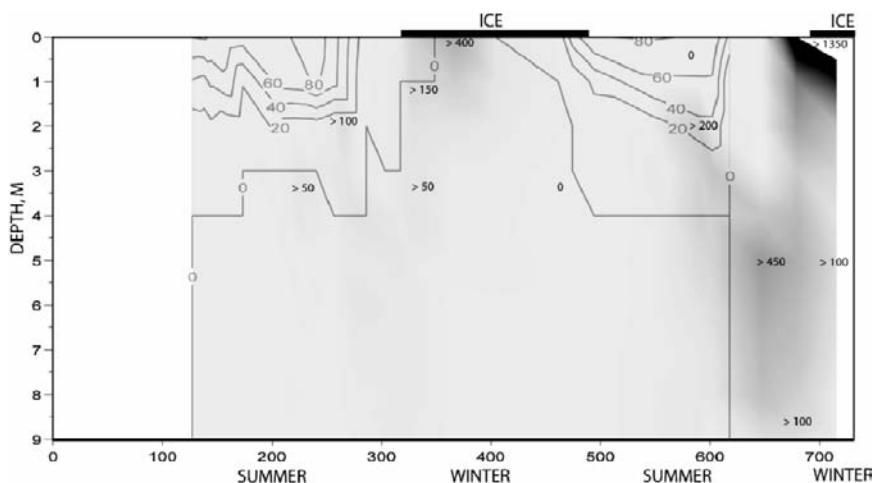


Figure 4. Plot of density (cells/mL) indicated as shades of grey “calibrated” by numbers (in black) from 0 to >1350 cells/mL, of *Distigma striato-granulatum* Skuja, in lake Vesle Bakketjern through the years 1975–1976 (day number since 31 December, 1974 (=day 0) along abscissa). Cell density is plotted upon the unsmoothed isopleth diagram of oxygen saturation (gray, numbers indicate % saturation) through the sampling period.

Gloeotila curta Skuja (1956) (Planta, phylum Chlorophyta, Ulothricales). This green alga was described from the hypolimnion of a holomictic lake (Munkbosjön) and the depths of meromictic lakes (Lushavet, Storacksen) in Sweden, and from the upper monimolimnion (depth interval 20–40 m) of lake Blankvann in Norway (cf. Table 1). According to Skuja (1956), this is a hypolimnetic form (he rarely used the terminology developed for meromictic lakes, so the exact position in the Swedish meromictic lakes is uncertain since he refers to their appearance at the surface during periods of circulation). There may be several more green algae tolerating and thriving under anoxic conditions in the monimolimnia for extended times. There are ssu rRNA data from two species of *Gloeotila* in GenBank (AY422074 and AY195976).

Metopus sp. (Protozoa, phylum Ciliophora) is the ciliate genus typifying the *Metopetum* community (Foissner and Berger, 1996) that possibly may visit or inhabit strictly anoxic zones of some meromictic lakes (but present in some holomictic lakes with pronounced seasonal anoxia, e.g., Esthwaite Water, see Bark (1981) for an instructive visual presentation). These ciliates are devoid of mitochondria, but have hydrogenosomes and tolerate permanent exposure to H_2S . Other genera and species represented in the *Metopetum* may be *Caenomorpha* spp., *Loxodes* spp., *Epalkxella* spp. and *Saprodnium* spp., and species like *Plagiophyla nasuta* and *Pseudoconilembus pusillus* a.o. may be present (Foissner and Berger, loc. cit., cf. also Lauterborn, 1901, 1916). Genomic information on *Metopus* is available from GenBank.

2.3. PELOBIONTS

Among pelobionts count the strictly aerobic populations of many species (e.g., upon and within the sand and silt sediment surface of some clear-water oligotrophic lakes), through microaerophilic and nitrate-reducing species (e.g., Finlay et al., 1983; Psenner and Schlott-Idl, 1985) under conditions of very low oxygen tension, to the extremely anaerobic communities occurring under highly reducing conditions within the sapropel of hypereutrophic or highly polluted lakes (“Die sapropelische Lebewelt” by Lauterborn, 1901, or “the Metopetum” of Foissner and Berger, 1996). Within this gradient, the meromictic lakes may be extremes within two directions: hyperanoxia due to H_2S (mainly due to sulphate reduction in saline environments), or hyperanoxia in the absence or with low levels of H_2S (mainly due to control of sulphide by iron, supplied from the watershed – cf. Davison and Finlay, 1986). Here, only two extremes are noted, see Lauterborn (1901, 1916), Foissner and Berger (1996) for more complete lists:

Gymnodinium aeruginosum Stein. (Protozoa, phylum Myzozoa). May represent the population most loosely associated with pelobionts, since *G. aeruginosum* occurs above the oxygenated sapropel in the littoral of lakes (“– or immediately above the sapropel where life conditions may be different from those of the free water” – Schnepf et al., 1989). May also be found above the oxygenated littoral-sublittoral

sediment in meromictic forest lakes (like those discussed by Hakala, 2004) – but is also not uncommon in samples from the pelagial of brown-water lakes (cf. Skuja, loc. cit.). This interesting species is easy to recognize by microscopy, but is not yet documented in GenBank.

Pelomyxa palustris Greef (Protozoa, phylum Amoebozoa). A pelobiont *par excellence*, inhabiting lake sediment, sometimes in large numbers (e.g., Lauterborn, 1901, Finlay et al., 1988). It has been found that *Pelomyxa* inhabit more than half of the investigated freshwater sites that release gas from organic mulch, in the south-eastern USA (Griffin, in Whatley and Chapman-Andresen, 1990). Although *Pelomyxa* may survive for months under anaerobic conditions (e.g., Lindeman, 1942), it is not a strict anaerobe, as it also seems to survive under low oxygen tension (at least in the overlying water, cf. Whatley and Chapman-Andresen, 1990. See also this latter contribution for a review). AF320348 a.o. in GenBank.

3. Discussion

At the end of their remarkable review, Tyler and Vyverman (1995) call attention to the algae at the chemocline. My brief sequel here can only support and extend the appeal to include protists generally – and to more lake types, particularly those with a deep and unlit chemocline (and pycnoclines, where sedimenting particulates may accumulate). From Table 1, it may be deduced that there may be combinations of depth of chemocline (lighted, or chemocline in permanent darkness) and chemical gradients across the chemocline (salinity; e.g., limnetic/limnetic with or without H₂S (controlled by iron), limnetic/mesohaline with H₂S (from seawater sulphate), mesohaline/polyhaline with H₂S. Under labile conditions as in productive ponds and lakes during summer stagnation, an intermediate layer where oxygen is diminishing and nitrate may serve as electron acceptor for respiratory processes, may be identified by chemical (e.g., Hongve, 1974) or biological (e.g., Finlay et al., 1983) methods. In a meromictic lake, this layer may occur well above, and not directly related to, the “permanent” chemocline in meromictic lakes (Hongve, 1974).

“Thiophilie” is a German term used to characterize freshwater algae occurring in water where the smell of H₂S usually was convincing (e.g., Huber-Pestalozzi, 1968), but the availability of some nutrients may also become more favourable under such reducing conditions. Thus, the tolerance to or preference for H₂S by some algae (*Cryptomonas rostriformis*, *Euglena proxima*) may either satisfy nutrient requirements, or simply reflect a low fitness to “normal” conditions where competition is fierce. The possible permutations of conditions occurring in stagnant and meromictic lakes still outnumber the actual types investigated with regard to tolerant (*Coleps*, *Euglena* spp.) or archaic (*Loxodes*) clinobionts and visiting or well-adapted monimobionts among the eukaryote protists. We are here dealing with environments of very limited spatial distribution (related to the “Phasengrenzschichten” in the terminology of Pringsheim, 1964)

where investigation requires and deserves unusual creativity and special skills (e.g., Perfilev and Gabe, 1969; Baker 1970; Blakar 1978, 1979; Lindholm 1979; Tyler and Vyverman, 1995, a.o.). Of particular interest, and a technical challenge, may be the chemoclines located in some very deep Norwegian lakes, like lake Salsvatn (464 m deep) with a limnetic – polyhaline chemocline located at 405 m depth (Bøyum, 1973).

The success of members of the Euglenozoa close to and within the anaerobic realm is conspicuous, and further studies of these should be pursued. In one respect, the anaerobic environments favour euglenoids since these appear to use reduced nitrogen compounds preferentially (Leedale, 1967) and may be deficient in nitrate reductase (Smillie, 1968). In the literature, interesting observations may be recognized from the studies of Skuja (1956), from field-studies by Christen (1958, 1959) and others. The comparable success of certain ciliates is remarkable as well, as the absence of mitochondria in *Metopus* and the alternative function of the same in *Loxodes* allows a permanent existence under anaerobic or microaerobic conditions. The evolutionarily acquired or conserved bacterial endosymbionts in certain amoebae like *Pelomyxa* also permit an existence under reducing conditions. Fortunately, the genetic tools developed have now reached a level of sophistication where they may be of great help, and leading research groups are to an increasing extent combining modern and classical microscopy with genetic tools (Jakobsen et al., 2002; Stoeck et al., 2003; Shalchian-Tabrizi et al., 2006, a.o.). This combination of techniques will be of great value and significance for the next generation of environmentalists, when morphology and microscopical characters will be even more informative.

The lack of precise characterization of habitats and environmental conditions is probably the greatest present obstacle to further learning from observations in nature. Several fine and detailed observations have been made in seasonally or periodically stagnant lakes, where conditions changes through time or seasons allowing production and consumption of biochemical intermediates like nitrite and N₂O (cf. Cohen, 1978), possibly by bacteria and eukaryotes. But in truly meromictic lakes at the level of the chemocline and below, these redox-related changes may be absent, and the anaerobic conditions are severe and may last for millennia. The protistologists must be schooled in basic limnology to be able to distinguish the meromictic lakes from the seasonally or intermittently stagnant ones, and to describe the chemical environments precisely everywhere in order to learn more about the eukaryote inhabitants of these interesting localities, and to develop tools and methods for their further study in the laboratory.

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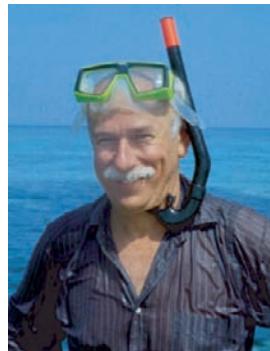
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MARINE PHOTOTROPHS IN THE TWILIGHT ZONE

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1. Introduction

Phototrophs (photolithoautotrophs) are organisms that use light as their energy source to synthesize organic compounds. These organisms include some bacteria, cyanobacteria, algae, and plants. They harvest light by various pigments, the main of these being chlorophylls, and its energy is transferred to the photosynthetic reaction centers. Even though phototrophs depend on light for their survival, some of these grow under very low light.

In general, the terrestrial light flux, even under the most intense sunlight is too low for single chlorophyll molecules to sustain photosynthesis, since the arrival of photons would be so slow that the S states (Kok et al., 1970, Falkowski and Raven, 1997) would decay spontaneously, not allowing generation of oxygen or carbon reduction. In reality, light is harvested in the photosynthetic apparatus by “antennae,” consisting of hundreds of pigment molecules embedded in the thylakoids or similar membranes. The antennae have a far larger cross section, σ , or probability of intercepting a photon than single pigment molecules. The energy intercepted by the antennae migrates as excitation energy to the few chlorophyll molecules in the photosynthetic reaction centers.

Even under the brightest light, such as is common at noon of a summer day on the shores of the Red Sea, of $\sim 2000 \text{ }\mu\text{mole q m}^{-2} \text{ s}^{-1}$, a single chlorophyll *a* molecule would be hit some 0.1 sec apart, a rate too slow to sustain photosynthesis. The *in vivo* optical cross section of chlorophyll *a*, a^* (see Dubinsky, 1992, for definitions) is on the order of $\sim 0.005 \text{ m}^2 \text{ mg}^{-1}$. Therefore, in many algae, not all, acclimation to light intensity involves major changes in antenna size, as it increases under dim light, and decreases when exposed to intense radiation (Dubinsky et al., 1986, Falkowski and Raven, 1997). In other algae and higher plants the same light-harvesting adjustment is accomplished by changes in the number, not in the size of photosynthetic units (PSUs).

The antennae of phototrophic organisms are built to capture light. The absorption spectra of chlorophylls *a*, *b*, *c*, *d*, and *e*, and of bacteriochlorophyll have two bands, one in the red and another in the blue (blue-green), the precise peak depending on the chlorophyll type, and on its position in the light-harvesting chlorophyll complexes. In addition, accessory photosynthetic pigments that are not part of the reaction centers, such as phycobiliproteins, some carotenoids and xanthophylls are integral components of the light-harvesting antennae in

different taxa, and broaden the spectrum of light available for photosynthesis (Jeffrey et al., 1997).

Photoacclimation and photoadaptation are the responses of phototrophs to changes in light intensity. By nearly universal usage, photoacclimation consists of phenotypic adjustment within the boundaries of the genotype, whereas the term adaptation is currently reserved for evolutionary or selective changes in organism's genotypes. Photoacclimation is accomplished rather rapidly; on hours to few days time scales, in the same cell, while adaptation requires generations and may take years to millennia. Transferal to low light results in an increase in the cellular concentrations of photosynthetic pigments, and thereby of the total light absorbed by the organisms. Such increase in pigmentation is a self limiting strategy, since the increase in pigment concentration invariably enhances the intracellular self shading and cross-section overlap, the so-called "packaging effect," thereby decreasing the *in vivo*, chlorophyll *a* specific, spectrally averaged, effective optical cross section, a^* (Berner et al., 1989). Additional facets of low-light photoacclimation are the reduction in the light-saturated photosynthesis, P_{\max} , and respiration rates (Falkowski and Owens, 1978).

In total, extended darkness, phototrophs can survive by extreme reduction of metabolism in the cold, where respiration is extremely reduced. Only the subzero temperatures in the circumpolar waters allow for the survival of phytoplankton that will initiate the spring bloom as days lengthen (Tilzer and Dubinsky, 1987). An additional, common mechanism is the formation of resistant cysts (dinoflagellates), spores (diatoms and chlorophytes) or akinetes (cyanobacteria) all of these capable of surviving for months and decades in the dark.

However, in the nondormant state some light is essential to support life processes, unless the organism is capable of switching to heterotrophy, or at least operate as an obligate or facultative mixotroph. Overmann and Garcia-Pichel (2005), based on physiological parameters, calculated a minimum irradiance of $2 \mu\text{mol q m}^{-2} \text{ s}^{-1}$ for survival of phototrophic cells. In this review we shall present a gallery of phototrophs "living on the edge," including anoxygenic phototropic bacteria, living below this threshold by exploiting unique opportunities present in their environment by means of unusual physiological mechanisms and devices.

Obviously, life in the twilight zone is opted for only when factors other than light are more favorable there than in more illuminated niches. These may be nutrient availability, temperature, competition pressure, and predation.

2. Low-Light Photoacclimation

The photoacclimation process allows plants to adjust to changes in light intensity in order to avoid photodynamic damage under strong light, while sustaining life processes in deep shade. This process is of paramount importance for phytoplankton as vertical mixing exposes cells to orders of magnitude changes in irradiance. The dynamic photoacclimation process takes place on the time

scale of hours to days, however, in the case of the organisms discussed in the present work, a permanent feature of all organisms, superimposed on inherited properties. As far as the mechanisms and eventual results in the case of organisms living in semidarkness, photoacclimation and photoadaptation are undistinguishable.

The photoacclimation of phytoplankton consists of a coordinated series of adjustments on the ultrastructural, biophysical, biochemical, and physiological levels. These changes result in maximization of harvesting of scant impinging photons, their efficient utilization with parsimonious allocation to respiration, allowing survival and growth in otherwise too dark niches. Photoacclimation to low light involves increases in light-harvesting pigments, including the various ubiquitous chlorophylls, phycobilins in cyanobacteria and rhodophytes, and light-harvesting carotenoids such as peridinin in the dinoflagellates and fucoxanthin in chromophytes. This enhanced absorptivity increases the flow of excitation energy to reaction centers. Concomitantly photoprotective pigments like β carotene and astaxanthin as well as the light dissipating pigments associated with the xanthophylls cycle, all decrease, minimizing waste of precious light energy. The increases in pigmentation are accomplished in coordination with an increase in thylakoid area, providing the needed space to accommodate pigments and light-harvesting proteins (Berner et al., 1989). These changes in absorptivity are self-limiting, since further increases in pigmentation would result in pigment molecules within the cell being shaded by the outer ones, thus even > 200 fold decreases in ambient light, at most, led to a $\times 12$ increase in pigmentation. The low light induced raise in light harvesting is accompanied by major improvement in the efficiency of light utilization, namely, Φ , the quantum yield of photosynthesis, as energy dissipation processes are reduced (Dubinsky et al., 1986, Dubinsky, 1992).

3. Deepest Record of Seaweeds

Littler et al. (1985) found photosynthesizing coralline red macroalgae (not described) on a seamount at 268 m in San Salvador Island, Bahamas. Littler et al. (1985, 1986) described a four-zone assemblage on that seamount over the depth range of 81–268 m: a) *Lobophora* (Phaeophyta) dominated group at 81–90 m; b) *Halimeda* (Chlorophyta) group at 90–130; c) *Peyssonnelia* (Rhodophyta) group 130–189 m; and d) an unidentified crustose coralline zone 189–268 m (Fig. 1).

In addition, the chlorophyte, rock boring alga *Ostreobium* sp., was found from 210–240 m. *Ostreobium* is common as a borer in coral skeletons and other marine aragonite substrata (see below for endolithic algae). At the Lee Stocking Island, Bahamas a Corallinales/*Peyssonnelia* group was abundant from 60 to 120 m, where below 90 m it shared dominance with the chlorophyte *Ostreobium*. In that study this was the only alga observed below 150 m and remained

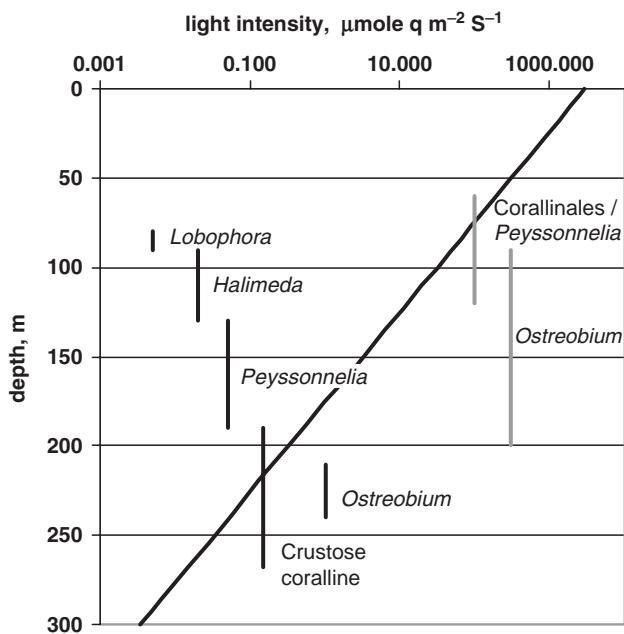


Figure 1. Light intensity and seaweed distribution in the San Salvadore Island, Bahamas based on Littler et al., (1985) and At the Lee Stocking Island, Bahamas (based on Aponte et al., 2001).

abundant below 200 m (Aponte and Ballantine, 2001), Fig. 1. Seaweeds were recorded at 200 m near Hawaii (Doty et al., 1974) and in Jamaica, crustose red and filamentous green algae were found at 175 m (Lang, 1974) and in other reef area (see Littler et al., 1986).

It should be noted that all deepest records of seaweeds are associated with tropical oligotrophic waters, Case 1 waters, as defined by Morel and Prieur (1977), and Jerlov's (1976) type II. These low nutrient "blue deserts" support very little phytoplankton growth resulting in very transparent clear water in which the 1% depth may be as deep as 150 m ($K_d = 0.03 \text{ m}^{-1}$, (Kirk, 1994)). In these seas and such depths it is mostly the blue wavelengths that penetrate (Fig. 2).

The coralline red macroalgae at 268 m algae are exposed to 0.015–0.025 $\mu\text{mol q m}^{-2} \text{s}^{-1}$ during full sunlight at its maximum zenith, which amounts to only ~0.0005–0.001% of subsurface light (Littler et al., 1985, 1986). The physical support provided by cell wall calcification presumably allows coralline algae to develop extremely thin tissues that can be fully illuminated by whatever light reaching them, thus enabling them to live at extreme depths in the water column (Littler et al., 1985, Chisholm, 2003).

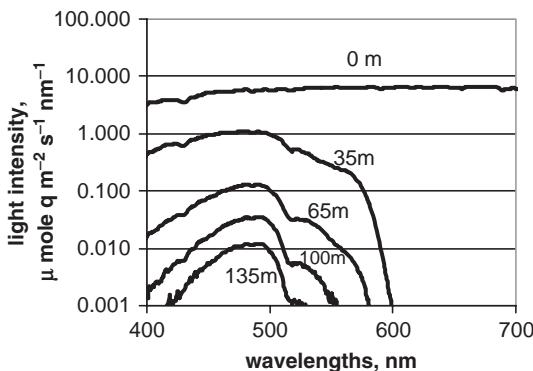


Figure 2. Spectra of downward irradiance at 6 depths at the Atlantic ocean during summer DCM located at about 100 m, (Stambler, unpublished data).

4. Deep Chlorophyll Maximum

The deep chlorophyll maximum (DCM) occur at depths below 50 m, and in tropical, temperate Atlantic and Arctic waters, usually at 80–150 m (Kiefer et al., 1976, Estrada et al., 1993, Owrid et al., 2000). The DCM is found at the bottom of the photic zone, at light intensities of 0.1–1% of subsurface light. At that depth it is related to depletion of one or more of the major inorganic nutrients (nitrogen, N; phosphorus, P; silicic acid, Si) in the overlying water column. Thus, the depth at which DCMs occurs coincides with the nutricline, where the concentration of the limiting nutrient element increases sharply with depth (Kiefer et al., 1976), Fig. 3.

Prochlorococcus (chloroxybacteria), which is the most abundant photosynthetic organism in the oceans, dominates the DCM (Partensky et al., 1999). That organism is one of the smallest picophytoplankters in the ocean with volume of only 0.11–0.38 μm^3 (0.6–0.9 μm). It is smaller than the ubiquitous *Synechococcus*, 0.52 μm^3 (1 μm), and much more so than the smallest eukaryotic algae (4–10 μm). The small size of *Prochlorococcus* is an advantage under oligotrophic conditions, as high surface-to-volume ratios give it a distinct edge for nutrient uptake (Chisholm, 1992), as well as for maximizing light harvesting per unit pigment (Kirk, 1986, 1994). These algae that also have the capability of utilizing nitrite, are “locked” between the light and nutrient gradients typical of oligotrophic waters during stratification (Fig. 3).

Low-light-adapted genotypes preferentially thrive under dim light at the bottom of the euphotic zone (80–200 m), but in a relatively nutrient-rich environment (Dufresne et al., 2003). *Prochlorococcus* populations grow in the lowest part of the photic zone, where light is enriched in the blue domain. It is adapted to absorbing the blue part of the spectrum by means of its photosynthetic apparatus consisting of Chl a_2 and Chl b_2 (which are unique to this genus), divinyl

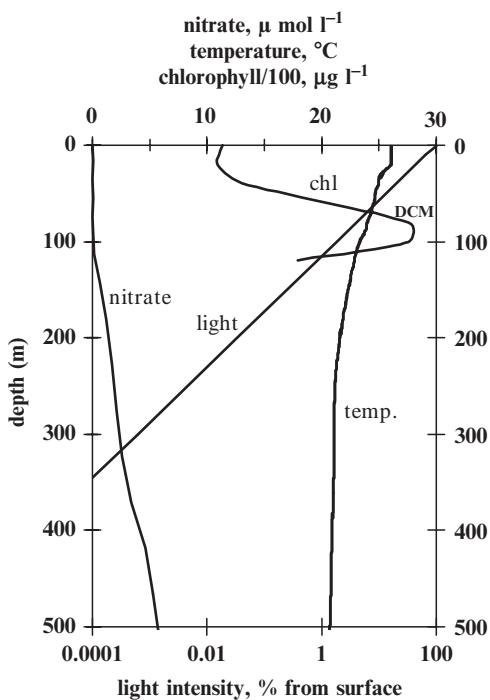


Figure 3. Temperature, salinity, nitrate, and light percent as a function of depth at the Gulf of Eilat (Aqaba) during summer (Stambler, 2006).

chlorophylls *a* and *b*, zeaxanthin, *a*-carotene, and small amounts of a Chl *c*-like pigment possibly Mg, 3–8 divinyl phaeoporphyrin *a*₅, (Partensky et al., 1999). *Prochlorococcus* lacks phycobilisomes, the large extrinsic multisubunit light-harvesting complexes found in typical cyanobacteria. These complexes are replaced by Chl *a*₂-*b*₂-binding proteins called Pcb's, which are analogous in function, but are structurally and phylogenetically distinct from the light-harvesting complexes of higher plants (Dufresne et al., 2003, Steglich et al., 2003). Phycourobilin is the major chromophore in low-light-adapted *Prochlorococcus* ecotypes (Steglich et al., 2005). It possesses divinyl chlorophyll *a* and *b* that are bound to distinct chlorophyll *b*-binding proteins, the major antenna proteins. These proteins form a concentric ring around photosystem I (Bibby et al., 2001) and have functionally fully replaced phycobilisomes as the light-harvesting system in this group (see Steglich et al., 2003).

The maximum of the absorption spectrum of *Prochlorococcus* ranges from 440 to 480 nm where the probability for photons of being absorbed by its cell exceeds that of being scattered (Morel et al., 1993). The *Prochlorococcus* absorption capabilities per chlorophyll unit are not far from being maximal, due to the small size of their cells thereby reducing the package effect (Morel et al., 1993).

5. Survival in the Dark

Phototrophs find themselves in darkness for periods varying from hours to days, as a result of mixing and water mass movement. When different phytoplankton species were exposed to prolonged darkness of 10 to 12 days, two distinct types of metabolic response to darkness were described. *Brachiomonas submarina* (Chlorophyta), *Pavlova lutheri* (Prymnesiophyta), and *Chrysosochromulina hirta* (Prymnesiophyta) adapted by reducing their metabolism to a lower level of activity within few days, whereas *Prymnesium parvum* (Prymnesiophyta), *Bacteriastrum sp.* (Bacillariophyta) and an unidentified pennate diatom continued respiration at unchanged rates. Neither of the studied species displayed signs of resting spore formation (Jochem, 1999). It is not clear for how long these algae could survive.

In another experiment by Murphy and Cowles (1997) *Thalassiosira weissfloggii* survived 2 months of darkness without spore formation and commenced exponential growth upon re-illumination. They suggest that both the photochemical apparatus and biochemical carbon oxidation pathways remained functional and > 80% of cells remained viable. From measurements of particulate organic carbon they further assumed that *T. weissfloggii* utilized organic carbon during dark survival (Murphy and Cowles, 1997).

6. Polar Algae

Each winter, polar marine microalgae face total darkness for periods of up to 6 months. Zhang et al., (2003) show that after 161 days in the dark, species dominance in the algal assemblage shifted from pennate diatoms to small phytoflagellates. The mean algal growth rate was 0.01 d^{-1} and while diatom species had negative growth rates, phytoflagellate abundance increased. Resting spore formation during the dark period was observed in less than 4.5% of all cells and only for dinoflagellates and the diatom *Chaetoceros* spp. The authors assume that facultative heterotrophy and vernal energy storage are the main processes enabling survival during the dark Arctic winter (Zhang et al., 2003). As little as two hours of daylight suffice to initiate the phytoplankton bloom, mainly due to the low respiration rates in the still cold waters, -2.5°C (Tilzer and Dubinsky, 1987).

The acclimation response of the chloroplast was studied in the green marine microalgae *Koliella antarctica* exposed to a simulated austral night of 90 days. During treatment, the organism underwent substantial structural and functional reorganization of the plastid, resulting in the formation of a chlorochromoplast-like structure, suitable for the storage of products and components coming from the breakdown of the preexisting plastid constituents (Baldisserotto et al., 2005).

In the austral spring, at the onset of the bloom of microalgae, irradiance under the ice is generally less than 0.1–1% of irradiance incident on the surface. Despite the limiting light, standing stocks of sea ice microalgae peaked at 10^9 cells m^{-2} ($300\text{ mg chl }a\text{ m}^{-2}$). Among these algae, the prymnesiophyte *Phaeocystis*

pouchetti and diatoms were found to be highly shade adapted, with photosynthesis saturating at 2–10 $\mu\text{mol q m}^{-2} \text{s}^{-1}$ (see Soohoo et al., 1987).

7. Ice Coralline Algae

In Antarctic waters, following the long winter of total darkness, days lengthen and the algal spring bloom is triggered. At the beginning of the growth season, the algae are below a thick layer of sea ice. The ice strongly reflects and attenuates the light reaching the algae; in addition, there might be wind-blown snow on top of the ice that further reduces and modifies the light reaching the algae (Samsonoff and MacColl, 2001).

Coralline algae covered 4–60% of the seafloor from water depths of 13–26 m in the McMurdo Sound (Ross Sea) in Antarctica. At this site algae are covered by 2.5 m of sea-ice through which only ~0.5% of incident irradiance penetrates, amounting to below 2 $\mu\text{mole q m}^{-2} \text{s}^{-1}$. The coralline algal crusts showed light-saturated rates of photosynthesis from 9.4 to 20 mmole $\text{O}_2 \text{ m}^{-2} \text{ thallus d}^{-1}$ and dark respiration rates from 0.15 to 0.96 mmole $\text{O}_2 \text{ m}^{-2} \text{ thallus d}^{-1}$. The average irradiance at which the onset of light saturated photosynthesis occurred (E_k) was ~3 $\mu\text{mole q m}^{-2} \text{s}^{-1}$. The light compensation for photosynthesis (E_c) was estimated as 0.10 $\mu\text{mole q m}^{-2} \text{s}^{-1}$ (Schwarz et al., 2005). It is noteworthy for comparison that in shallow water reef-building crustose coralline algae E_k is ~30–200 $\mu\text{mole q m}^{-2} \text{s}^{-1}$ (Chisholm, 2003).

In ice covered periods, irradiance at 20 m depth averaged 1 $\mu\text{mole q m}^{-2} \text{s}^{-1}$, with daily maxima of 2–3 $\mu\text{moles q m}^{-2} \text{s}^{-1}$. During the open water season, E_d at 20 m depth averaged 7 $\mu\text{mole q m}^{-2} \text{s}^{-1}$ with daily maxima of 30 $\mu\text{mole q m}^{-2} \text{s}^{-1}$. The crustose coralline algae *Phymatolithon foecundum* and *Phymatolithon tenue* from NE Greenland are low light adapted, with compensation irradiances (E_c) averaging 0.7–1.8 $\mu\text{mole q m}^{-2} \text{s}^{-1}$ and (E_k) values averaging 7–17 $\mu\text{mole q m}^{-2} \text{s}^{-1}$ (Roberts et al., 2002).

The Antarctic red alga *Phyllophora antarctica* grows under ice in cold dim light conditions. These algae have in addition to chlorophyll *a* a unique combination of biliprotein pigments which are: R-phycoerythrin IV, phycocyanin and allophycocyanin. Light energy absorbed by the biliproteins transfers to chlorophyll and then to the photosynthetic reaction center where it turns into chemical energy. The R-phycoerythrin of *P. antarctica* has absorption bands at 565, 545, and 495 nm. The 565 and 545 bands are from phycoerythrobilin and 495 nm from phycourobilins.

R-phycoerythrin has the lowest ratio of phycoerythrobilin to phycourobilins ($A_{545\text{nm}}/A_{495\text{nm}} = 0.7$, $A_{565\text{nm}}/A_{495\text{nm}} = 0.99$). This unique absorption spectrum gives the alga an improved ability to harvest blue light, which may enhance its survival in its blue, light-deprived, habitat (Samsonoff and MacColl, 2001). *P. antarctica* and *Acaryochloris marina* both live in very low light and have in addition to chlorophyll phycocyanin and allophycocyanin (Marquardt et al., 1997, Samsonoff and MacColl, 2001).

8. Zooxanthellae Corals Growing in Deep Water

Reef building, or hermatypic (Schumacher and Zibrowius, 1985) corals harbor in their tissues endocellular dinoflagellate microalgae, the zooxanthellae (Brandt, 1883). The symbionts provide by their photosynthesis a major fraction of the metabolic requirements of the animal host, which decreases as light becomes limited with depth (Muscatine, 1990). This dependence on photosynthesis sets depth limits to zooxanthellate corals restricting most species to the photic depth, with reef growth and species diversity declining with light. The photic (also euphotic, trophogenic) depth is usually set at the 1% of subsurface light. The platelike coral *Leptoseris fragilis* grows in the upper twilight zone between 95–145 m depth. This is in contrast to other hermatypic corals and reefs which peak in the upper 30 m and decrease in cover and diversity until their total disappearance at about 80–100 m. On the average, *L. fragilis* grows when exposed to maximum light intensity of 0.5–10 $\mu\text{mole q m}^{-2} \text{s}^{-1}$ (at noon time) for example, 0.15–1.7% of surface light. Due to absorption by water and other components, in addition to the exponential decrease of light intensity, the irradiance spectra shifted towards the blue with depth. Most of the irradiance reaching *L. fragilis* is in the short wavelengths of the spectrum (380–500 nm) (Schlichter and Fricke, 1991).

The saturating light intensity, E_k , for *L. fragilis* zooxanthellae is 10–20 $\mu\text{mole q m}^{-2} \text{s}^{-1}$ (Schlichter and Fricke, 1991) compared to 400 $\mu\text{mole q m}^{-2} \text{s}^{-1}$ needed for zooxanthellae from shallow water to reach maximum photosynthesis. The compensation light intensity, the intensity when respiration equals photosynthesis, was 2–5 $\mu\text{mole q m}^{-2} \text{s}^{-1}$ (Schlichter and Fricke, 1991). This is up to ten times lower than the light intensity needed for compensation by shade adapted *Stylophora pistillata* (Porter et al., 1984). In addition to the flat like shape of the coral, large intercellular spaces in the oral epidermis facilitate light penetration to the algae that are found only in the coral gastrodermis (Schlichter and Fricke, 1991). The fluorescent granules in this species were found in gastrodermal chromatophores underlying the symbiotic algae. These granules contain pectinoporine pigments and GFP (green fluorescent proteins) and may function as photoreceptors and also support photosynthesis by converting UV radiation into visible light (Bassham et al., 1978). Photoreceptors to sense blue moon light were found in the host animal of symbiotic corals. However, so far these were only implicated in the timing of spawning. The threshold of the photoreception sensitivity is as low as $\sim 1.2 \times 10^{15}$ quanta $\text{m}^{-2} \text{s}^{-1}$ ($\sim 0.002 \mu\text{mole q m}^{-2} \text{s}^{-1}$) (Gorbunov and Falkowski, 2002).

9. Endolithic Algae Within Corals

Endolithic algae are algae that live within skeletal material, or nonliving calcareous matrices. A unique situation is of endolithic algae living in the skeleton of the scleractinian corals (Odum and Odum, 1955), see (Shashar and Stambler, 1992).

It is noteworthy that in terrestrial environments algae have been reported to photosynthesize under very low light under flint stones in deserts (Berner and Evenari, 1978).

These endolithic filamentous algae, usually siphonaceous chlorophytes, are of the genus *Ostreobium* (Jeffrey, 1968). These algae can appear as dense green bands or zones within the coral skeleton, underneath the living animal tissue (Odum and Odum, 1955, Halldal, 1968, Jeffrey, 1968) or can be found throughout the perforate skeleton as is the case in the coral *Porites compressa* (Shashar and Stambler, 1992). Light intensity inside the coral's aragonite skeleton is extremely low, and only about $1 \text{ } \mu\text{mole q m}^{-2} \text{ s}^{-1}$ is actually available for the endolithic algae (Halldal, 1968, Shibata and Haxo, 1969). These survive at such low light intensities having adapted to function in light highly enriched in far-red wavelengths unused by the zooxanthellae (Halldal, 1968, Fork and Larkum, 1989). The low light limits the photosynthesis of the endolithic algae (Kanwische and Wainwrig, 1967) restricting both respiration and photosynthesis of the endolithic algae to the extremely low $\sim 3.5\%$ of the total metabolism of the coral colony (Shashar and Stambler, 1992).

It should be noted that some endozoic algae, Chromophyta (mainly belonging to the dinoflagellates) and some chlorophytes, found within shelled gastropods in shallow parts of Red Sea coral reefs are exposed to much higher light. These algae are found in the hepatopancreas and gonad tissues that are permanently concealed within the upper whorls of the shell. Only 5–15% of the incident light that reaches the shell penetrates it. Since these gastropods, for example, *Strombus tricornis*, live in very shallow water at depth of up to 0.5 m, the intensity of the light reaching the algae is $50\text{--}100 \text{ } \mu\text{mole q m}^{-2} \text{ s}^{-1}$ (Berner et al., 1986a,b).

Likewise, the diploid, sporophyte *Conchoecelis* phase of the rhodophyte *Porphyra*, bores into mollusk shells, where light intensity is bound to be very low, however, no data are available (Cole and Sheath, 1990).

10. Symbiotic Algae in Sponges

Symbiotic cyanobacteria and unicellular algae in sponges, such as *Theonella swinhonis* and *Clionavastifica* sp. from the Red Sea grow under dim light ranging from 5 to $50 \text{ } \mu\text{mole q m}^{-2} \text{ s}^{-1}$ (Beer and Ilan, 1998). The photosymbionts are restricted to sponge surface cell layers where these are exposed to maximum light (Beer and Ilan, 1998). The symbiotic algae photosynthesize and contribute towards the sponge's energy needs (see Beer and Ilan, 1998, Steindler et al., 2002). In the Mediterranean Sea the sponges *Cliona viridis* and *Cliona nigricans* associate with dinoflagellate symbionts adapted to low light. These bioeroding sponges live cryptically within calcium carbonate substrates, including live coral skeletons, which absorb and reflect most incident light. In both sponges the dinoflagellates reach light compensation and saturation at similar light levels with means close to 10 and $30 \text{ } \mu\text{mole q m}^{-2} \text{ s}^{-1}$, respectively, indicative of high efficiency of light capture and low respiration rates (Schonberg et al., 2005).

11. Symbiotic Algae and Chloroplasts in Foraminifera

Symbiont-bearing foraminifera populate the euphotic zone, where the symbionts are exposed to light levels sufficient for photosynthesis. The planktonic foraminifer *Orbulina universa* has a swarm of hundreds of dinoflagellate endosymbionts that show a diurnal migration pattern. During the day, the dinoflagellates spread out on the rhizopodial network between the spines, while at night they withdraw into their shells. No significant self-shading of the dinoflagellate cells inside the swarm was observed. Light saturation irradiances (E_k) were 75 and 137 $\mu\text{mole q m}^{-2} \text{ s}^{-1}$, in two specimens that were studied in detail (Rink et al., 1998). The benthic foraminifer, *Nonionella stella*, retains (plastids) chloroplasts. Down to the depth of 600 m, where as little as one photon every 120 sec per PSU might be available. Nevertheless, the two carboxylating enzymes RuBisCO and phosphoenol pyruvate carboxylase retained their catalytic activity. The majority of the pigments found in *N. stella* chloroplasts are associated with proteins to form complexes containing Chl *a*, *c*, fucoxanthin and the chlorophyll degradation products, chlorophyllide *a* and pheophytin. Based on spectroscopic data it was suggested that the plastids are capable of energy transfer given a photon source. They examined the potential for primary charge separation using fast repetition rate fluorometry and found that the enslaved plastids retained photochemical activity. It is highly unlikely that the interval between photon arrivals would allow the system to ever proceed beyond S3. This implies that the plastids are unlikely to function photoautotrophically, and it was proposed that they are involved in nitrogen metabolism (Grzymski et al., 2002).

12. *Acaryochloris*-like Phototrophs

The *Acaryochloris*-like cyanobacterium is a unique phototroph living underneath minute coral-reef didemnid ascidians in Heron Island reef, the Great Barrier Reef, forming biofilms between the animal and the substrate. They thrive as free-living organisms in a shaded niche, enriched in near infrared (NIR). This group of organisms may be found in many localities and sites around the world. A similar, *Acaryochloris*-like organism grew in enrichments from an artificial, eutrophic, saline lake in California (USA). *Acaryochloris marina* exploits light environments depleted of visible radiation and enhanced in NIR. It was found growing as an epiphyte on the stripes of certain red algae in Japanese waters (Larkum and Kuhl, 2005). *Acaryochloris* like phototrophs use chlorophyll *d* (Marquardt et al., 1997) as their principal light-harvesting pigment, rather than chlorophyll *a*, the form commonly found in plants, algae, and other cyanobacteria (Kuhl et al., 2005). Chl *d* has a light-harvesting role and might also replace Chl *a* in the special pair of chlorophylls in both reaction centers of photosynthesis (Larkum and Kuhl, 2005). PS II, in *A. marina*, utilizes Chl *d* and not Chl *a* as primary electron donor and that the primary electron acceptor is one of two molecules of pheophytin *a* (Chen et al., 2005).

Far-red light penetrated efficiently through the ascidian tissue, and was enhanced relative to the incident light owing to the light harvested by the exosymbiont *Prochloron didemni* residing in the ascidians (Lewin and Withers, 1975) overlaying them (Larkum and Kuhl, 2005). Under all ascidians, visible light (VIS) was strongly depleted resulting in a 10–20 fold increase in the NIR/VIS ratio. This provides an ideal niche for *Acaryochloris*-like phototrophs with a maximum absorption at 700–720 nm of chlorophyll *d*. Even though *Acaryochloris*-like cells thrive in nature in extreme shade, they are capable of acclimation to strong light as when they were exposed experimentally to the relatively high light of 585 $\mu\text{mole q m}^{-2} \text{ s}^{-1}$ (Kuhl et al., 2005).

13. Green Sulfur Bacteria

Green sulfur bacteria are anaerobes that utilize light for growth by the oxidation of sulfur compounds to reduce CO_2 to organic carbon, and are capable of photosynthetic growth at extremely low light intensities. Chlorobiaceae are obligate anaerobic phototrophic prokaryotes that exist both as benthic and as planktonic forms. They contain bacteriochlorophylls (BChls) *c*, *d*, or *e* as their main light-harvesting pigment, while chlorobactene and isorenieratene are the main carotenoids in green- and brown-colored species, respectively (Blankenship et al., 1995, Airs et al., 2001). The BChl molecules are located inside the chlorosomes, cigar-shaped vesicles attached to the inner side of the cytoplasmic membrane, forming rod-like structures that result from spontaneous aggregation by chromophore–chromophore coupling without the involvement of proteins (Holzwarth et al., 1992, Airs et al., 2001). Interactions between the bacteriochlorophylls themselves govern the absorptive properties of the photosynthetic antennae in green sulfur bacteria (Blankenship et al., 1995). The dense packaging of BChls allows optimal functioning of the chlorosomes as antenna complexes under low light intensities (Olson, 1998). Including some that grow at 80 m below the surface of the Black Sea. One of these isolates, the Green sulfur bacterial strain MN1 grows at light intensities as low as 0.25 5mole quanta $\text{m}^{-2} \text{ s}^{-1}$ (Overmann et al., 1991). These bacteria trap every single photon reaching them, and as such are among the most efficient photosynthesis phototrophs known (Bohannon, 2005).

14. Green Sulfur Bacteria from Deep-Sea Hydrothermal Vents

Deep-sea hydrothermal vents, such as black smokers are associated with mid-ocean ridges at the bathyal to abyssal depths of 1500–4000 m, far below the photic zone in the oceans (Van Dover, 2000). Around them a unique microbial and invertebrate ecosystem exists, living off organic material generated by CO_2

reduction by chemotrophic bacteria that oxidize inorganic compounds emanating from vents (Van Dover, 2000, Beatty et al., 2005).

In addition, it has been recently suggested that some of the organisms actually are “phototrophs.” In general the geothermal light intensity detected at hydrothermal vent peaks at wavelengths in excess of 700 nm, are likely to be thermal or blackbody radiation (VanDover et al., 1996, Van Dover, 2000, White et al., 2002, Beatty et al., 2005, Marris, 2005). Radiation emitted from 350°C blackbody heat source peaks at long wavelengths in the mid- to far-infrared, but the tail of the spectrum extends into visible wavelengths, as far as 400–650 nm. Digital imaging and photometric data confirm that most of the light produced by the black smoker is indeed in the near infrared (750–1050 nm) however, there is a small but detectable flux at shorter wavelengths (VanDover et al., 1996, Van Dover, 2000). But the source of light cannot be explained only by the thermal radiation from the vents alone as too much of the light is in the visible part of the spectrum. This deep-sea illumination is too weak to be detected by the human eye even though it has a frequency well into the visible spectrum. The mechanism by which the vents generate this deep-sea illumination is not clear and remains a mystery (Bohannon, 2005, Marris, 2005). Possible suggested sources include sonoluminescence, in which imploding bubbles emit a brief flash of light, sulfide oxidation by bacteria (Marris, 2005), crystalloluminescence, whereby light emitted as the 2°C seawater encounters the 350°C brine, causing dissolved minerals to crystallize and drop out of the solution, or chemiluminescence, in which energy is released by chemical reactions in the vent water (Reynolds and Lutz, 2001).

Indeed it has been demonstrated that the photon flux emitted by high temperature fluids is sufficient to support facultative photosynthetic bacteria using bacteriochlorophyll or other long wavelength light capturing pigment systems (VanDover et al., 1996, Van Dover, 2000). An obligatory aerobic anoxygenic photosynthetic bacterium has been isolated from black smoker plumes. These bacteria were found to be facultative phototrophs under culture and contain bacteriochlorophyll *a* with an *in vivo* absorption maximum at 867 nm (Yurkov and Beatty, 1998).

Recently, Beatty et al. (2005) reported the discovery of a previously unknown green sulfur bacterial species, GSB1, from a deep-sea hydrothermal vent, where the only source of light is geothermal radiation. The *in vivo* absorption spectrum of the hydrothermal vent bacteria was found to be similar to that of all green sulfur bacteria, with a major peak at 750 nm, indicating the presence of light-harvesting bacteriochlorophyll (BChl) *c*, and absorption in the 450 nm region due to a BChl Soret band and light-harvesting carotenoid pigments. The *in vivo* fluorescence emission spectrum contained a major peak at 775 nm, indicative of BChl *c*. The GSB1 pigments were extracted into an organic solvent, resolved in HPLC, and determined to be very similar to the pigments of a *Chlorobium tepidum* control. On the basis of absorption fluorescence spectra and HPLC elution times the major chlorophylls of GSB1 are BChls *c*, and mass spectrometry indicated that the major carotenoid is chlorobactene (Beatty et al., 2005).

The photon flux at 750 ± 50 nm (corresponding to the long wavelength absorption peak of light-harvesting BChl *c* in GSB1) at the orifice of a 370°C black smoker was 108 photons $\text{cm}^{-2} \text{ s}^{-1} \text{ sr}^{-1}$; the flux in the 400–500 nm range (short wavelength BChl and chlorobactene absorption peaks) was 104 photons $\text{cm}^{-2} \text{ s}^{-1} \text{ sr}^{-1}$ (6×10^{13} photons $\text{cm}^{-2} \text{ s}^{-1} = 1 \text{ mole q m}^{-2} \text{ s}^{-1}$). Within 1–2 cm of 332°C flange pools on black smoker chimneys, the total photon flux ($\sim 10^{11}$ photons $\text{cm}^{-2} \text{ s}^{-1} \text{ sr}^{-1}$) over the 600–1,000 nm range was estimated to be of the same order of magnitude as the solar photon availability for a green sulfur bacterium living at 80 m depth in the Black Sea (Beatty et al., 2005).

Beatty et al. (2005) conclude in their paper “This discovery expands the range of possible environments that could harbor life forms which use light energy to drive endergonic biochemical reactions and frees the thinking of the scientific community from the constraint that any form of life that depends on light energy is necessarily limited to solar illuminated habitats.”

15. Summary

1. Phototrophs of diverse taxa successfully colonize extensive dim light marine domains.
2. Their carbon assimilation depends on extremely efficient light harvesting and utilization.
3. In some cases there is an indication of capability to utilize UV and IR, rather than visible light, as sources of energy for carbon assimilation.

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PART 3: PHOTOTROPHS IN THE MARINE ENVIRONMENT

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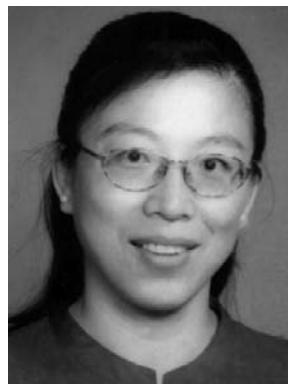
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BIOLOGY OF THE CHLOROPHYLL D-CONTAINING CYANOBACTERIUM *ACARYOCHLORIS MARINA*

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1. Introduction

Oxygenic phototrophs (cyanobacteria, algae and higher plants) primarily absorb solar energy in the visible spectral (400–700 nm) region by use of various chlorophylls, while anoxygenic phototrophs are bacteria, which can absorb infrared wavelengths (>700–1100 nm) by use of different bacteriochlorophylls (Overmann and Garcia-Pichel, 2004). Each of the groups also has a variety of characteristic antenna pigments and other accessory pigments that can enhance light capture and/or provide protection against excess actinic light and UV-radiation in specific habitats. However, amongst these broadly defined groups there are outlier organisms exhibiting atypical photopigmentation. Amongst the oxygenic phototrophs, the most conspicuous are:

1. The endolithic green alga *Ostreobium* sp. that inhabits coral skeletons and thrives under extreme shade below the coral tissue due to possession of a special Chl *a* antenna absorbing in the far-red region around 700–730 nm (Halldal, 1968; Fork and Larkum, 1989; Koehne et al., 1999), that is a region of the solar spectrum which is not absorbed by the overlying coral tissue (Magnusson et al., 2007);
2. The prochlorophytes present three independent lineages of cyanobacteria, that is the genera *Prochlorococcus*, *Prochlorothrix* and *Prochloron*. *Prochlorococcus* contains unique divinyl-Chl *a* and divinyl-Chl *b* photopigments and only minor amounts of phycobiliprotein (PBP) pigment, while *Prochlorothrix* and *Prochloron* are the only prokaryotes containing Chl *b* (Partensky and Garczarek, 2003).

Prochloron, which lives mainly as an exosymbiont in the outer test and exhalant canals of didemnid ascidians, has so far resisted any cultivation attempt (Kühl and Larkum, 2002). However, during one such attempt to isolate *Prochloron* from ascidians, the perhaps most unique oxygenic phototroph was cultivated by a Japanese group (Miyashita et al., 1996), viz. the Chl *d*-containing cyanobacterium *Acaryochloris marina* (Miyashita et al., 2003). In this review we summarize the

current knowledge about *A. marina* including a brief account on the discovery and properties of Chl *d* (see also Larkum and Kühl, 2005).

2. Discovery of Chl *d* and *A. marina*

Chl *d* was first found in pigment extracts from red algae (Manning and Strain, 1943). The chemical structure of Chl *d* is only different from Chl *a* by the presence of a 3-formyl group, which replaces the vinyl group on ring I (Fig. 1) (Holt and Morley, 1959). But this structural change causes a pronounced red-shift of the long-wavelength absorption maximum (Q_y) of Chl *d* by about 30 nm relative to Chl *a* (Fig. 1), that is into the near-infrared (NIR) spectral region with an *in vivo* absorption peak at 710–720 nm (Chen et al., 2002a). However, after its discovery the new chlorophyll could not be assigned to a specific organism and it was shown that Chl *d* could also be formed as an intermediate byproduct from other chlorophylls during pigment extraction (Holt, 1961). Consequently, the biological relevance of Chl *d* remained unresolved until the discovery of a Chl *d*-containing microorganism in 1996 (Miyashita et al., 1996).

This microbe was first isolated from didemnid ascidians, when a Japanese research group attempted to isolate the ascidian symbiont, that is the prochlorophyte *Prochloron*. While *Prochloron* resisted isolation, another pigmented microorganism was isolated, which turned out to contain large amounts of Chl *d*. The organism was named *A. marina* and analysis of its 16S rRNA gene later showed that it belonged to the cyanobacteria (Miyashita et al., 2003). This assignment has later been supported by additional phylogenetic analysis of genes encoding proteins such as the light-harvesting protein prochlorophyte Chl *a/b* (Pcb) (Chen et al., 2005b). Cultures of *A. marina* are easy to keep in the laboratory, and such cultures have been subject to detailed biochemical and photophysiological studies. Furthermore, the genome of *A. marina* is currently being sequenced (see <http://genomes.tgen.org/index.html>), and this will soon reveal a much more detailed picture of its phylogenetic position and functional characteristics.

3. Cell Biology of *A. marina*

A. marina is a unicellular non-motile cyanobacterium with a spheroidal/ellipsoidal shape, $1.8\text{--}2.1 \times 1.5\text{--}1.7 \mu\text{m}$ in size (Miyashita et al., 1996). It appears dull yellow-greenish under the microscope, and TEM shows the presence of 6–12 layers of thylakoid membranes arranged peripherally in the cells (Fig. 2). In contrast to other cyanobacteria (prochlorophytes aside) it does not have phycobilisomes (Miyashita et al., 1997; Marquardt et al., 2000), but has PBPs (see Section 4.1.1).

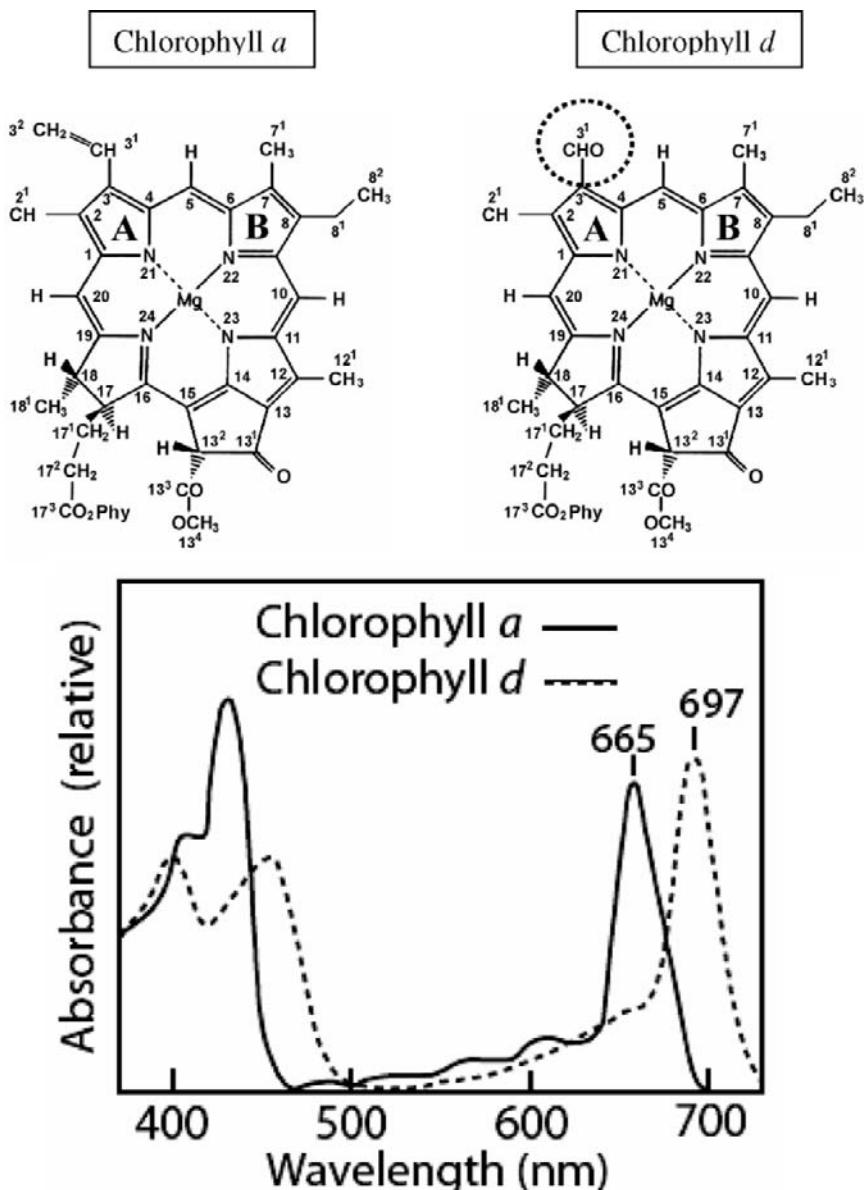


Figure 1. Structure of Chl *a* and Chl *d* and their in vitro absorbance spectra in acetone extracts. A divinyl group in ring A of Chl *a* is replaced by a formyl group in Chl *d* (dotted circle). This shifts the Q_y absorption maximum of Chl *d* about 30 nm towards the infrared relative to Chl *a*.

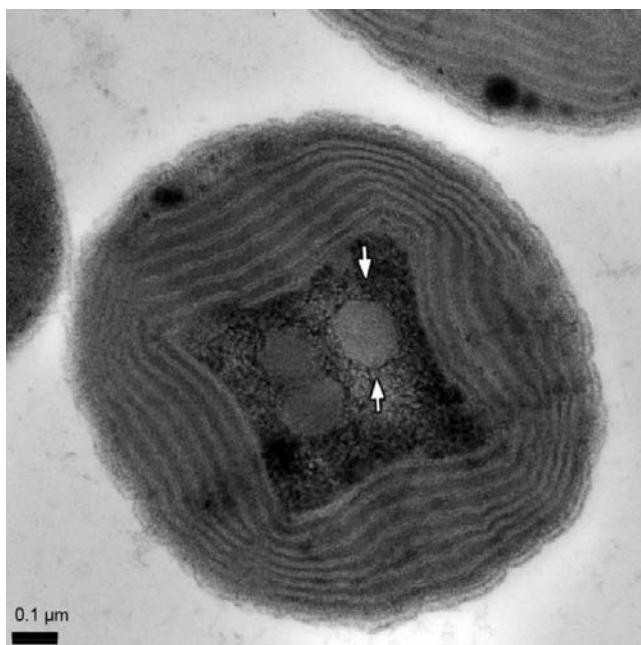


Figure 2. TEM picture of *A. marina*. Ultrathin section (~60 nm). Cells were fixed with glutaraldehyde (1%, 45 min), followed by potassium permanganate (1%, 10 min) and embedded in Spurr's resin. Arrows indicate the position of carboxysomes. Scale bar indicates 0.1 μ m (provided by Dr Martin Hofman and Prof. Robert E. Blankenship, Arizona State University).

Thylakoids in *A. marina* show appressed regions predominantly with PSI trimers (Chen et al., 2005c) and more separated thylakoid regions containing PSII and small rod-shaped PBP complexes (Marquardt et al., 1997; Hu et al., 1999). This lateral heterogeneity is not present in phycobilisome-containing cyanobacteria. The thylakoid membrane stacks in *A. marina* are perforated by channel-like structures connecting central and peripheral cell portions. This ultrastructural feature has not been found in other photosynthetic organisms, and its function is unresolved (Marquardt et al., 2000).

Like most other cyanobacteria, *A. marina* contains carboxysomes (Fig. 2), that is polyhedral inclusions in the cell containing ribulose-1,5-bisphosphate carboxylase/oxygenase (*Rubisco*), the key enzyme involved in inorganic carbon fixation. Whether *A. marina* possesses similar carbon concentration metabolism as other cyanobacteria remains to be shown. The *Rubisco* large subunit gene sequence (*rbcL*, AB065004) of *A. marina* shows high homology (83% identity) with other cyanobacteria. Investigation on metabolic pathways in *A. marina* is still at an early stage. Besides the unique photopigmentation, so far no other unique biochemical characteristics were detected in *A. marina* based on the limited information.

4. Photosynthesis Driven by Chl *d*

For decades, it was believed that Chl *a* is an essential chlorophyll in the reaction centres for oxygenic photosynthesis because it is the only chlorophyll that can form the special pair of chlorophylls needed to generate the redox potential span to split water into molecular oxygen. Bacteriochlorophylls can also form special pairs in the reaction centre but absorb longer wavelengths, that is lower energy radiation, and therefore the excited state energy and redox potential spans generated by those photopigments are significantly less than Chl *a* and only functional in anoxygenic photosynthesis.

A. marina has exchanged most of its Chl *a* with Chl *d*, both in its antenna and the reaction centres, allowing it to harvest far-red wavelengths (700–730 nm) for oxygenic photosynthesis. The exact *in vivo* absorption and fluorescence emission maxima of *A. marina* are known to show some variability. Schiller et al. (1997) showed the presence of various spectral forms of Chl *d* in *A. marina*, and showed that two phenotypes (one with low Chl *a* and another with somewhat higher Chl *a*) had different *in vivo* absorbance spectra in the far-red. Furthermore, Chen et al. (2002a) showed that variations in fluorescence emission maxima of *A. marina* depended on iron-availability. No unusual photochemical processes are detected for isolated Chl *d* molecules in solvent (Nieuwenburg et al., 2003) and this is inconsistent with the postulate that uphill energy transfer may occur between Chl *d*-antenna and a Chl *a*-reaction centre (Mimuro et al., 2000, 2004).

Chl *d*-containing organisms have puzzled biologists and the bioenergetic features of *A. marina* have been debated (Blankenship and Hartman, 1998). How and why does *A. marina* use Chl *d* as its major photopigment? The current evidence, provided by fluorescence resonance energy transfer (FRET) analysis, demonstrated that the coordination reaction of Chl *d*, that is the chlorophyll binding to the imidazole of histidine by the fifth coordination bond of Mg, is similar to that of Chl *a*, but not to that of Chl *b* (Chen et al., 2005d). The basic functional properties of the photosynthetic apparatus in *A. marina* are similar to Chl *a*-containing oxygenic phototrophs. There are two photosystems, PSI and PSII, and preliminary protein sequence data show that the polypeptide composition of both PSI and PSII complexes of *A. marina* are similar to that of well-known Chl *a*-containing analogues (Hu et al., 1998; Miyashita and Sasaki, 2001) despite its unique light-harvesting antenna systems.

4.1. PIGMENT COMPOSITION AND ANTENNA SYSTEMS OF *A. MARINA*

A. marina contains five photopigments of which Chl *d* is the major pigment amounting to up to 99% of the total lipid-soluble pigment of the cell and more than 2% of the cell dry weight. Chl *a* is a minor constituent and its quantity varies

with culture conditions from 1% to 10% of the total chlorophylls (Miyashita et al., 1997; Akiyama et al., 2001, 2002). Under high-light conditions, the ratio of Chl *a/d* is increased either due to additional Chl *a* synthesis in the cells (Mimuro et al., 1999; Chen et al., 2002a), or due to inhibition of Chl *d* biosynthesis under high light. Enriched iron in the culture medium caused a low ratio of Chl *a/Phe a* of 0.68 in *A. marina*, which is significantly less than the ratio of 1.43 found under iron-stressed culture (Swingley et al., 2005). This recent finding is inconsistent with the previous suggestion of a minimum Chl *a/Phe a* ratio of 1 (Mimuro et al., 2004), used as a major argument for the proposal that PSII in *A. marina* contains two or more Chl *a* molecules along with two pheophytin (Phe) *a* molecules. Minor photopigments in *A. marina* include a Chl *c*-like pigment, zeaxanthin and α -carotene (Miyashita et al., 1997). α -carotene is an unusual carotenoid for cyanobacteria, which typically have β -carotene, and besides *A. marina*, α -carotene is otherwise reported in certain taxa of eukaryotic algae. There is only a small difference between these carotenes as α -carotene has ten conjugated double bonds while β -carotene has 11. However, there has been no investigation on the physiological significance and effects of these differences.

4.1.1. Phycobiliproteins

A. marina has two light-antenna systems, PBPs and Pcb-bound Chl *d* complexes (Chen et al., 2002b). Phycobilins are present in most cyanobacteria, as well as some eukaryotic algae, that is glaucocystophytes, rhodophytes and cryptophytes, where the bilin chromophores are attached in variable numbers to polypeptides to form PBPs. PBPs are often associated with linker polypeptides to form a supramolecular antenna-complex, the phycobilisome, on the outside surface of thylakoid membranes. Phycobilisomes are the primary light-harvesting antennae in cyanobacteria, glaucocystophytes and rhodophytes allowing these organisms to utilize the spectral region from green to orange, where only a few other light-harvesting systems are active. Cyanobacteria can exhibit ontogenetic complementary chromatic adaptation, whereby they express those PBPs best able to exploit a particular light climate (Larkum and Barrett, 1983). There are no phycobilisomes reported in *A. marina*, but some PBPs (phycocyanin and allophycocyanin) are present and organized in rods of four hexameric units that can act as antenna for light harvesting (Hu et al., 1999; Marquardt et al., 2000). The PBP content of *A. marina* is normally very low, but a recent investigation showed that replete iron-availability in the growth medium caused higher ratio of PBPs to Chl *d* (Swingley et al., 2005).

How the PBPs link with the photosystem-reaction centre in *A. marina* is uncertain. Action spectra of intact cells of *A. marina* indicate that the PBPs transfer energy to PSII with somewhat higher efficiency than to PSI (Boichenko et al., 2000). Excitation energy transfer studies with time-resolved fluorescence spectroscopy indicated that the elemental structure of PBP in *A. marina* provides efficient energy transfer from PBPs to Chl *d* in PSII with a time constant of 70 ps, which is about three times faster than energy transfer from phycobilisomes to PSII in the Chl *a*-containing cyanobacteria (Petrasek et al., 2005).

4.1.2. Pcb-bound Chl d

The main light-harvesting antenna system in *A. marina* consists of Pcb-bound Chl d complexes. Pcb are Chl *a/b*-binding proteins, which are found in the three different prochlorophyte lineages of cyanobacteria. The surprising discovery of Pcb-protein bound Chl d in *A. marina* (Chen et al., 2002b) showed that Pcb-bound Chl d-protein pigment complexes can function as the major light-harvesting protein. There are two Pcb genes in *A. marina*, *pcaA* and *pcaC*. Phylogenetic analysis supports that they may be functionally associated to PSII and PSI, respectively, under different nutrient conditions (Chen et al., 2005a–c); however, recent genomic information indicates that there are multiple copies of the Pcb gene in *A. marina* (M. Chen and R. Blankenship, unpublished data).

4.2. PHOTOSYSTEM I

Because PSI complexes are more stable and easier to isolate, the properties of PSI in *A. marina* have been resolved in more detail than the properties of PSII and its connected O₂ evolving reactions. Sequence comparisons indicated that PsaA/PsaB of *A. marina* has 85–87% homology to the typical cyanobacterium *Synechocystis* sp. PCC 6803. There are PsaA,-B,-C,-D,-E,-F,-L,-K and two unidentified polypeptides of < 6 kDa peptides in isolated PSI complexes. It was suggested that the primary electron donor of PSI in *A. marina* is a special pair of Chl d, and it was shown by laser spectroscopy that the oxidation of the primary donor of PSI occurs at 740 nm and this caused the *Acaryochloris* PSI reaction centre to be named P740 (Hu et al., 1998; Akiyama et al., 2001). The size of PSI and PSII and their pigment content is still not fully resolved. Hu et al. (1998) showed that isolated PSI had a Chl d/a ratio of 180, which is six times higher than in intact cells, and a Chl d/P740 ratio of 150. However, an antenna size of 80–90 Chl d per PSI reaction centre has also been suggested (Boichenko et al., 2000).

Laser spectroscopy, ENDOR and FTIR studies (Hastings, 2001) indicated that the basic structure and environment of P740 is similar to that of P700. The (P740+ to P740) IR spectrum band pattern was very similar to that of (P700+ to P700) from the cyanobacterium *Synechocystis* sp. PCC 6803. This indicates that P740 is probably a dimer consisting of a special pair of chlorophylls in the reaction centre similar to P700, but with a pair of Chl d molecules instead of Chl a. Extraction and reconstitution of the phylloquinone molecule was performed on the PSI particle, and the function of quinone was assumed to be similar to that observed in isolated PSI complexes from other organisms (Itoh et al., 2001).

The primary electron acceptor is also identified as Chl d (Akiyama et al., 2001, 2002). This is unique among all photosynthetic organisms including anaerobic photosynthetic bacteria that use a Chl a-type pigment as the primary acceptor. Chen et al. (2005a) revealed that Pcb-PSI super-complexes were formed when *A. marina* was grown under iron-limitation (Bibby et al., 2001; Boekema et al., 2001). In vivo action spectra of PSI in *A. marina* demonstrated that a low but reliable efficient energy transfer happened from the PBPs to PSI (Boichenko et al., 2000; Fig. 4).

4.3. PHOTOSYSTEM II

PSII characteristics in *A. marina* have not yet been fully resolved and since its discovery it has been debated whether Chl *d* has replaced the primary photochemical role of Chl *a* in the PSII reaction centre. PSII reaction centre polypeptides D1, D2, cytochrome b559 and CP 43 and 47 were reported for PSII in *A. marina* (Hu et al., 1999) and the sequence of D1/D2 shows high (up to 93%) homology with polypeptides in Chl *a*-containing cyanobacteria.

Action spectra of PSII activity showed an efficient and preferential energy transfer from PBP to PSII (Boichenko et al., 2000; Fig. 4), which agrees with the ultrastructural evidence for a close physical connection between PSII complexes and PBPs (Hu et al., 1999). However, isolated mega-antenna-reaction centres of the PSII super-complex showed an incompatible ultrastructure arrangement of PSII reaction centres and its antenna systems (Chen et al., 2005b). The isolated PSII-antenna super-complexes consisted of tetrameric PSII reaction centres surrounded by decamer Pcb along each side of the tetrameric reaction centre (Fig. 3). This unique subunit arrangement could explain experimental results showing that the effective optical cross-section of O₂ evolution in *A. marina* is

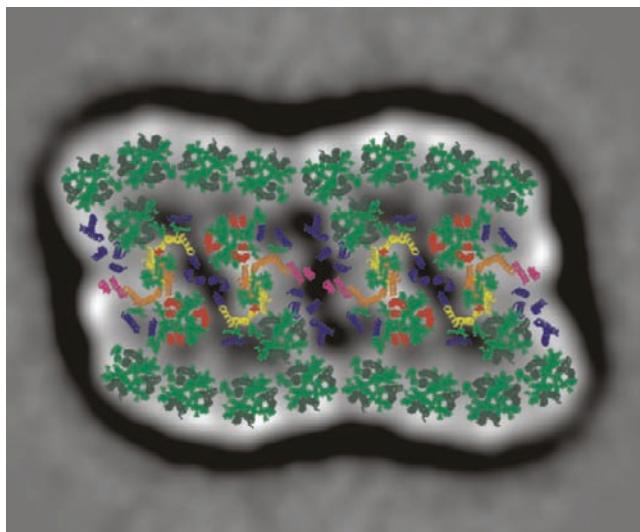


Figure 3. Modelling of the high-resolution X-ray structure of PSII and CP43 (Ferreira et al., 2004) into the Pcb-PSII super-complex isolated from *A. marina* viewed from its luminal surface. The 36 Chls and 14 Chls of each PSII monomer and Pcb subunit, respectively, are shown in light green. Other colours represent transmembrane helices of D1 protein (yellow), D2 protein (orange), CP47 (red), CP43 and Pcb proteins (dark green), cytochrome b559 (pink), low molecular weight subunits (blue). Maximum length of the negatively stained particle is 385 Å, inclusive of the detergent shell (model by Dr J. Nield, Imperial College).

about 65 Å², that is two times larger than with a dimeric-reaction centre arrangement. In addition, the functional antenna size of PSII in *A. marina* indicates at least the double number of CP43/47 subunits per reaction centre as compared to other cyanobacteria, that is that one reaction centre is supported by one CP43/47 and 2–3 Pcb subunits as its antenna systems (Boichenko et al., 2000).

4.4. UPHILL ENERGY TRANSFER IN *A. MARINA*

It is conventional to consider that energy flows from a higher energy source to a lower energy sink. In terms of light this means that energy flows, pigment-wise, from the blue to the red end of the spectrum, and the final sink is the special pair of chlorophylls in either reaction centre (PSI or PSII). In *A. marina*, this question has caused some debate because the special pair in PSII may turn out to be either Chl *a* or Chl *d*. If it is Chl *a* then light absorbed by light-harvesting Chl *d* would have to flow “uphill” from as far out as 750 nm to P680 at 680 nm. If it were Chl *d* then light would still have to flow from 750 nm to the hypothetical P715 at 715 nm. However, it has been shown clearly that this does not pose a problem in terms of quantum mechanics (Nieuwenburg et al., 2003; Trissl, 2003) and there are experimental indications for uphill energy transfer in *A. marina* (Mimuro et al., 2000). In fact, this phenomenon is already known to happen in “conventional” algae; for example, it has long been known that the endolithic alga *Ostreobium queckettii*, found in the skeleton of living corals, has a light-harvesting protein, containing Chl *a* and Chl *b*, that can harvest light up to at least 750 nm (Halldal, 1968) and pass this light on to P680 at 680 nm. The mechanism for this transfer process is similar to that which operates in *A. marina* (Trissl, 2003; Wilhelm and Jakob, 2006).

4.5. ACTION SPECTRUM AND PHOTOSYNTHETIC O₂ PRODUCTION

Light intensity and quality are known to play an important role for regulating the PSI/PSII ratio and the size of the light-harvesting antenna in oxygenic phototrophs (Razeghifard et al., 2005). The function of oxygenic photosynthesis in *A. marina* is similar to other oxygenic phototrophs, that is the turnover of the O₂-evolving system under flash excitation follows the S-state cycles with a period-4 oscillation, although the O₂ release is slower than in classical Chl *a*-containing cyanobacteria (Boichenko et al., 2000). Action spectra of photosynthetic oxygen production showed that Chl *d* and PBPs act as antenna for both PSI and PSII, and that both far-red and blue wavelengths can drive photosynthesis effectively in *A. marina* (Boichenko et al., 2000; Fig. 4). Interestingly, the contribution of PBPs to photosynthesis decreased when the original culture was grown under white light or under far-red light pointing to some ability of *A. marina* to optimize its pigmentation towards ambient light conditions.

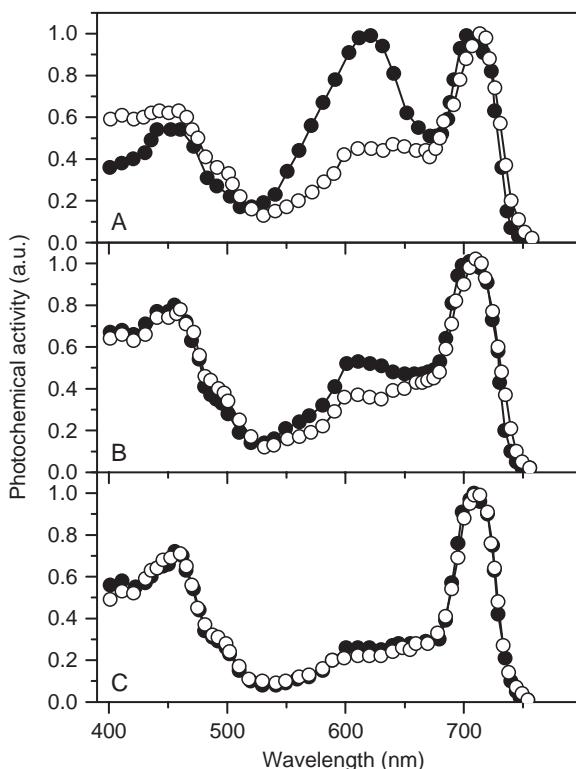


Figure 4. Action spectra of PSI (open symbols) and PSII (solid symbols) activity in a *A. marina* culture (A), and in the same culture kept under white light (B) and red light (C), respectively. Redrawn after Boichenko et al. (2000).

Oxygen production as a function of irradiance has been investigated in cultures of *A. marina* by several groups (e.g. Miyashita et al., 1997), showing a remarkable ability of *A. marina* to cope with high irradiance. The O_2 activity of cells was significantly increased compared to the typical activity of $70\text{--}80 \mu\text{mol O}_2 (\text{mg of Chl})^{-1} \text{ h}^{-1}$ when *A. marina* was grown in white light at an irradiance of $60 \mu\text{mol photons m}^{-2} \text{ sec}^{-1}$ provided by a metal-arc lamp. Such high-light conditions caused the PSI content to decrease to half and the light harvesting complex (LHC) component decreased, therefore, O_2 activity (based on chlorophyll concentration) was increased (Razeghifard et al., 2005). In a recent study (M. Kühl et al., unpublished data) we used a new microrespirometry system (Unisense A/S, Denmark) to measure photosynthesis and respiration as a function of irradiance (Fig. 5). In line with earlier reports, *A. marina* showed a remarkable tolerance of high irradiance up to $> 700 \mu\text{mol photons m}^{-2} \text{ sec}^{-1}$ without any sign of photoinhibition. Maximum rates of gross photosynthesis reached $> 200 \mu\text{mol O}_2 (\text{mg}$

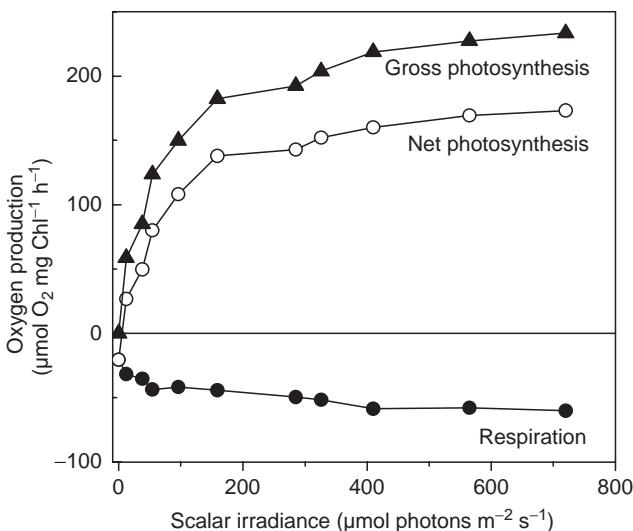


Figure 5. Photosynthesis and respiration as a function of scalar irradiance in a culture of *A. marina*, as measured with an oxygen microelectrode respirometry system (Unisense A/S, Denmark). (M. Kühl, unpublished data).

$\text{Chl}^{-1} \text{h}^{-1}$. Furthermore, we found a pronounced post-illumination respiration reaching 300% of the dark respiration level at the highest irradiance. The mechanism behind this needs further investigation and there evidently is a need for more detailed ecophysiological analysis of *A. marina* cultures, for example with respect to effects of pH, inorganic carbon, salinity, temperature and nutrient regimes on the photosynthetic performance and growth. Micronutrients such as iron may also play an important role (see Swingley et al., 2005).

5. Evolutionary Relationships of *A. marina*

A. marina is a cyanobacterium, according to small-subunit (SSU) rRNA analysis (Miyashita et al., 2003; Fig. 6). According to morphological and cytological classification of cyanobacteria (Castenholz, 2001), *A. marina* is accommodated in the *Chroococcales*-group of unicellular cyanobacteria, but the molecular data show that *A. marina* has no close relationship to any of the unicellular subgroups. *A. marina* is placed in the middle of the cyanobacterial lineage and diverges independently from the other cyanobacterial subgroups. The possession of Chl *d* of *A. marina* poses an interesting question concerning the presence of this unique chlorophyll. Why is it only found in *A. marina* and none of its distant relatives? The answer to these questions can be only speculative at present and would be

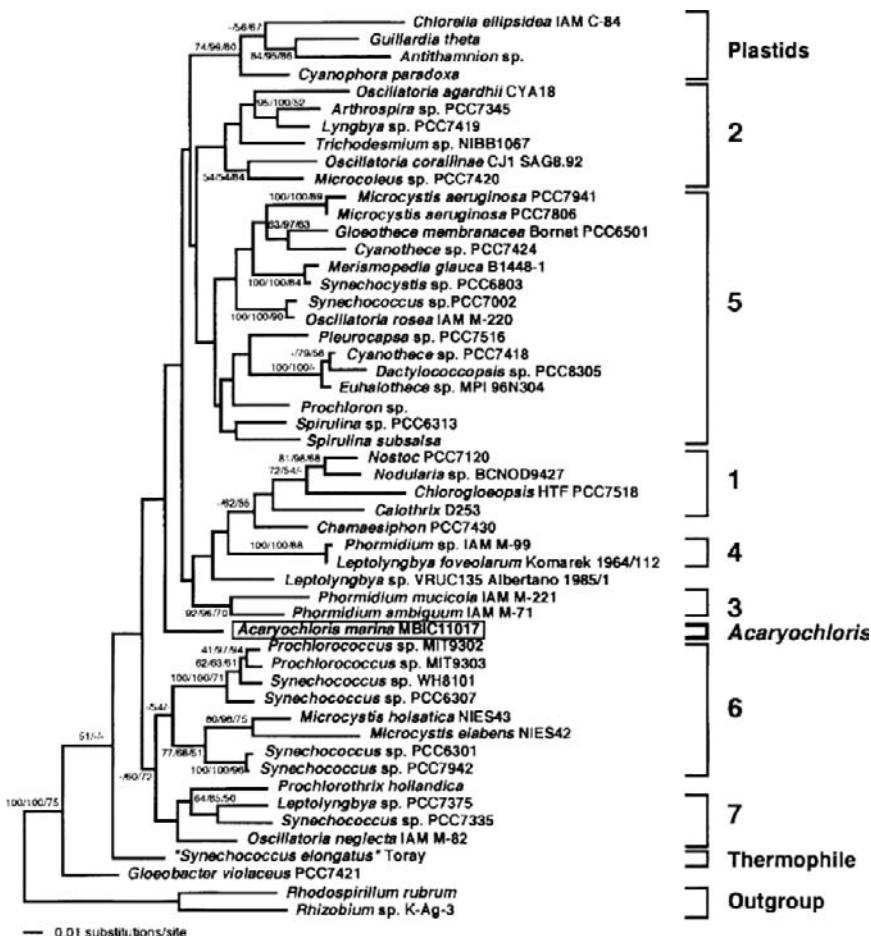


Figure 6. Evolutionary relationship of *A. marina* to other cyanobacteria based on SSU rRNA analysis (from Miyashita et al., 2003).

much less so if we had: (i) the whole genome of *A. marina*, which is currently being sequenced at the University of Arizona (Blankenship, see above), and (ii) the biosynthetic pathway for Chl *d*.

In the absence of these vital pieces of evidence, we can fall back on the weaker evidence from the light-harvesting protein that binds Chl *d* (Chen et al., 2005c). It is clear that *A. marina* has the same or similar light-harvesting chlorophyll protein as prochlorophytes and some classical cyanobacteria. In some classical cyanobacteria an iron-stress induced protein A (isiA protein) is inducible under low iron-conditions (Ting et al., 2002). In prochlorophytes Chl *b* is present, in addition to Chl *a*, and attached to a LHC, the so-called pcb protein, which is, phylogenetically, closely related to isiA proteins. It is clear that the LHC of

A. marina belongs to the same class as those of the *isiA* and *pcb* LHCs (Chen et al., 2005e).

In those *Prochlorococcus* species, for which a whole genome analysis has been done (Dufresne et al., 2003; Rocap et al., 2003), it is clear that there are wide differences in the genetic structure but these organisms all have a small number of genes allowing them to express *pcb* LHCs and to make Chl *b*; note that Chl *a* oxygenase, the key enzyme in forming Chl *b* in *Prochloron*, algae and plants is not present in *Prochlorococcus* species, so some other gene(s) must be responsible. These species may thus be joined in their ability to express *pcb* LHCs, while showing great evolutionary diversification in other characters (Ting et al., 2002); although other evidence suggests that *Prochlorococcus* species may be a real taxonomic group (A.W.D. Larkum et al., unpublished data).

The other prochlorophytes, *Prochloron* and *Prochlorothrix* may have gained entry to this group of organisms merely by the lateral transfer of the suite of Chl *b* synthesis and *pcb* genes (Chen et al., 2005e) and a similar reasoning may be put forward for *A. marina*, although we know nothing of the biosynthetic mechanism of Chl *d*. Reinforcing this view is the fact that in phylogenetic analyses of *Prochlorococcus* species, three “classical” cyanobacteria, with no Chl *b* but possessing PBPs, that is *Synechococcus* CC9605, *Synechococcus* CC9902 and *Synechococcus* WH8102 are found within the *Prochlorococcus* branch (Rocap et al., 2003), suggesting that these are closely related. Furthermore, it is also known that a “classical” cyanobacterium without Chl *b* and with PBPs, *Synechocystis trididemni*, is closely related to *Prochloron* in terms of SSU rRNA (Shimada et al., 2003).

6. Natural Habitats and Niche of *A. marina*

A. marina was first isolated during an attempt to isolate *Prochloron* from the didemnid ascidian *Lissoclinum patella* (Miyashita et al., 1996, 2003), and it was for several years regarded a symbiont of didemnid ascidians. Cultures of *A. marina* are easy to keep in the laboratory, and such cultures have been subject to detailed biochemical and photophysiological studies, but the actual niche and habitat of this unique cyanobacterium remained unknown until 2004/2005, when it became clear that *A. marina* is more widespread than previously thought. Murakami et al. (2004) reported *A. marina* growing as small epiphytic patches on the red macroalga *Ahnfeltiopsis flabelliformis*, and they obtained several strains from such habitats. Miller et al. (2005) isolated *A. marina* in enrichments from a benthic sample taken in the eutrophic and saline Salton Sea, the largest lake in California. These two studies clearly showed that *A. marina* could be free-living, so what about the initial claim of *A. marina* being a symbiont in didemnid ascidians? A thorough investigation of several didemnid ascidians showed that *A. marina* was indeed not living as a symbiont inside the ascidian but formed dense cell patches in biofilms growing below the animal (Kühl et al., 2005; Fig. 7A). By the help of microscopy, microspectrometry, and variable chlorophyll

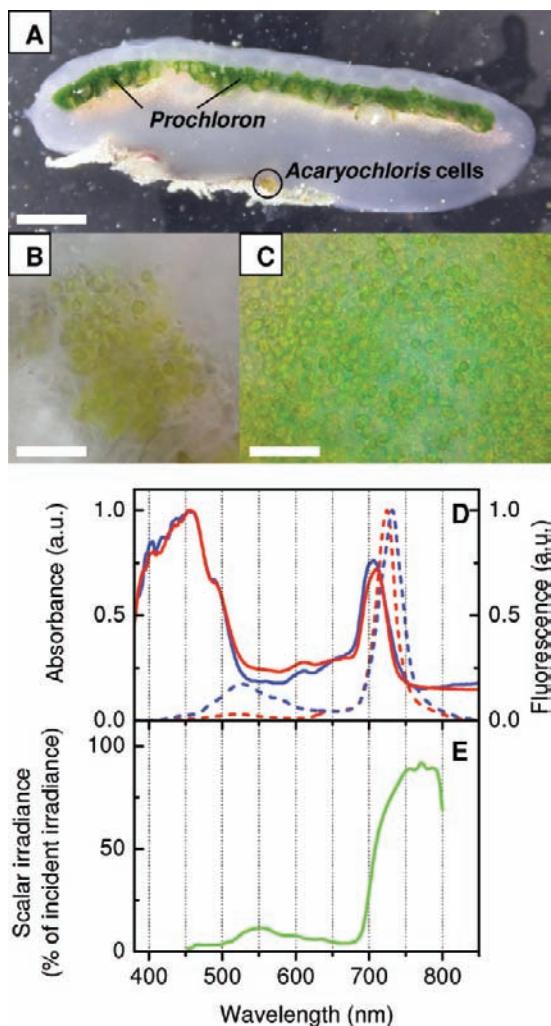


Figure 7. Microscopic observation and spectral analysis of Chl *d*-containing cells associated with the didemnid ascidian *Diplosoma virens*. A. Vertical section through the ascidian showing symbiotic *Prochloron* sp. (green colour) in the cavities of the transparent test and patches of biofilms with Chl *d*-containing cells growing on the underside of the ascidian (scale is 2 mm). B. Chl *d*-containing cells within the circled biofilm area in panel a (scale is 10 μm). C. Cells from an *A. marina* culture (scale is 10 μm). D. Spectral absorbance (solid lines) and UV-excited fluorescence (dashed lines) of cells growing in the biofilm (circled area in A, blue lines) and of an *A. marina* culture (red lines). Data were normalized to the maximal absorbance and fluorescence, respectively. E. Transmission of spectral scalar irradiance in tissue of *D. virens*, as measured 1.3 mm below the upper surface of the ascidian. Data are expressed in percent of the downwelling irradiance at the tissue surface (from Kühl et al., 2005).

fluorescence imaging both the distribution, spectral characteristics and the photosynthetic activity of *A. marina* could be studied in situ, and this enabled the first description of the niche that *A. marina* inhabits.

6.1. HABITAT CHARACTERISTICS AND IN SITU ACTIVITY OF *A. MARINA*

Contrary to the long held belief that *A. marina* is a symbiont of didemnid ascidians, we were not able to demonstrate the presence of Chl *d*-containing cells in the test or internal cavities of the ascidians. However, the underside of didemnid ascidians harbours a dense and diverse biofilm of phototrophic microorganisms and here we found patches of *A. marina*-like cells with morphology and spectral characteristics similar to cells in a *A. marina* culture (Fig. 7B–D). The spectral light field below ascidians is characterized by a strong depletion in visible wavelengths, while far-red light is, relatively, much more abundant (Fig. 7E) and can support the photosynthesis of *A. marina*. Using variable chlorophyll fluorescence imaging, we were able to perform the very first in situ photosynthetic activity measurements of *A. marina* in its natural habitat below the didemnid ascidians harbouring a dense internal population of *Prochloron* (Fig. 8), showing a high maximal quantum yield of PSII ranging from 0.7 to 0.8 and adaptation towards high irradiance.

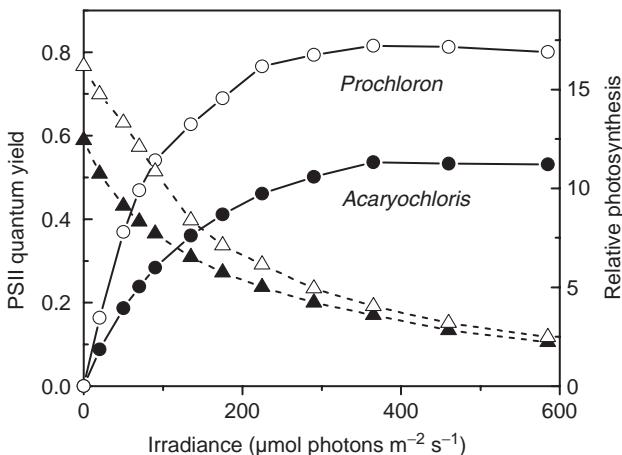


Figure 8. Effective quantum yield of PSII (dotted curves, triangles) and derived relative photosynthesis (=yield × irradiance × absorptivity) (solid curves, circles) measured as a function of irradiance with an imaging PAM fluorometer equipped with blue LED's (470 nm). Measurements were done on intact didemnid ascidians harbouring *Prochloron* symbionts (open symbols) in their internal cavities and a biofilm with *A. marina* (solid symbols) on their underside. Data are from specific areas of interest shown by microscopy and spectroscopy to be predominated by *Prochloron* and *A. marina*, respectively (from Kühl et al., 2005).

There is an apparent paradox of *A. marina* growing in extreme shade in situ and at the same time showing characteristics of high-light adaptation. However, *A. marina* is de facto adapted to high levels of far-red light that for this organism is photosynthetically active due to the presence of Chl *d* as the major photopigment. In this context it is important that: (i) far-red light absorbed by Chl *d* drives both PS I and PS II (Boichenko et al., 2000), (ii) Chl *d* also absorbs substantially in the blue, which we made use of to assess PS II quantum yield via PAM fluorometry (Schiller et al., 1997; Kühl et al., 2005) and (iii) for photoacclimation of the cells it is irrelevant whether a high electron transport rate is driven by far-red or blue light. Interestingly, a photosynthesis (measured as oxygen production) versus irradiance experiment with *A. marina* cultivated under $80 \mu\text{mol photons m}^{-2} \text{ sec}^{-1}$ (Miyashita et al., 1997) showed a similar high-light adaptation, but of course such culture experiments are strongly dependent on the actual growth conditions used and the authors did not quantify far-red intensity.

While blue light is rather insignificant in the niche of *A. marina* due to the strong absorption in the overlaying ascidian tissue, the strong inherent absorption of Chl *d* in the blue spectral region allowed us to use PAM fluorometry with blue excitation light. Blue and far-red light have been shown to be almost equally efficient in the action spectra of both photosystems in *A. marina* cultures (Boichenko et al., 2000) and the equivalence of blue and far-red should be even more pronounced in situ due to the pigment flattening effect in the highly scattering biofilm. This allowed us to determine the high-light adaptation towards far-red light on the basis of PAM fluorometry with blue light (Fig. 8). In an analogous example, a Chl *b*-containing phototroph (e.g. a green leaf) will also become high-light adapted if it is irradiated with light depleted of, for example 480 nm. Once this adaptation has taken place, it would also be possible to demonstrate high-light adaptation from active fluorescence measurements using 480 nm as actinic light, although this wavelength was absent during the adaptation period.

Our laboratory analysis of the microhabitat below didemnid ascidians has clearly shown that *A. marina* can thrive here in extreme shade provided sufficient NIR radiation is available. To demonstrate the latter, we performed in situ measurements of the spectral light field within the dense patches of dead corals, which were colonized by didemnid ascidians (Fig. 9). Far-red light around 700–730 nm, that is the range of the in vivo absorption maximum of Chl *d*, and further NIR wavelengths predominated the spectral light field in the natural habitat. This indicates that the high Chl *d* content of *A. marina* may indeed be adaptive to the ambient spectral irradiance in the environment where it occurs, as hypothesized by Blankenship and Hartman (1998).

6.2. DISTRIBUTION OF *A. MARINA*

Since our initial discovery of *A. marina* growing in photosynthetic biofilms below didemnids harbouring *Prochloron* (Kühl et al., 2005), we have also found *A. marina*

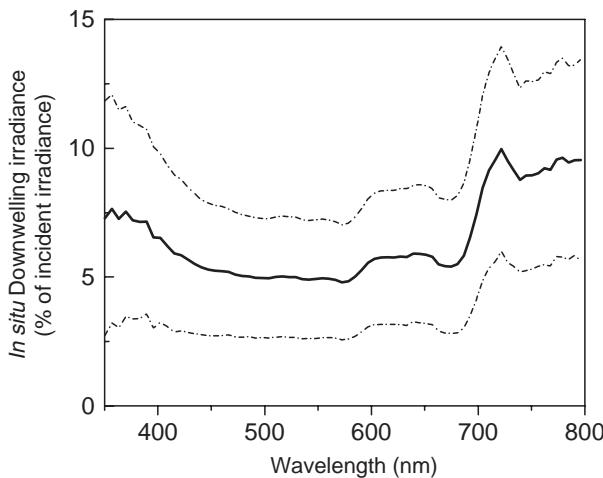


Figure 9. In situ measurements of downwelling spectral irradiance a few cm below the surface of dead coral patches on the reef flat at Heron Island (GBR) harbouring didemnid ascidians with *Prochloron* symbionts and external biofilms of *A. marina*. Solid line indicates the mean, while dotted lines indicate mean \pm standard deviation, respectively ($n = 10$). Data were obtained with an Ocean Optics spectrometer (USB2000) equipped with a 600 μm diameter quartz fiber and a cosine collector (Ocean Optics CC-3-UV). Data were normalized to the downwelling irradiance measured just below the water surface. (M.J. Durako et al., unpublished data).

biofilms below white didemnids growing without photosynthetic symbionts on dead coral branches. Together with the finding of *A. marina* as red algal epiphytes (Murakami et al., 2004) and in enrichments from biofilm samples taken from a hypertrophic salt lake (Miller et al., 2005), our data indicate that Chl *d*-containing cyanobacteria may be more widespread in shallow-water niches, where visible wavelengths are strongly depleted and sufficient NIR prevails.

So far, all free-living *A. marina* have been found in biofilms growing on biotic or abiotic substrates, and *A. marina* seems adapted to a surface-associated lifestyle. There is now a need for a more careful screening of such habitats to assess the abundance of Chl *d*-containing phototrophs. There may also be other yet undiscovered microenvironmental conditions, besides the light microclimate, that shape the niche of *A. marina*-like organisms. For example, *A. marina* has neither been found in endolithic habitats or in microbial mats, both of which are characterized by a strong depletion of visible wavelengths by microalgae and cyanobacteria in the surface layers and efficient penetration of NIR wavelengths into deeper layers (e.g. Kühl and Fenchel, 2000; Magnusson et al., 2007).

In the coral skeleton, the spectral range where Chl *d* is absorbing is also covered by the special antenna pigments of the predominating endolithic green alga *Ostreobium*. In microbial mats, NIR is absorbed by various bacteriochlorophylls present in dense subsurface layers of anoxygenic phototrophs, but the spectral

window where Chl *d* absorbs is not affected by these pigments, and based on the light microclimate this seems an ideal niche for Chl *d*-containing phototrophs. But microbial mats are also characterized by extremely steep and strongly fluctuating light-dependent gradients of chemical variables such as oxygen (zero to almost pure oxygen), pH (<6 to >9.5) and poisonous hydrogen sulfide (zero to several mM). We know nothing about how *A. marina* can cope with such extreme conditions, or whether the habitats where it has been found exhibit similar characteristics.

Does *A. marina* have an efficient carbon concentrating mechanism allowing for efficient carbon fixation under high pH? Can *A. marina* tolerate sulfide, or even use it for anoxygenic photosynthesis as many phycobilisome-containing cyanobacteria are able to? Can *A. marina* fix nitrogen, like some other unicellular cyanobacteria? These are but a few open questions that call for more detailed in situ and in vitro physiological investigations of *A. marina* in concert with data mining of its genome for hitherto unknown metabolic capabilities.

7. Summary

The cyanobacterium *A. marina* is the only known oxygenic phototroph with Chl *d* as its major photopigment. In *A. marina* Chl *d* is the major light-harvesting pigment along with a minor amount of PBPs, and Chl *d* has replaced the primary photochemical role of Chl *a* in the PSI reaction centre (and maybe also in PSII). The cell biology and photophysiology has been studied in cultures of *A. marina* and its genome is currently being sequenced. However, the in situ habitat and ecophysiology of this unique phototroph is underexplored. Oxygen and fluorescence-based measures of photosynthesis show that *A. marina* is high-light adapted and does not suffer from photoinhibition at high-irradiance levels. While initially regarded a symbiont of didemnid ascidians, it is now clear that *A. marina* is free-living and grows in biofilms associated with biotic and abiotic surfaces (red algae, didemnid ascidians, and in the hypersaline Salton Sea), where it occupies a niche depleted of visible wavelengths but enriched in far-red light. Thus *A. marina* is probably widespread and can be found in a range of habitats yet to be explored.

8. Acknowledgements

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PHYLOGENETICS, MOLECULAR BIOLOGY AND ECOLOGICAL IMPACTS OF A GROUP OF HIGHLY UNUSUAL PROTISTS: *The Dinoflagellates*

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1. Introduction

Dinoflagellates are well-known and readily recognized protists, consisting of ~4,000 named extant and fossil species (Fensome et al., 1993). Dinoflagellates exhibit great diversity in many ecological parameters such as niche exploitation, and show extreme idiosyncrasy in their ultrastructural and molecular biological characteristics. The human impact of sudden dinoflagellate proliferation, in the form of harmful algal blooms (HABs), as well as the impact of the sudden loss or senescence of dinoflagellates, in the form of coral bleaching, have become an increasing focus of concern in recent years. For these reasons, studies of the molecular evolution, ecology, diversity and physiology of dinoflagellates have increased dramatically, and have revealed ever more interesting features. This review will explore the significance of recent findings in the phylogenetics, evolution, molecular biology and ecology of this intriguing group.

2. Diversity, Phylogeny and Evolution

Dinoflagellates are a well-supported clade within the group, the alveolates (Gunderson et al., 1987; Sogin, 1989; Lenaers et al., 1991). They are clearly related to other alveolates, which constitute the apicomplexan parasites, the perkinsids, including the genera *Parvilucifera* and *Perkinsus*, *Oxyrrhis marina* and the colpodellids, as well as the slightly more distantly related ciliates (Leander and Keeling 2004). The alveolates may appear at first glance to be very variable, as they include obligate parasites, autotrophic phytoplankton and predators, however, the addition of more data has only confirmed the monophyly of the alveolates (Fast et al., 2002). Morphologically, alveolates share some features, in particular, the presence of alveoli, a pattern of membrane-bound organelles, which form a continuous layer beneath the cell membrane. More recently, a close relationship between alveolates and stramenopiles has been proposed, based on evidence of plastid genes, including plastid targeted GAPDH, (Fast et al., 2001; Yoon et al., 2002; Harper and Keeling, 2003). A new eukaryotic ‘super group’,

(‘kingdom’, sensu Simpson and Roger, 2005), the ‘Chromalveolata’, has been proposed. Molecular studies based on nuclear genes have not yet confirmed the data from plastid genes.

2.1. DIVERSITY

Although extremely diverse in many morphological features, all dinoflagellates share some characteristics. All dinoflagellates exhibit a ‘closed’ type of mitosis, meaning that the nuclear envelope does not break down during cell division. The majority of Dinophyceae, those constituting the group Dinokaryota (Table 1,

Table 1. Classification of dinoflagellates to family, modified after Fensome et al. (1993).

Division	Subdivision	Class	Order	Family
Dinoflagellata	Dinokaryota	Dinophyceae	Gymnodiniales	Gymnodiniaceae
			Ptychodiscales	Polykrikaceae
				Warnowiaceae
				Actiniscaceae
				Dicroerismaceae
			Suessiales	Brachydiiniaceae
				Amphitholaceae
				Ptychodiscaceae
			Gonyaulacales	Symbiodiniaceae
				Suessiaceae
			Peridiniales	Rhaetogonyaulacaceae
				Mancodiniaceae
				Cladopyxiaceae
				Scriniocassiacae
				Lotharingiaceae
				Pareodiniaceae
				Goniodomaceae
				Pyrocystaceae
				Gonyaulacaceae
				Areoligeraceae
				Ceratocoryaceae
				Ceratiaceae
				Heterodiniaceae
				Crypthecodiniaceae
				Heterocapsaceae
				Peridiniaceae
				Pfiesteriaceae
				Protopteridiniaceae
				Podolampaceae
				Lessardiaceae
				Glenodiniaceae
				Comparodiniaceae
		Unknown		Tovelliaceae
		Nannoceratopsiales		Nannoceratopsiaceae

(Continued)

Table 1. Classification of dinoflagellates to family, modified after Fensome et al. (1993)—cont'd.

Division	Subdivision	Class	Order	Family
		Dinophysiales	Oxyphysiaceae Dinophysiaceae Amphisoleniaceae	
		Prorocentrales	Prorocentraceae	
		Desmocapsales	Desmocapsaceae	
		Phytodiniales	Phytodiniaceae	
		Thoracosphaerales	Dinocloniaceae Thoracosphaeraceae	
	Blastodiniphyceae	Blastodiniales	Blastodiniaceae Protoodiniaceae Cachonellaceae Oodiniaceae Haplozoaceae Apodiniaceae	
	Noctiluciphyceae	Noctilucales	Noctilucaceae Kofoidiniaceae Leptodiscaceae	
Syndinea	Syndiniophyceae	Syndiniales	Duboscquellaceae Syndiniaceae Amoebophryaceae Sphaeriparaceae	
		Unknown	Arpyloraceae	

after Fensome et al., 1993), contain cells with a unique type of nucleus (the 'dinokaryon'), containing permanently condensed chromosomes, that lack true histones (Rizzo, 1987). All motile cells possess two dissimilar flagella. One of these is a ribbon-like flagellum that beats in a wave pattern, perpendicular to the cell's dorso-ventral axis, in a groove referred to as the 'cingulum'. The second is a conventional flagellum which beats with few waves, longitudinally towards the posterior of the cell, generally in a second groove referred to as the 'sulcus'. Dinoflagellates possess pusules, a unique organelle associated with the flagella, whose role is not yet known. The dinoflagellate cell membrane consists of a continuous outer membrane over a layer of alveoli, the amphiesmal vesicles, which may be empty or contain cellulosic plates of varying thickness (Dodge and Gruet, 1987). The pattern of cellulosic plates and amphiesmal vesicles is currently the most common feature used for differentiating amongst dinoflagellates below the class level (Taylor, 1987; Fensome et al., 1993; Taylor, 1999). Taxa with only a few rows of amphiesmal vesicles usually also contain cellulosic plates within the vesicles, and are said to be 'thecate'. The pattern of cellulosic plates is referred to as thecal tabulation. In most groups of dinoflagellates, the theca forms a pattern which is conservative among closely related taxa (Taylor, 1987, 1999) and is extensively used in dinoflagellate systematics.

There are considered to be 56 described families of dinoflagellates, including those of purely fossil taxa, and about 210 genera (Table 1, updated from Fensome et al., 1993). This classification should be treated as only indicative of the true

diversity and evolutionary relationships of dinoflagellates, as these are incompletely known, as will be described in the following section. In recent years, studies of dinoflagellate diversity, including those of direct sequencing of environmental samples, have blossomed. Novel dinoflagellate lineages have been recorded, as well as a novel sister clade to dinoflagellates (López-García et al., 2001; Moon van der Staay et al., 2001). In 2005–2006 alone, eight new genera and one new family of dinoflagellates were recognized: *Galeidinium* Tamura et al., 2005; *Jadwigia* Moestrup et al., 2006; *Pileidinium* Tamura and Horiguchi, 2005; *Pseudopfiesteria* Litaker et al., 2005; *Pseudothecadinium* Hoppenrath and Selina, 2006; *Pyramidodinium* Horiguchi and Sukigara 2005; *Rhinodinium* Murray et al., 2006; *Tovellia* Moestrup et al. 2006, and the Tovelliaceae, Moestrup et al. 2006. The rate of analysis of dinoflagellate diversity appears to be increasing, probably because of the increased research effort into the group, due to their human impacts. Some of the newly recognized genera have unusual morphological features, including non-motile main life-cycle stages and unusual thecal plate patterns, making it difficult to determine their relationship to existing dinoflagellate families.

2.2. CRYPTIC DIVERSITY AND INTRA-SPECIFIC COMPARISONS OF MOLECULAR GENETIC SEQUENCES

Since the pioneering studies in the 1980s of intra-specific divergence in multiple clonal dinoflagellate cultures, using sexual compatibility and allozyme profiles (Beam and Himes, 1987), it has been realized that dinoflagellate taxa, as defined by their morphological features, may conceal considerable amounts of diversity. During the last 15 years, it has been possible for the first time to conduct detailed studies on intra-specific diversity using molecular genetic techniques. Studies of the coral symbiont genus *Symbiodinium* revealed a much greater level of diversity than had been previously thought (Rowan, 1998; LaJeunesse, 2001). From being originally considered a monotypic genus with a single species, *Symbiodinium microadriaticum*, it is now thought that seven clades and up to 23 uniquely evolving lineages of *Symbiodinium* exist, with differences in the SSU rDNA sequences of about 0.6% among some lineages (McNally et al., 1994; Rodriguez-Lanetty, 2003).

Equally high levels of cryptic diversity have been found within other dinoflagellate taxa. Within *Cryptocodinium cohnii*, Beam and Himes (1987) recognized 54 reproductively isolated ‘sibling species’, which differed in allozyme profiles. Within species of toxic *Alexandrium* such as *Alexandrium catenella*, *Alexandrium fundyense* and *Alexandrium tamarense*, several clades have been found that appear to be more closely related to the geographic origin of the strains than to the morphological features used to identify the taxa (Scholin et al., 1994). In the genus *Amphidinium*, multiple genotypes of several species have been found, including three clades of the species *Alexandrium carterae*, with differences of up to 1.7% in the D1–D6 regions of LSU rDNA (excluding the hypervariable D2) (Murray et al., 2004). The cyst forming taxon *Scrippsiella trochoidea* is known to encompass

several distinct clades or genotypes (Montresor et al., 2003), as is the planktonic dinoflagellate *Peridinium volzii* (Hayhome et al., 1987). Inter-strain differences in most dinoflagellate taxa have not been studied, and it may be found that considerable diversity has commonly been overlooked within the group.

2.3. PHYLOGENY

Despite a multitude of studies, phylogenetic relationships within the dinoflagellates are not yet resolved. There is considerable variation among phylogenetic trees that have been inferred, and some of the major lineages appear to be not consistently supported as monophyletic at the order or family level (see Table 1, Saunders et al., 1997; Litaker et al., 1999; Daugbjerg et al., 2000; Saldarriaga et al., 2001, 2003; Murray et al., 2005; Skovgaard et al., 2005; Yamaguchi and Horiguchi, 2005). The parasitic Synidiales appears to be well supported as the sister group to the Dinokaryota, based on SSU rDNA sequences (Skovgaard et al., 2005). The Gonyaulacales have consistently appeared to be monophyletic. However, the inclusion of the genus *Cryptothecodium* within the clade remains uncertain (Murray et al., 2005). The Suessiales, including the symbiotic *Symbiodinium*, the free-living genera *Polarella*, some species of *Woloszyskia* and some fossil taxa, appears to be monophyletic. The newly described family the Tovelliaceae, containing the genera *Tovellia* and *Jadwigia*, appears to be monophyletic (Lindberg et al., 2005). The monophyly of almost all other dinoflagellate orders and families have not been investigated in any detail. Of those others that have been investigated, the Gymnodiniales, the Peridiniales and the Phytodiniales are almost certainly polyphyletic to some extent (Daugbjerg et al., 2000; Horiguchi et al. 2000; Yamaguchi and Horiguchi, 2005), and the monophyly of the Prorocentrales is uncertain.

There may be several other reasons for the lack of support for the topology inferred in many phylogenetic studies of dinoflagellates. Genes from fewer than 10% of dinoflagellates have been sequenced, and of those, the vast majority are photosynthetic strains available in culture collections. Most estimates of phylogeny have been based on a single gene or gene region. The most common genes used have been parts of the ribosomal DNA (rDNA) array, which codes for ribosomal RNA, including the small subunit rDNA (SSU rDNA or 18/16S) (i.e. McNally et al., 1994; Saunders et al., 1997; Saldarriaga et al., 2001; Yamaguchi and Horiguchi, 2005), the large subunit rDNA (LSU rDNA or 28/26S), including the D1–D6 domains (Daugbjerg et al., 2000), internal transcribed spacer regions (ITS) (LaJeunesse, 2001), and the D8 domain (Lenaers et al., 1991). The D1–D6 regions of the LSU rDNA has a substitution rate that is 4–8% faster than that of the complete SSU rDNA (Murray et al., 2005). Smaller scale studies have also inferred phylogenies based on protein coding genes: α - and β -tubulin, heat-shock protein 90 (Leander and Keeling, 2004), Cytochrome B (Zhang et al., 2005), cox 1 and actin (Saldarriaga et al., 2003). Cladistic studies based on matrices of

morphological, ultrastructural, life-history or other characters have occasionally been performed (Roberts, 1991; Roberts and Roberts, 1991; Flø Jørgensen et al., 2004a; Leaw et al., 2005), but may be better suited to the analysis of single clades or sub-groups than the whole class.

It remains to be determined whether the uncertainty of the topology of the deeper branches of the dinoflagellate tree is an artefact related to the use of rDNA for inferring phylogenies, or whether it indicates broader issues in recovering the evolutionary history of the group. Phylogenetic signal in ribosomal DNA in dinoflagellates is affected by its different rates of evolution in different regions, and the fact that sites in stem regions do not evolve independently. In addition, it may be significantly compositionally heterogeneous and has a high level of homoplasy (Murray et al., 2005). Broader issues that may impact phylogenetic signal include a rapid or recent radiation of some groups of dinoflagellates, a high rate of gene transfer, reticulation, duplication or nucleotide substitution. Other potential causes of uncertainty in dinoflagellate phylogenies are distortions caused by unrepresentative taxon sampling and uncertainty over taxon identity. Several options exist for overcoming these problems, and these will ensure that future studies may be increasingly useful in determining evolutionary relationships amongst this group.

3. Molecular and Cell Biology

One of the most remarkable features of dinoflagellates is their vast, haploid genome, containing from 1.5 to 200 pg DNA cell⁻¹ (Lin, 2006), up to two orders of magnitude larger than the human genome. The smallest dinoflagellate genomes, at around 1.5–5 pg DNA cell⁻¹, appear to be present in species with the smallest cell diameters, which include picoplanktonic dinoflagellates and species of the genus *Symbiodinium* (LaJeunesse et al., 2005, Lin, 2006). Up to 60% of the genome may consist of repeat sequences. Dinoflagellate DNA has a high G + C content and many modified and very rare bases: 5-hydroxymethyluracil, N⁶-methyladenine, and 5-methyl-C (Rizzo, 1987; Diaz de la Espina et al., 2005). Of these, 5-hydroxymethyluracil, which replaces up to 70% of the thymidine in dinoflagellate DNA, is otherwise only known from prokaryotes. Histones are absent from dinoflagellate nuclei, and the protein/DNA ratio is therefore considerably lower than that in other groups of eukaryotes at 1:10, rather than 1:1. (Rizzo, 1987). With such a low ratio, unusual mechanisms of regulating and packing genes into the nucleus is required, which is likely to affect gene transcription and translation mechanisms.

While the size of the dinoflagellate genome has, to date, prevented whole genome sequencing projects, several genes and EST datasets from dinoflagellates have been sequenced. Apart from those previously mentioned that were sequenced for the purpose of phylogenetic analyses, genes that have been characterized include those of the luciferase protein family, iron superoxide dismutase, glyceraldehydes-3-phosphate dehydrogenase, redox-regulated genes and some

genes that are differentially expressed during various periods of the cell cycle in species of toxic *Alexandrium* (Li and Hastings, 1998; Okamoto et al., 2001; Okamoto and Hastings, 2003). A feature that has been noted in all genes so far sequenced is that a consensus sequence or TATA box is absent from the promoter region of the gene (Li and Hastings, 1998). A possible novel promoter has been found in the intergene spacer region of the luciferase protein family (Li and Hastings, 1998).

3.1. PLASTIDS

Plastids, and the genes that regulate them, are a highly unusual feature in dinoflagellates. Plastids are estimated to be present in about 50% of the group. The most common type of plastid in dinoflagellates contains peridinin, a carotenoid unique to the group, as a major pigment, and is surrounded by three membranes. This plastid may have been acquired relatively early in the evolutionary history of dinoflagellates, or may have been inherited from a common 'chromalveolate' ancestor, through a secondary endosymbiosis, possibly from a red algal lineage (Yoon et al., 2002; Harper and Keeling, 2003).

Some taxa appear to have recently acquired plastids from other algal lineages (Schneppf and Elbrächter, 1999). At least two lineages of dinoflagellates contain another, aberrant type of plastid: bound by three membranes, without peridinin, but instead containing 19'-hexanoyloxy-fucoxanthin or 19'-butanoyloxy-fucoxanthin as accessory pigments (Chesnick et al., 1997; Yoon et al., 2002). These plastids appear to have been inherited through a tertiary endosymbiotic event from haptophyte and diatom endosymbionts, respectively (Chesnick et al., 1997; Yoon et al., 2002). At least one lineage of dinoflagellates contains a green plastid containing the pigment prasinoxanthin, and lacking peridinin. This plastid appears to have been acquired from a prasinophyte endosymbiont (Watanabe et al., 1990). Finally, some species of dinoflagellates appear to contain 'kleptoplastids', or temporary plastids that have been taken from prey cells (Schneppf and Elbrächter, 1999). Typical examples are species of the genus *Dinophysis*, which contain 'plastids', or possibly prey items, that appear to have a cryptophyte origin and remain photosynthetically active in the host (Takishita et al., 2002), for at least some time.

Genes in the most common plastid type, that which contains peridinin as a major carotenoid, are highly unusual. Plastids genomes have been found to be hugely reduced, and they now occur as a series of plasmids or minicircles, that each contain only a single gene (Zhang et al., 1999). These minicircles appear to encode only a fraction of the number of genes generally found on plastid genomes (Zhang et al., 1999; Barbrook and Howe 2000; Laatsch et al., 2004). The rest of the genes normally found within the plastid appear to have been lost to the dinoflagellate nucleus (Bachvaroff et al., 2004; Hackett et al., 2004). In some dinoflagellates, it appears that the minicircles are present in the nucleus, rather than the plastid (Laatsch et al., 2004). Interestingly, it appears that genes from plastids more

recently acquired through tertiary endosymbiosis may have fused with genes from the older ‘typical’ dinoflagellate peridinin-containing plastid in some taxa (Patron et al., 2006).

4. Ecological Impacts of Dinoflagellates

4.1. SYMBIOTIC SPECIES AND CORAL BLEACHING

Some dinoflagellate taxa live in symbiotic associations with a diverse array of invertebrates and other protists, including foraminifera, radiolarians, flatworms, anemones, jellyfish and corals (Trench, 1987). These include a paraphyletic group of dinoflagellates, comprising at least four orders (Banaszak et al., 1993). Of these, the most significant and widespread is the relationship between members of the genus *Symbiodinium* and the reef-building corals. This relationship has been shown to contribute greatly to the success of the host coral (Trench, 1987), as it allows for the transfer of significant amounts of photosynthate, in the form of glycerol, to the host, which in turn affects growth parameters of the coral such as calcification rates (Marshall, 1996). Although less well studied, *Symbiodinium* are thought to benefit from the symbiosis through increased nutrient availability, compared to that available in the generally oligotrophic tropical waters (Mitchelmore et al., 2002).

Environmental perturbations, in the form of increased temperatures and increased solar radiation, can lead to the death of *Symbiodinium* or their loss from coral hosts. This phenomenon is known as coral bleaching. Coral bleaching can eventually cause coral death and reef degradation (Lewis and Coffroth, 2004). It is possible that in some circumstances, free-living *Symbiodinium* may recolonize corals after a bleaching event, leading to successful recovery of the coral (Lewis and Coffroth, 2004). The exact mechanism/s of the affect of environmental changes on *Symbiodinium* spp are imprecisely known, and research is currently focused on determining more clearly the causes and consequences of coral bleaching.

4.2. HARMFUL ALGAL BLOOMS

Harmful algal blooms (HABs) are a common and growing phenomenon in coastal countries, and are caused by the sudden and excessive proliferation of certain species of toxic or nuisance planktonic micro-algae (Hallegraeff, 1993). Most HAB-causing species are dinoflagellates. The most common affects of HABs are poisoning events, resulting in the deaths of marine organisms, or the accumulation of toxins in the marine food chain, leading to eventual human poisoning. Human poisoning syndromes with a dinoflagellate origin are: Paralytic Shellfish Poisoning (PSP), Diarrhetic Shellfish Poisoning (DSP), Neurotoxic Shellfish Poisoning (NSP), Ciguatera Fish Poisoning (CFP), and Azaspiracid Shellfish Poisoning (AZP). Other, less well-studied toxins with human and animal impacts have also been found. The

toxicity of some species, for example, species of *Pfiesteria*, has been suggested, but appears doubtful (Vogelbein et al., 2002). Other species (i.e. *Cochlodinium polykrikoides*) can cause mass deaths of marine organisms through simply consuming all available oxygen in the water column, sometimes causing spectacular water discolourations ('red tides') in the process (i.e. *Noctiluca scintillans*). Because of the potential for shellfish poisoning via HABs, monitoring for dinoflagellates in aquaculture growing areas has become mandatory in most countries.

CFP is the most commonly reported marine toxin disease in the world, (25–50,000 cases per annum, Ragelis et al., 1984). The toxins responsible for CFP are produced by the benthic gonyaulacoid dinoflagellate, *Gambierdiscus toxicus*, which lives epiphytically on corals and macro-algae. Ciguatoxins (CTX) are concentrated through the food web as herbivorous fish consume toxic *G. toxicus*, and are in turn consumed by carnivorous fish. The ephemeral nature of the appearance of CTX, the epi-benthic growth of *G. toxicus* and the fact that toxic fish may be found some distance from the site of *G. toxicus* proliferation, have all made it more difficult to monitor and control CFP than most other HAB phenomena (Lehane, 2000). Despite the widespread occurrence of CFP in the Pacific Ocean, western Indian Ocean and Caribbean Sea, the ecology of *G. toxicus*, as well as the physiological and biochemical pathways involved in the synthesis of the ciguatoxin structural polyether congeners are poorly understood (Lehane, 2000; Villareal et al., 2002).

Almost all HAB-causing dinoflagellates are photosynthetic, and the majority are planktonic. Many studies have been conducted into the physical and chemical factors that trigger growth and bloom formation of particular HAB species, both *in vitro* and *in vivo* in particular locations. Models of bloom dynamics have been determined for several HAB species, in particular for PSP-causing *Alexandrium* species in Japan and the USA (Yamamoto et al., 2002), for PSP-causing *Pyrodinium bahamense* (Villanoy et al., 2006) and for species of *Karenia* in the USA and Japan (Yanagi et al., 1995). Calm conditions with less wind and tidal mixing were found to be important for the formation and maintenance of *Alexandrium tamarense* blooms in Hiroshima Bay, Japan (Yamamoto et al., 2002), and this has also been observed in other HAB-causing dinoflagellates (Franks, 1997).

4.3. ECOLOGICAL ROLE OF NON-PHOTOSYNTHETIC DINOFAGELLATES

In the past, the importance of heterotrophic dinoflagellates in the marine planktonic food web was little understood. It is now known that heterotrophic dinoflagellates can constitute a substantial part of the planktonic biomass, from 13–77% in one study (Hansen, 1991). Many heterotrophic dinoflagellates are predators, and use one of several mechanisms to find and ingest their prey. Many species engulf intact prey through the sulcus at the posterior end of the cell. The may

alter their shape in order to ingest large prey items (Gaines and Elbrächter, 1987). Another group of heterotrophic dinoflagellate engulf their prey using a pseudopodium that extends through the flagellar pore, known as a pallium. In some heterotrophic species such as *Katodinium fungiforme* and *Peridiniopsis berolinensis* food is taken up by suction through a feeding tube and, often only part of their prey is ingested (Hansen and Calado, 1999). These species can often use chemosensitivity to locate injured or dying prey. Some dinoflagellates are selective in their choice of prey, while others can consume a wide variety of unicellular and even metazoan prey items (Hansen and Calado, 1999).

Dinoflagellates of the subdivision Syndinea are well-known as parasites of unicellular and multicellular organisms, including fish, copepods, radiolarians, ciliates and even other dinoflagellates (Drebes, 1984; Skovgaard et al., 2005). In the genus *Syndinium*, the parasite develops a multicellular plasmodium inside its host, a copepod, until it finally occupies nearly the entire body cavity. At maturity the host is killed and numerous free-swimming zoospores escape (Skovgaard et al., 2005). Parasitism by dinoflagellates may be a significant influence on the marine pelagic food web. It has been estimated to exert a very similar pressure on ciliate numbers as the grazing pressure from metazoan predators (Coats et al., 1996).

5. Summary

Dinoflagellates exhibit extreme diversity in almost all ecological factors, including their modes of nutrition, habitat, life style and the presence or type of their plastids. This diversity belies the several unique ultrastructural and molecular biological characteristics that all dinoflagellates appear to share. Determining with certainty the phylogeny within the group has proved a challenge, and new approaches are probably required in order to progress. Studies of the evolution, ecology, diversity and physiology of dinoflagellates will continue to increase with the increasing awareness of human impacts of these organisms, and will no doubt continue to reveal more fascinating anomalies specific to this group of protists.

6. References

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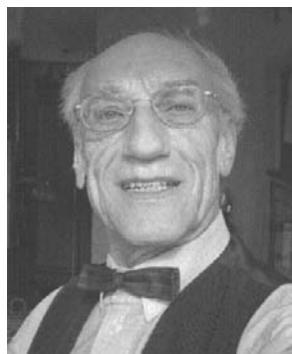
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Biodata of **Frithjof Sterrenburg, Richard Gordon, Mary Ann Tiffany, and Stephen S. Nagy**, authors of the chapter “*Diatoms: Living in a Constructal Environment*”

Frithjof Sterrenburg became intrigued by diatoms and the limitations of the optical microscope while wrestling with his father’s A.D. 1850 Oberhäuser microscope as a kid. Despite this handicap, he then studied electronics and medicine, and for over 40 years was an independent consultant to hi-tech industries and research centers, ranging from medical electronics and environmental science to communications technology and defense. Other activities included membership of organizations involved in postgraduate training and the editorial board of a professional electronics journal. He began publishing on diatoms in the early 1970s and is now working on the Kinker diatom collection for the National Natural History Museum of the Netherlands.

Dr. Richard Gordon is a theoretical biologist whose endeavors range from AIDS prevention to breast cancer imaging on the medical side and from the effects of microgravity on amphibian embryos to the delights of diatom motility and morphogenesis on the basic science side. He inadvertently wrote the first paper on diatom nanotechnology. He has a B.Sc. in Mathematics from the University of Chicago (1963) and a Ph.D. in Chemical Physics from the University of Oregon (1967), and is now a Professor of Radiology, Computer Science, and Electrical and Computer Engineering at the University of Manitoba.



Frithjof Sterrenburg



Richard Gordon

Dr. Mary Ann Tiffany was raised by two scientist parents who imbued her siblings and her with an atmosphere of curiosity and excitement about nature. At a young age she enjoyed peering at the antics of creatures from a local pond through a microscope, preferring this to other entertainment. She doubted about her possessing any artistic ability, until discovering a natural talent for capturing the beauty of diatoms and other algae using the scanning electron microscope. She prefers a session at the SEM to other pastimes, often discovering something exciting and new to science. Presently she is pursuing a doctorate in ecology at San Diego State University under Stuart Hurlbert, studying the phytoplankton of California's largest lake, the saline Salton Sea.

Dr. Stephen S. Nagy was fascinated by optical microscopes as a teen, and realized that he was literally surrounded by freshwater fossil diatomite when living in Southern Oregon. In 1999 he became a student of Klaus Kemp, learning the diatomist's slide-making art at long distance, taking lessons by e-mail and telephone. He received his undergraduate degree from Yale College (1973), his M.D. from The Medical College of Pennsylvania (1977), and completed his residency training at Pennsylvania Hospital and the Institute of Pennsylvania Hospital (1981). He is a Diplomate of the American Board of Psychiatry and Neurology in Adult Psychiatry (1982), and is certified by the American Society of Addiction Medicine (1990). He lives in Montana's capital city, and is a Staff Psychiatrist at VA Montana Healthcare, Fort Harrison.



Mary Ann Tiffany



Stephen S. Nagy

DIATOMS:

Living in a Constructal Environment

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1. Introduction

Diatoms (Bacillariophyta) are diploid eukaryotic unicellular algae with a wide range of regular and decorative shapes (Plate 1) that are now placed in the Heterokontophytes by botanists or the Stramenopiles by zoologists (Medlin et al., 1997). Using oxygenated carotenoids as light-harvesting pigments, they are generally photo-autotrophic, although there are N-heterotrophic species. Diatoms occur in very large populations in both freshwater and marine environments, in all climatic zones. They have been a very successful group from an early moment in their history. While claims of observations in Permian or even in Carboniferous deposits (Zanon, 1930) were spurious, the group's origin is generally placed in the Jurassic, as in Round et al. (1990). The oldest fossil records are of marine species – some hardly different from modern ones, others without surviving relatives – and in deposits from the Cretaceous both species diversity and number of specimens can already be impressive (Harwood and Nikolaev, 1995).

The outstanding feature of diatoms is their siliceous “shell” or frustule, which can be preserved for millions of years. In this manner, fossil deposits of microscopic diatom shells were built up as thick layers of “diatomaceous earth” extending over several miles, such as the deposits near Lompoc, California, USA, and such processes continue today in all oceans and lakes. Diatoms are of major importance in the aquatic food chain and as a source of atmospheric oxygen. As regards current diversity in the group, estimates vary between 10^5 and 10^6 species – a strong indication of the prevailing uncertainty, but equally strong evidence of a rich genetic repertoire. For this overview of diatoms, we have used a variety of fossil and recent materials from our personal collections. The light-microscope photomicrographs (LM) and arranged diatom slides were made by Stephen Nagy using a Kemp micromanipulator, a Zeiss Universal microscope with Nikon CFN planapo and Olympus S-planapo objectives, and a Canon Powershot G6 camera. Scanning-electron micrographs (SEMs) were made by Mary Ann Tiffany using

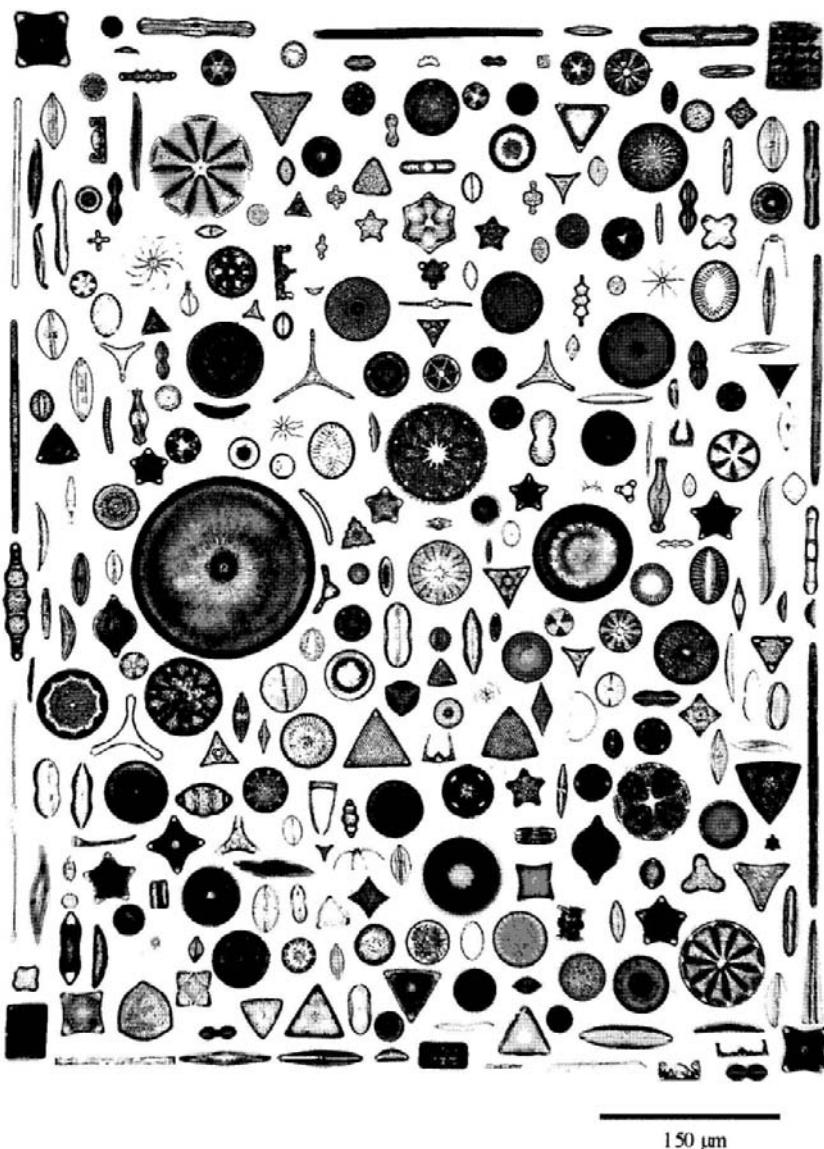


Plate 1: In the Victorian craftsman's tradition: slide of 300 diatoms mounted by Stephen Nagy; recent and fossil, freshwater and marine, from the UK, Holland, France, New Zealand, Sulawesi, Caribbean, Indian Ocean, Florida, Maryland, Oregon, Montana, Nevada, British Columbia, California, Alaska, Honolulu, and Russia. Bar = 150 µm.

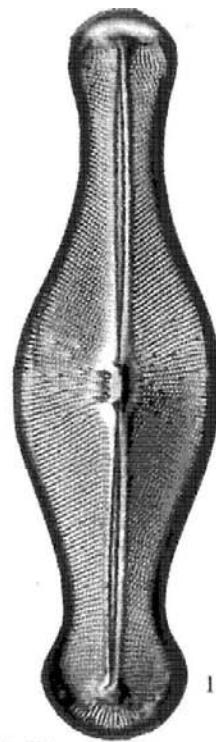
the von Stosch method (Hasle and Syvertsen, 1997); specimens were sputter-coated and examined in a Hitachi S-2700 scanning electron microscope at an accelerating voltage of 10 kV.

2. Ecology

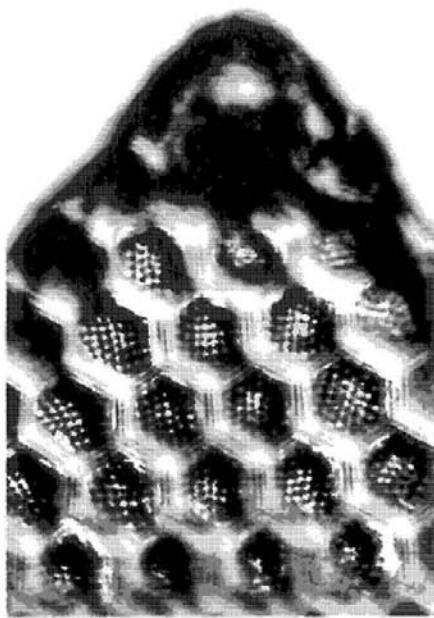
In the diatom literature of the nineteenth and early twentieth centuries, the observations were primarily related to the locality from which contemporary expeditions had collected samples, with only a freshwater or marine origin specified. Thus, the magnificent atlas by Schmidt et al. (Schmidt, 1874–1959) refers to tantalizingly exotic abodes like “Zanzibar” or “Nankoori,” and the numerous papers titled “Diatoms from . . .” tended to overemphasize locations. In the subsequent decades of the twentieth century, attention shifted to ecology rather than biogeography, because microscopic organisms might in principle be easily distributed worldwide to thrive wherever they find a suitable habitat. It became apparent that many species are fastidious as regards the pH, salinity, or degree of pollution of their environment and especially for routine water quality assessment, diatoms have now become valuable environmental indicators (see Stoermer and Smol, 1999).

More recently, it has nevertheless become increasingly evident that endemic diatoms do indeed occur (see, for instance, Metzeltin and Lange-Bertalot, 1998; Moser et al., 1998). In view of the vast number of diatom species and their minute morphological characters, taxonomy is difficult, and as taxonomic (especially electron-microscopic) investigations became more precise, it became clear that several morphologically and biogeographically distinct species had been ranked under single, supposedly “ubiquist” labels. Surprises in the field of biogeography and ecology continue to turn up, as shown by the following example.

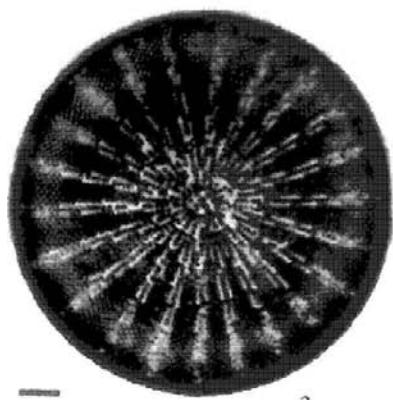
The diatom *Didymosphenia geminata* (Lyngbye) M. Schmidt in A. Schmidt (Plate 2, Fig. 1) is so characteristic that it is easily identified. In the freshwater flora of Krammer and Lange-Bertalot (1986), which is generally regarded as an authoritative publication, it is described as a “very rare species of boreal and alpine regions of Europe, Asia and North America, limited ecologically to oligotrophic, cold waters of low to moderate electrolyte content.” This profile certainly appears to be too restrictive, as the species has often been recorded from nonboreal regions (e.g., the UK, France, Spain, see Cleve, 1894–1896), but until very recently, the literature had not reported a phenomenon now observed: populations of this species have exploded in the form of blooms so massive that even the general public has started to complain (Kilroy, 2004). In streams of the USA, Vancouver Island, Europe, Iceland, and New Zealand, the bottom may be covered with thick colonies of this diatom over distances of many miles (Kilroy, 2004; Spaulding, 2006). Research presented at an International Symposium on *Didymosphenia* in Bozeman, Montana, USA at the time of writing, suggests that



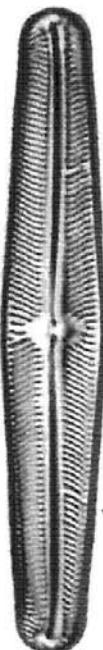
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4

the organism grows in a much broader temperature range than previously thought: 5–25°C (Bothwell et al., 2006). Because it survives for 5–10 days in a dark, wet environment, and for >40 days in a light, wet environment, the sudden occurrence of *Didymosphenia* in remote fishing rivers previously uncolonized may be caused by the mobility of international fly-fishermen and the relatively new popularity of the felt-soled wading boot, which traps and holds both the diatom and moisture, ready to be inoculated into a new ecosystem (Biggs, 2006).

3. Harsh Environments

Given a sufficient supply of sunlight, water, and minerals (especially silicon), floras of planktonic (suspended in the water), epiphytic (on plants), and benthic (on and in the sediment) diatoms can thrive in a wide variety of habitats. Some of these environments are “harsh”:

Example 1, thermophiles. Diatoms have been described in hot springs (Hustedt, 1937; Krasske, 1938; Van der Werff, 1941; Baudrimont, 1973; Owen et al., 2004). Although some of these studies verified that chloroplasts were still present in some frustules (excluding “empty” frustules of dead diatoms), it is not certain that these diatoms were still *alive* – cells from cooler areas may well have entered hotter areas by seepage, to be cooked there including the chloroplasts. Owen et al. (2004) specifically mention that at >50°C diatoms became scarce or absent in the samples studied. An upper temperature of 83°C is mentioned by Harwood and Nikolaev (1995), but this statement is not further elucidated and no reference is given. Clearly, culturing experiments are called for instead of floristic sampling for such investigations. Moreover, assuming that the identifications were correct, the floras mentioned in the papers referred to earlier appear to mainly or exclusively contain quite ordinary species that are normally found in cooler waters, for example, *Craticula cuspidata* (Kützing) D.G. Mann (Round et al., 1990), *Achnanthes exigua* Grunow, or *Cocconeis placentula* Ehrenberg.

Example 2, acidophiles. Peat bogs are characterized by floras of typical acidophilic and oligotrophic diatoms, of the genus *Eunotia*, for instance, which are not seen in other environments. A recent study (Witkowski et al., 2007) describes an environment of truly “extreme” pH (2.5), but this is indeed unusual. In a



Plate 2:

Figure 1. *D. geminata* (Lyngbye) M. Schmidt in A. Schmidt. Recent, freshwater, Rock Creek at Red Lodge, Montana. Scale bar = 10 µm.

Figure 2. *T. favus* Ehrenberg. Recent, marine, Hoofdplaat, Holland. DIC to illustrate sieve plate. Scale bar = 10 µm.

Figure 3. *Actinocyclus ehrenbergii* Ralfs in Pritchard. Fossil, marine, Dunkirk, Maryland. Scale bar = 10 µm.

Figure 4. *Pinnularia* species. Recent, brackish, Great Salt Lake, Layton, Utah. Arrows indicate Voigt faults. Scale bar = 10 µm.

review of the diatom taxa reported in acidic environments, DeNicola (2000) concluded that there may be a threshold between pH 4.5 and 3.5 below which many species are unable to maintain a population.

Example 3, halophiles. Many diatom species tolerate high salt concentrations. In extreme cases, salt concentrations may even be so high that NaCl crystals grow inside the silicalemma (Gordon and Brodland, 1990). Massive diatom blooms occur in very saline and highly polluted waters, for example, the Salton Sea, California, USA, with a salinity of 45–50 g l⁻¹ (Sterrenburg et al., 2000; Lange and Tiffany, 2002; Tiffany et al., 2007) and Farmington Bay of the Great Salt Lake, Utah, USA, highly polluted by sewage from Salt Lake City with a salinity varying from about 40 to 110 g l⁻¹ (Aldrich and Paul, 2002). A collection by Tiffany from Farmington Bay in January 2003 at 90 g l⁻¹ (unpublished data) revealed a relatively rich flora consisting of diatoms from the genera *Nitzschia*, *Navicula*, *Anomoeoneis*, *Amphora*, *Entomoneis*, *Cyclotella*, and *Thalassiosira*. Several species recorded in the publications referred to earlier are also found in less extremely saline environments, so they are euryhaline rather than halophilic. An interesting (and as yet unsolved) puzzle is the presence of rich crops of the diatom *Gyrosigma wormleyi* Sullivant in the very saline Salton Sea. In a type study (Sterrenburg, 1994), this diatom was verified to be a freshwater species and the Salton Sea specimens are a full morphological match for the type population (Sterrenburg et al., 2000). As the Salton Sea originated from an inadvertent detour of the Colorado River, an initially freshwater population of *G. wormleyi* may have evolved to a halophilic profile as the water became increasingly saline over the past century or so.

Finally, massive and varied populations of diatoms were collected (Sterrenburg, unpublished data) from the Reese River, Nevada, USA, so strongly alkaline that the river bank was encrusted with salt crystals. Again, some of the species present (e.g., *C. cuspidata*) are also common in freshwater, others (e.g., *Rhopalodia* sp.) still await definitive identification.

Example 4, "shock-proof diatoms." On mudflats in the tropical marine littoral, severe osmotic shock and extreme changes in temperature within brief intervals of time constitute a hazard (see Sterrenburg, 1989). Secretion of copious mucilage tubes may have a protective effect in this case, but the species examined in the study (*Gyrosigma obliquum* Grunow) is also found in less variable environments.

Example 5, xerophiles. Many diatoms can tolerate temporary desiccation, for example, in protective mucilage envelopes or as resting spores. In this manner, they can be transported by or perhaps in (Bennett, 1989) migratory water fowl or storms (Spitz et al., 1965; Chalmers et al., 1996) over large distances. This does not yet mean that they are true xerophiles, however. For the diatom *Luticola* (*Navicula*) *mutica* (Kützing) D.G. Mann and some related species it has long been known that dry rocks and stone walls are a habitat (Bock, 1963, 1970) but again, *L. mutica* is commonly found also in all sorts of freshwater ponds, ditches, etc., so that it is "xero-tolerant" rather than a true xerophile.

Example 6, "obscurophiles." Diatoms, especially as spores, can survive total darkness under experimental and natural conditions (subduction in oceans) for at least months (Fryxell, 1983; Oku and Kamatani, 1995; Murphy and Cowles, 1997), but this applies to a resting phase able to tolerate darkness.

Example 7, cryophiles. In late winter or very early spring (end of February in the Northern hemisphere), the lower surface of the ice on ponds and ditches may be covered with an abundant growth of diatoms. Observations in the course of many years (Sterrenburg, unpublished data) have shown that such floras consist of ordinary species present throughout the year in the sediments. Attracted by the sunlight, they float upward and settle on the lower surface of the ice, but they are not true cryophiles or psychrophiles. The situation is different for the rich and typical floras described (e.g., Poulin and Cardinal, 1982a, b; Janech et al., 2006) from Arctic sea-ice, however, where true cryophiles are present. The ecology of such sea-ice diatoms is interesting from yet another perspective. In Janech et al. (2006) it is said that their environment is hypersaline, but for microorganisms one should think in the appropriate temporal and spatial dimensions (Sterrenburg, 2005).

On the lower surface of ice-floes, the diatoms will live in a *limes divergens* with saline ocean water on one side and salt-free ice on the other. Such an interface may be only a thin layer, but it offers ample *Lebensraum* to a microorganism and in view of the marked gradient in salinity, the actual condition under which the sea-ice diatoms live is probably not that simple. Verification would call for microprobe measurements at the seawater/ice interface.

Example 8, "food commuters." Singler and Villareal (2005) describe an interesting case of migration for nutrient acquisition in mats formed by the planktonic diatom of the genus *Rhizosolenia*. In the upper euphotic zone of the ocean, little or no nitrification appears to occur, nitrates being present in a nutrient sink at the base of the euphotic zone. *Rhizosolenia* cells are large and because of their low surface area/volume ratio are at a disadvantage when competing for scarce nutrients with the nanoplankton that dominates the phytoplankton. By extensive vertical migration, these diatoms thus exploit deep nitrate pools, acquiring nitrate at depth and returning to the euphotic surface for photosynthesis.

Example 9, "Aliens." In our perambulations through the diatom literature, we encountered a suggestion that diatoms might well be difficult to beat for extremophilia. Hoover et al. (1986) describe a close correspondence between the measured infrared properties of diatoms and the infrared spectrum of interstellar dust as observed in the Trapezium nebula (in the constellation Orion) and toward the infrared source near the galactic center GC-IRS 7. The same applies to the ultraviolet absorbance. The authors suggest that the findings are consistent with the concept of a cosmic microbiological system in which these or similar microorganisms might exist on comets, Jupiter's moon Europa, and in interstellar space. Although Tiffany's SEM photomicrographs have sometimes reminded the other authors of certain Star Trek episodes, we think that actual field studies in the Trapezium Nebula and GC-IRS7 are required for confirmation.

4. Are Diatoms Extremophiles?

Diatoms taken as a group cannot be regarded as “extremophiles.” The vast majority of diatoms are mesophilic photoautotrophs, quite in contrast to the Methanobacteria, for instance. For many diatom species living in the special habitats mentioned earlier, it would be more appropriate to say that they also *tolerate* harsh conditions, without being *dependent* on them. The ability to reproduce in an extreme environment does not per se imply that an organism is extremophilic if it also occurs in ordinary environments. After all, the ability of *Homo sapiens* to reproduce consistently in environments with temperatures ranging from +50 to -30°C. does not mean that humans are thermophilic as well as psychrophilic extremophiles! In our view, a true extremophile lives in an extreme environment that forms its *exclusive* habitat, with the black smoker communities as an outstanding example.

Given the extraordinary evolutionary success of the diatoms, it is not surprising, however, that some species have ventured into extreme ecological niches and have indeed become extremophiles sensu stricto. Such is the case for species limited to oligotrophic or even dystrophic peat bogs (Example 2), for instance. There are also good arguments for including sea-ice diatoms (Example 7) among the extremophiles, as the marine ice-floe diatom flora consists of very typical species. In contrast, the diatom floras seen on the lower surface of ice in freshwater ditches, etc. (Example 7) consist of species commonly present throughout the year that are apparently indifferent as regards temperatures.

Systematic investigations on the physiology and genetics of those diatom species that are able to live in the “harsh” habitats referred to earlier are only just beginning. In Janech et al. (2006), the sea-ice diatom *Navicula glaciei* VanHeurck is shown to produce an “antifreeze protein” resembling those produced by psychrophilic snow molds, for instance. But it is too early as yet to determine in how far these extremophilic species have adopted a special physiology to cope with the special circumstances and whether this is consistent for the species from widely different genera that do thrive in such harsh environments. The DNA sequencing of diatom genomes has just begun (Alverson et al., 2003; Fox and Sorhannus, 2003; Armbrust et al., 2004).

5. “Silicatophiles”

With very rare exceptions, diatoms are “extremists” in that they are capable of, and indeed depend on, handling one of the most refractory materials in Nature, silica – hydrated SiO_2 or Si(OH)_4 . In this they are not alone: Phanerogams like marram grass (*Ammophila*) and stinging nettles (*Urticaria*), Cryptogams like Silicoflagellates, Protozoa like Radiolarians, or animals like sponges also handle silica, but in diatoms this property has become a pivotal issue. From a metabolic

point of view, the silica frustule of diatoms might only be considered as “excreta,” but silica management has attained a dominant position in the diatom’s physiology and morphogenesis: even DNA synthesis is Si-dependent (Okita and Volcani, 1978). Silicon is thus essential to diatoms: an insufficient supply of Si in the environment will prevent diatoms from flourishing, or results in teratological specimens. On the other hand, the excreted silica is impermeable and a solid silica shell would be the diatom’s coffin. The silica shell has therefore to be perforated on the micro- or the nanoscale, while still maintaining the mechanical integrity the living cell within seems to require. A typical example is the structure of *Triceratium favus* Ehrenberg (Plate 2, Fig. 2) in which each cell of the honeycomb structure is covered by a perforated sieve membrane, also termed the “hymen.”

The standard explanation of the silica shell’s role is that it protects the diatom protoplast, for example, against predation (Hamm et al., 2003; Hamm, 2005), but nevertheless, at 25% of the earth’s biomass (Werner, 1977), diatoms are the prime food source for all sorts of animals, from Tunicata like salps to grazing Molluscs (snails and limpets) and filter-feeders like oysters. So, such protection is far from assured.

In the fine art of balancing the requirement for protection and the maintenance of unhindered communication between the living organism and its environment, diatoms have become a “constructal paradigm” analogue (Bejan, 2000, 2005; Wikipedia, 2007) – their morphology may begin to resemble that of inanimate natural constructions like snowflakes or crystals or human products like diadems, brooches, or other such baubles. A diatom’s environment appears to be under the aegis of a celestial Euclid: in the world of diatoms, physical and geometric rules are strongly expressed in the organism’s phenotype. As a result, their ordered shapes and decorative patterns have attracted diatomists from the very beginning and as microscopes became capable of revealing the finer details of diatoms after the mid-nineteenth century (Frison, 1954), esthetics became a major factor in the study of diatoms. This is evident from the artistic arrangement of diatom illustrations (all line drawings at that time) in the published works of the Victorian era and the decorative arrangements of diatoms as prepared slides of that period. The former practice is well illustrated in Haeckel’s (1904) magnum opus *Kunstformen der Natur*. The latter craft is still practiced by a few contemporary diatomists including one of us (Nagy), as shown in Plate 1.

In the second half of the twentieth century, the electron microscope led to a vast number of fundamental studies on diatom valve morphogenesis. A detailed survey is given in Pickett-Heaps et al. (1990), and although many papers on aspects of the subject have been published since, the general principles outlined there are still regarded as valid. Particularly as a result of the introduction of the scanning-electron microscope, diatoms have become of interest to architects (a good survey is Bach and Burkhardt, 1984) and nanotechnology (see Gordon et al., 2005).

6. Reproduction and the Diatom Shell

The diatom shell (frustule) consists of two interlocking boxes or valves (Plate 3, Fig. 1), both of which have girdles attached, the latter allowing expansion and overlap. During mitosis, new valves are formed within the existing ones, starting from a membrane-bound cytoplasm structure called the silica deposition vesicle (SDV) at the center of the cell and moving outward from there. The SDV fuses with the cell membrane externalizing its valve. The consequence is that one line

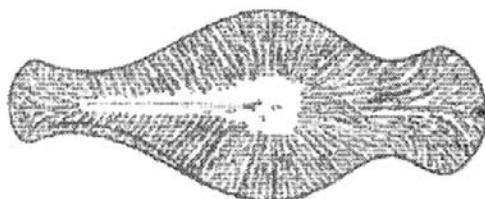
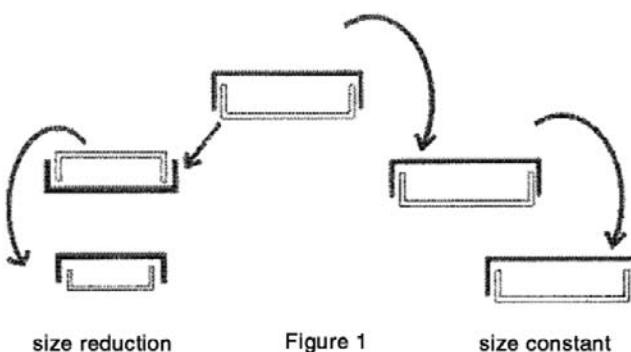


Plate 3:

Figure 1. On mitosis, the progeny that uses the larger valve (black) will have the original size, the progeny that uses the smaller valve (white) shows continuous size reduction.

Figure 2. Sequence of formation of the periphery of the valve in Pennates.

Figure 3. Teratology in *D. geminata*, loss of stria pattern organization secondary to malformation of raphe. Line drawing from notebook (Sterrenburg, 1973).

of the progeny will become smaller and smaller with each act of vegetative reproduction, and, although this can go on for some time, leading to a marked range of variation as regards size within a species (e.g., Ussing et al., 2005), a point is reached when the smallest offspring is no longer capable of further mitosis. Sexual reproduction then takes over and the resulting swollen auxospores form the start of a new line. The auxospores and initial cells differ considerably from the species' "normal" aspect, being more or less spherical or sausage-shaped (Plate 8, Fig. 1). Whereas during mitosis the old frustule serves as a mold because the new frustules are constrained within it, frustule morphogenesis after sexual reproduction has to start anew, and there have to be (as yet unclarified) reliable controlling mechanisms to ensure that on subsequent vegetative reproduction, the new line will faithfully reproduce the characteristic morphology of its ancestors. Typically, this may take a couple of cell divisions. The spatial confinement of the new valves within the old ones during mitosis implies that markedly aberrant shapes can only originate on sexual reproduction, to be transmitted through the subsequent vegetative divisions. For instance, there exist quadrangular forms of normally triangular *Triceratium* species. The relationship, if any, of this non-genetic form of inheritance to "cortical inheritance" in protozoans (Frankel, 1989; Grimes and Aufderheide, 1991) has not been investigated.

The main division in the Bacillariophyta is in radially symmetric forms (Centrics) (Plate 2, Fig. 3) and bilaterally symmetric forms (Pennates) such as *Pinnularia* (Plate 2, Fig. 4) or *Didymosphenia* (Plate 2, Fig. 1). The Centrics appear first in the geologic record and are regarded as ancestors of the Pennates (see Alverson and Theriot, 2005), but the difference between the two groups is not just one of symmetry – Centrics sexually reproduce by oogamy, Pennates by isogamy. A large group of Pennates possesses a longitudinal slit (raphe) in the "midrib" (raphe sternum) of the valve (Plate 8, Fig. 2, black arrow), which is an organ of locomotion (see Gordon and Drum, 1970; Gordon, 1987).

In the Centrics, silica for the new valves is deposited in a pattern radiating from the center, as is clearly seen in an early stage of the formation of an *Amphitetrax* valve (Plate 8, Fig. 3). In the Pennates, silica deposition starts from the "midrib" outward, which is thus already heavily silicified when the outer portions are still developing (Plate 4, Fig. 4). Formation of the valve's periphery then progresses in the manner shown in Plate 3, Fig. 2. Where the two developing sections of the periphery eventually meet, a slight "glitch," the Voigt fault, often disturbs the regular fine pattern of the valve. This is shown arrowed in LM in a *Pinnularia* (Plate 2, Fig. 3) and in SEM in *Caloneis* (Plate 4, Fig. 2, white arrows). In genera like *Cocconeis* (Plate 5, Fig. 1), the valves consist of a single layer, but in many others they have a complex three-dimensional structure. In genera like *Thalassiosira* or *Coscinodiscus*, valve formation progresses from the interior to the exterior surface. This is clearly shown in the series of developing valves of *Coscinodiscus granii* Gough (Plate 9, Figs. 1–4). In other genera, such as *Pinnularia*, however, valve formation is from the exterior to the interior surface. Computer simulations of a diffusion-limited aggregation model for diatom valve

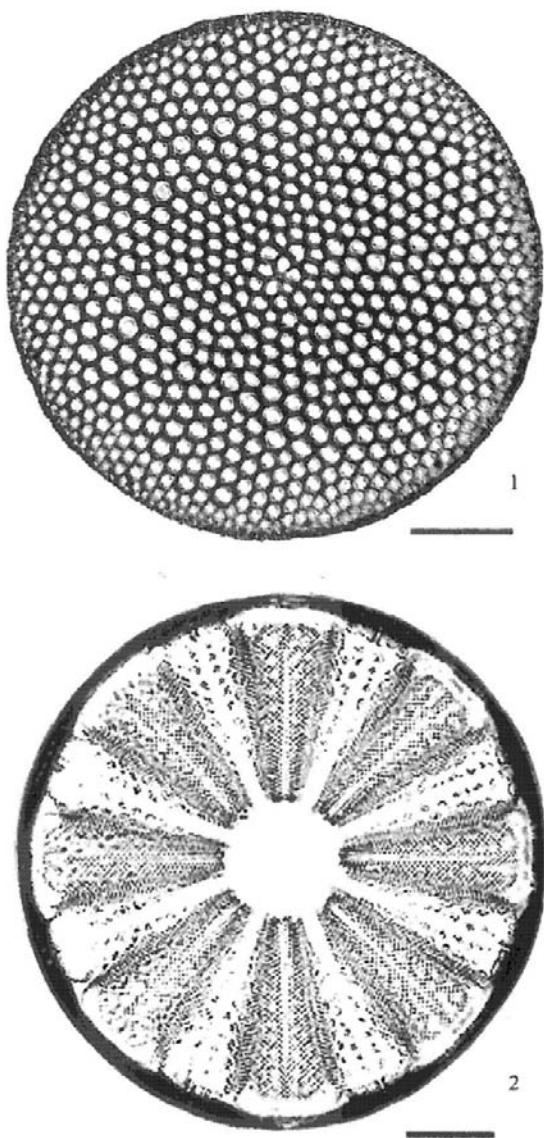
**Plate 4:**

Figure 1. *Coscinodiscus radiatus* Ehrenberg. Recent, marine, Sulawesi. Scale bar = 10 µm.

Figure 2. *Actinopytychus maculatus* (Grove and Sturt) A. Schmidt. Fossil, marine, Cormack's Siding, Oamaru, New Zealand. Scale bar = 10 µm.

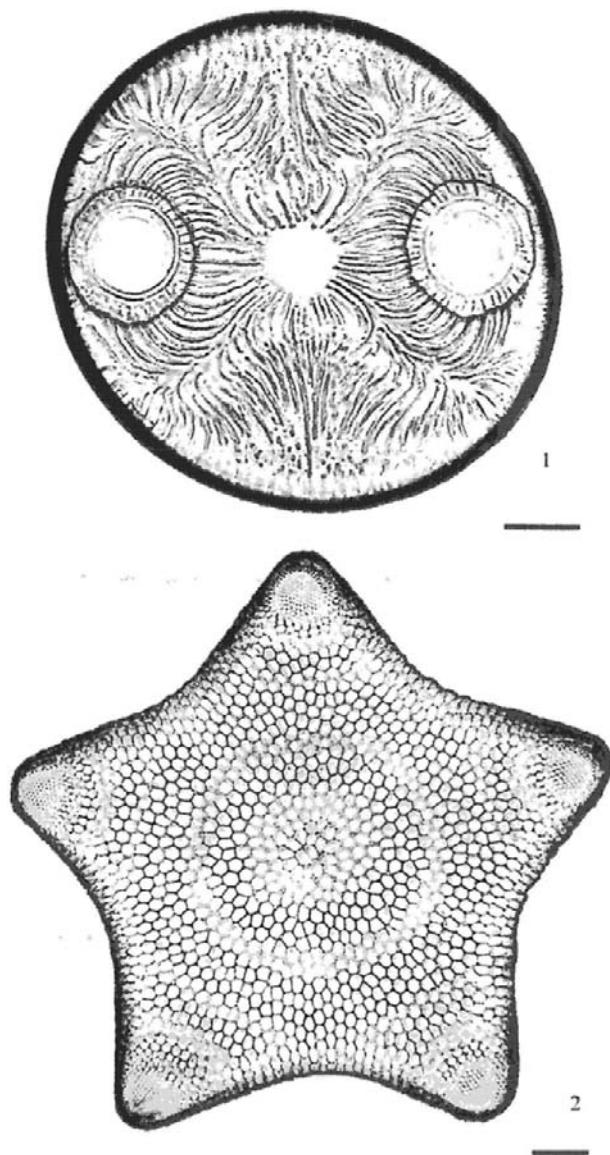
**Plate 5:**

Figure 1. *Auliscus elegans* var. *californica* (Grunow) Rattray. Fossil, marine, Newport Back Bay, California. Bar = 10 μ m.

Figure 2. *Triceratium formosum* var. *quinquelobata* (Greville) Hustedt. Recent, marine, Waikiki Aquarium, Honolulu, Hawaii. Scale bar = 10 μ m.

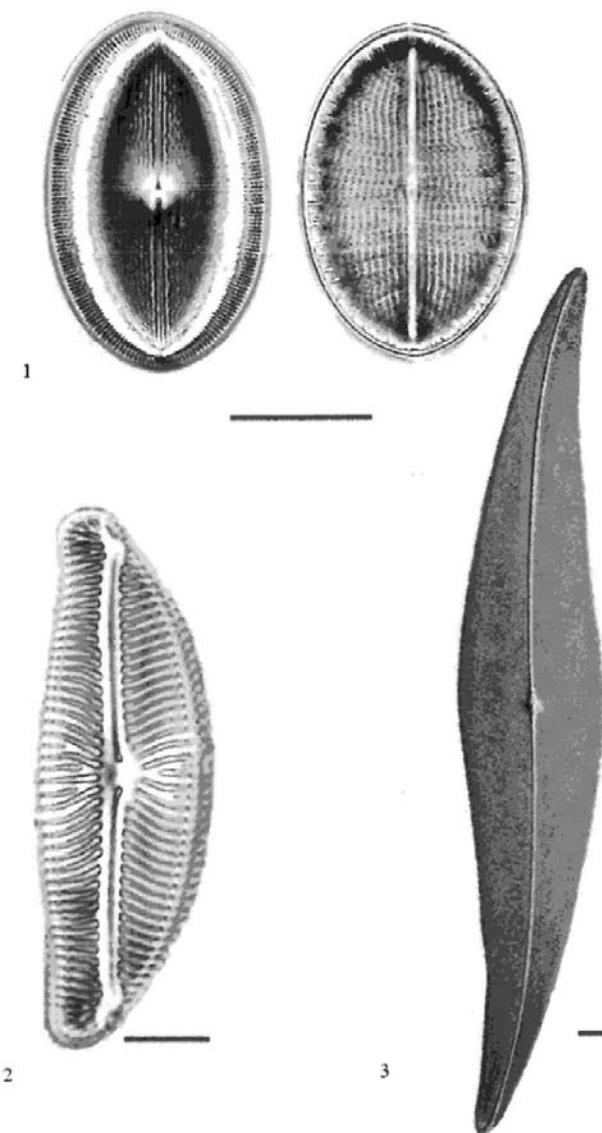
**Plate 6:**

Figure 1. *Cocconeis klamathensis* Sovereign. Recent, freshwater, headwaters of Wood River in Jackson Kimball State Park, Klamath County, Oregon. Bar = 10 µm.

Figure 2. *Cymbella prostrata* (Berkeley) Brun. Recent, freshwater, Sun River, Augusta, Montana. Bar = 10 µm.

Figure 3. *Pleurosigma angulatum* (Qukett) W. Smith. Recent, marine, Somerset, UK. Bar = 10 µm.

morphogenesis consistent with these modes of shell growth have been carried out (Gordon and Drum, 1994), though refinements including the supposed roles of microtubules are necessary to get anything at all resembling real diatoms (Parkinson et al., 1999). In many diatoms, the inner valve surface can display a structure totally different from that of the outer, as is the case in *D. geminata* (Plate 10, Figs. 1 and 2, Plate 2, Fig. 1). Mitochondria may play a role in this difference for some diatoms (Pickett-Heaps et al., 1990; Gordon and Drum, 1994).

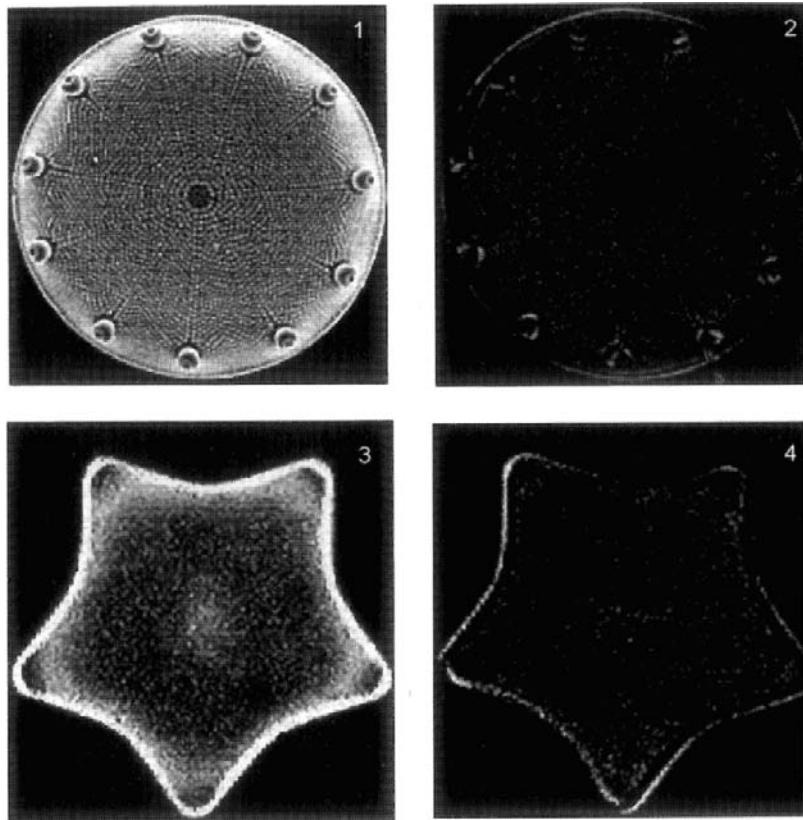


Plate 7:

Figures 1 and 2. The result of subtracting the SEM image of *A. oregonus* with 11-fold symmetry from its own image rotated by $360/11 \approx 32.7^\circ$. Fig. 2 would be all black if symmetry and centering were perfect. Where a near perfect match obtains, the image is nearly black.

Figures 3 and 4. Same for a LM image of *T. formosum* var. *quinquelobata* with fivefold symmetry and rotation by $360/5 = 72^\circ$.

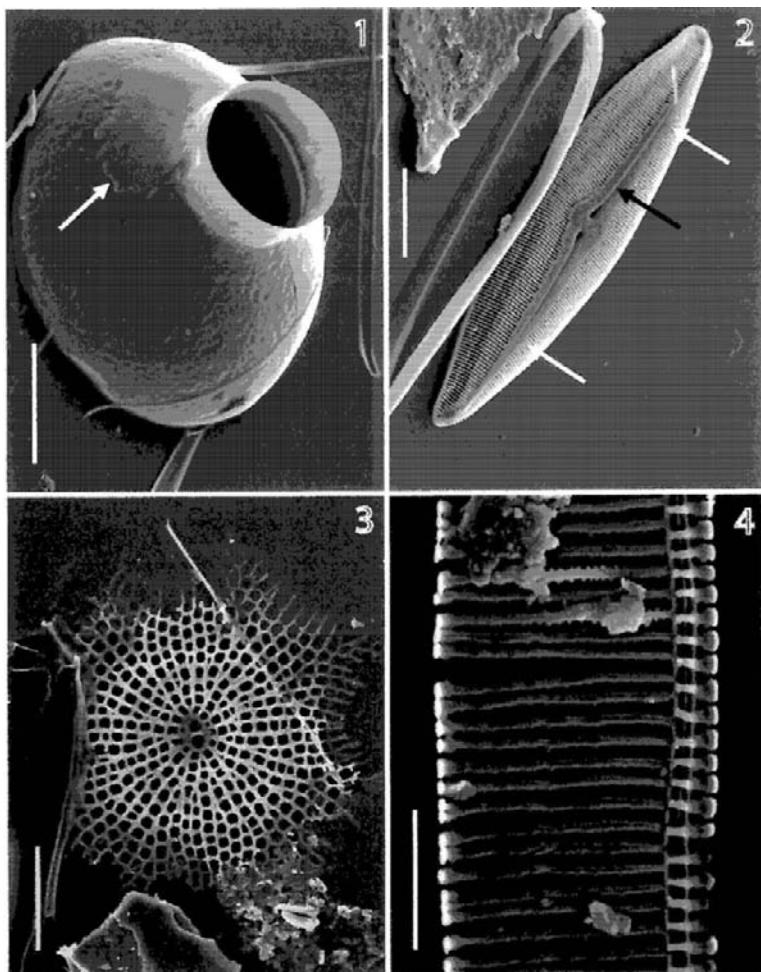
**Plate 8:**

Figure 1. *Pleurosira laevis* (Ehrenberg) Compère. Alvarado Creek, San Diego, California. Large epivalve of initial cell attached to smaller mother cell with remnants of auxospore scales attached (arrow). Scale bar = 50 μm .

Figure 2. *Caloneis amphisbaena* (Bory) Cleve. Garst Road, Salton Sea, California. Black arrow points to raphe, white arrows indicate Voigt faults. Scale bar = 20 μm .

Figure 3. Forming valve of *Amphitetrads antediluvianum* Ehrenberg. Point Dume State Preserve, California. Ribs radiate from central annulus. Scale bar = 20 μm .

Figure 4. Delicate forming valve of *Pseudonitzschia australis* Frenguelli. Mission Bay, San Diego, California. Raphe sternum is at top, with fibulae, structures spanning the raphe, already well formed. Bar = 5 μm .

7. “The Architectural Diatom”

The constructal principles controlling the remarkably “architectural” diatom morphology are apparent on both the microscale (the general morphology of the valves) and the nanoscale – the fine structure of the valves. These constructal principles appear to be aimed at generating a self-supporting structure with minimal consumption of silica, as this may be in scarce supply, for example, in the open ocean and in oligo- to dystrophic peat bogs. Also, the shell must be able to bear mechanical loads, not only from the outside, but especially also from within. The living cell is under turgor and this would push the two valves apart, exposing the protoplast. The valves are therefore held together by cytoplasmic strands, which are anchored at specific structures of the shell: the rimoportulae (“labiate processes,” Plate 7, Fig. 3) and fultoportulae (“strutted processes,” Plate 7, Fig. 4) in the Centrics and the raphe ribs and septa in Pennates. These anchoring sites are thus of cardinal importance to the spatial arrangement of the diatom shell and their precise location is essential to the shell’s morphology. For example, a teratological specimen of *D. geminata* (Plate 3, Fig. 3) showed total absence of one half of the raphe sternum (“midrib”) and an incompletely formed other half and raphe (Sterrenburg, 1973). The resultant gross anomalies in the stria pattern clearly demonstrate the role of the raphe sternum as a center of morphological organization.

As regards general morphology, various types of symmetry are observed. In the Centrics, symmetry has been generally called radiate, but besides truly radiate symmetry as in *Coscinodiscus* (Plate 4, Fig. 1), triradial or multiradial (*Actinoptychus*, Plate 4, Fig. 2 or *Triceratium*, Plate 5, Fig. 2) symmetry occurs. Around 1850, Ehrenberg described several species in the genus *Actinoptychus* depending on the number of sectors (e.g., *Actinoptychus quatuordenarius*), but these are now all ranked as a single species, *Actinoptychus splendens* (Shadbolt) Ralfs, where the number of sectors can vary over a wide range. A similar variability is seen in *Aulacodiscus oregonus* Harvey and J.W. Bailey (Plate 11, Figs. 1 and 2).

In many Centrics, however, the symmetry is not truly radiate. A notable exception is the genus *Auliscus* (Plate 5, Fig. 1), where radiate structure is combined with bilateral symmetry. Also, in the normally radiate genus *Cyclotella*, aberrant bilaterally symmetric specimens have been observed (Plate 11, Fig. 3).

How rigidly controlled a centric’s general morphology may be is illustrated by an experiment we carried out: subtracting a rotated image of two centric diatoms from the original image (Plate 7, Figs. 1–4). If the result looks horrid, that’s just what we hoped for: if the match were perfect, the image would be completely black and the small actual residue indicates that the match is excellent. The apparently high degree of perfection of this noncrystalline precipitate deserves quantification, which may prove comparable with the degree of imperfection of crystalline snowflakes (Libbrecht and Rasmussen, 2003).

In the Pennates, symmetry is basically bilateral in relation to the heavily silicified “midrib,” but it can be isopolar as in *Pinnularia* (Plate 2, Fig. 4) or markedly

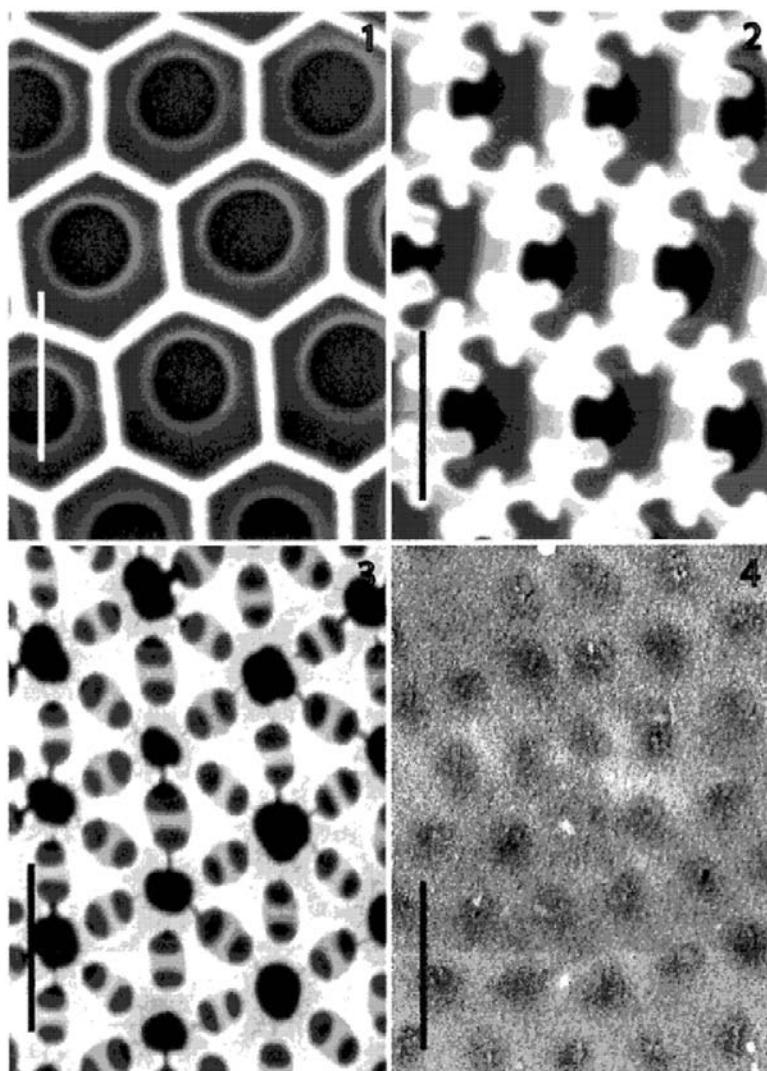
**Plate 9:**

Figure 1. Close-up of early forming valve of *C. granii* Gough. Mission Bay, San Diego, California, showing hexagonal chambers. Scale bar = 1 μm .

Figure 2. Close-up of a somewhat later stage in valve formation of *C. granii*. Mission Bay, San Diego, California, with struts at corner of hexagons, initiating the cribra, the external plates that cover the chambers. Scale bar = 1 μm .

Figure 3. Close-up of nearly complete valve of *C. granii*. Mission Bay, San Diego, California. Scale bar = 1 μm .

Figure 4. Close-up of mature valve of *C. granii*. Mission Bay, San Diego, California. Cribra (the outer covering) now completely encompass the chambers. Scale bar = 1 μm .

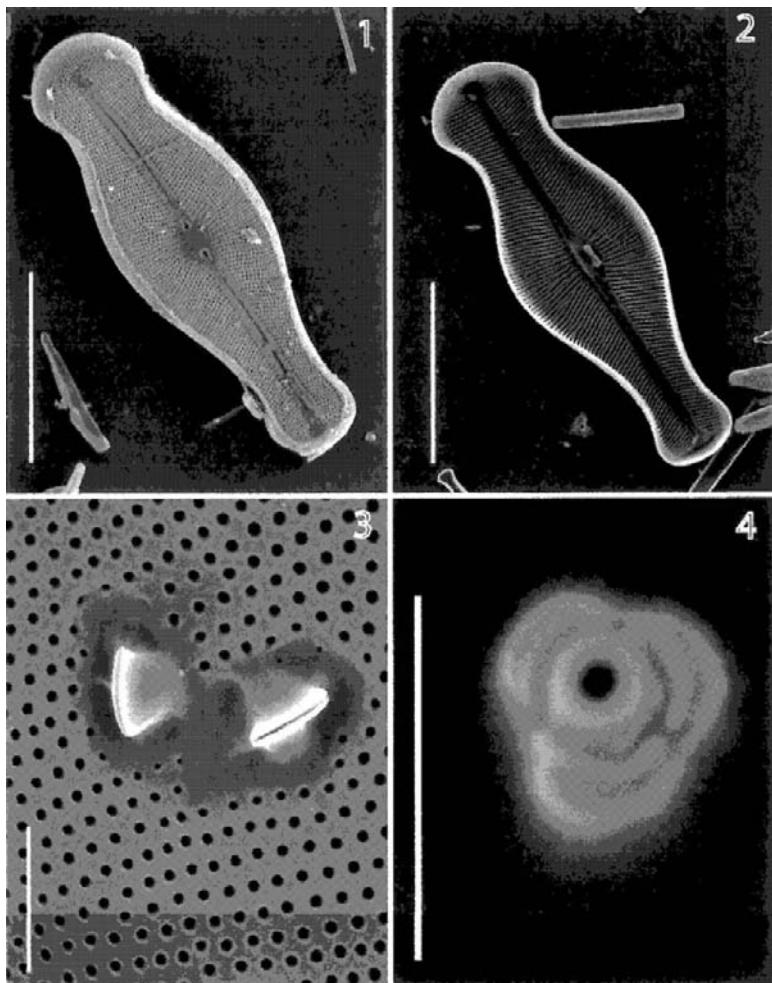
**Plate 10:**

Figure 1. External view of valve of *D. geminata*. Cache la Poudre River, Colorado. Bar = 50 µm.

Figure 2. Internal view of *D. geminata*. River Ardle, Scotland. Scale bar = 50 µm.

Figure 3. *P. laevis*. Alvarado Creek, San Diego, California. Several rimoportulae at center of inner surface of valve. Scale bar = 5 µm.

Figure 4. *Cyclotella meneghiniana* Kützing. Inlet at beach, San Clemente, California. Extreme close-up of a fultoportula on inner surface of valve. Scale bar = 1 µm.

heteropolar as in *Didymosphenia* (Plate 2, Fig. 1). When the raphe sternum is not straight, the two “top” and “bottom” halves of a valve can be mirror-symmetric, as in *Cymbella* species (Plate 6, Fig. 2), or symmetric, as in *Pleurosigma* (Plate 6, Fig. 3).

In an exploration of the geometry and topology of diatom shapes and overall patterns, Pappas (2005a, b) used three-dimensional parametric equations

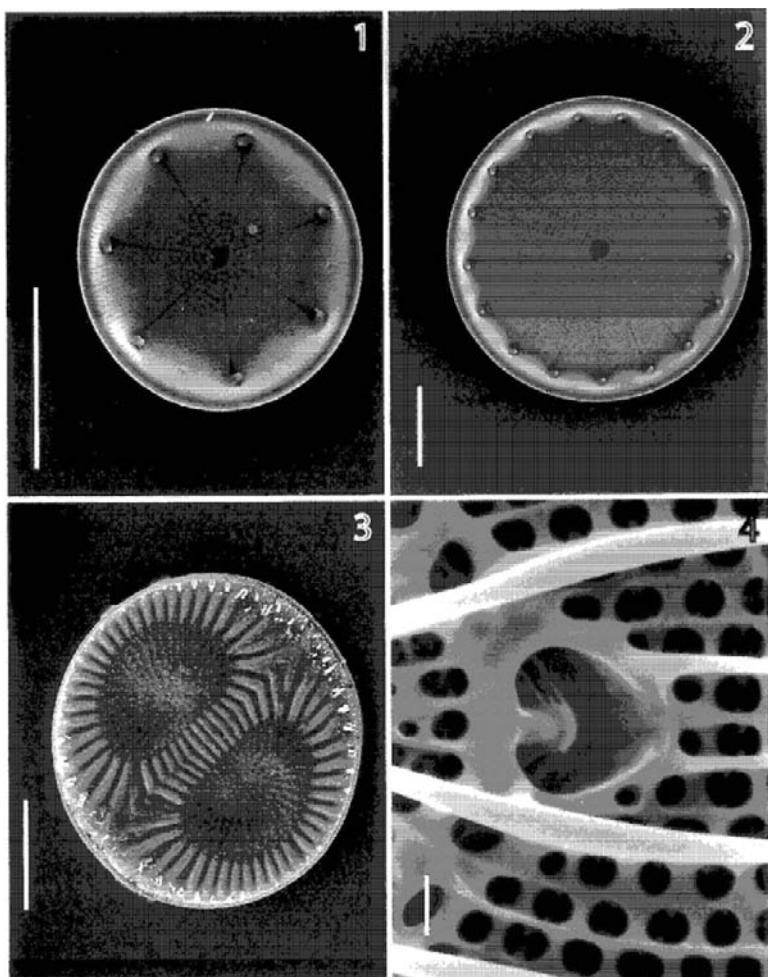
**Plate 11:**

Figure 1. *A. oregonus*. Cabrillo Beach, California. Valve with seven sectors. Scale bar = 50 μm .

Figure 2. *A. oregonus*. Point Dume State Park, California. Larger valve with 17 sectors. Bar = 50 μm .

Figure 3. *C. meneghiniana*. Inlet at beach, San Clemente, California. Abnormal valve with two pattern centers. Scale bar = 10 μm .

Figure 4. *Epithemia sorex* Kützing. Pond at San Marcos, California. Internal view showing central termination of raphe slits. Scale bar = 1 μm .

based on circular and hyperbolic functions to create “theoretical diatoms.” In this approach to diatom geometry, it was indeed shown that a series of naturally occurring diatom forms can be mathematically generated. Although the initial focus of interest (Pappas, 2005a) was the possible application to nanotechnology, this approach also provided preliminary insights into adaptation of mathematically derived morphologies to hypothesized phylogenies in certain pennate diatom lineages.

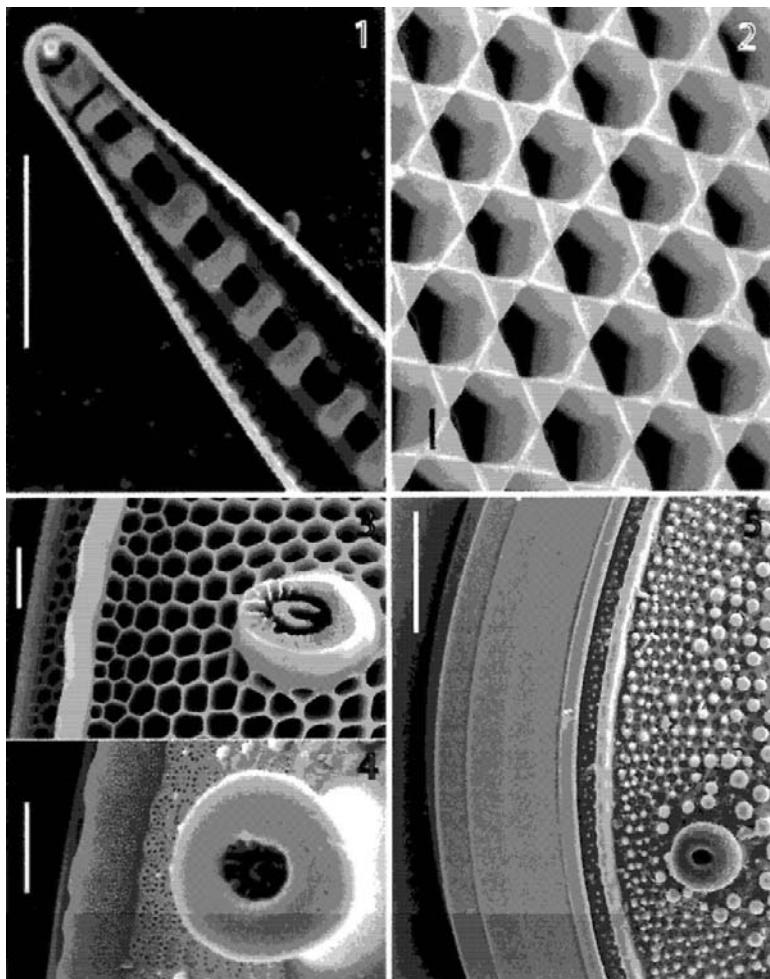
**Plate 12:**

Figure 1. *B. paxillifer*. Alvarado Creek, San Diego, California. Fibulae span the raphe adding structural strength. Scale bar = 5 μm .

Figure 2. *Coscinodiscus* sp. Mission Bay, San Diego, California. An interesting hexagonal pattern on the surface of a forming valve. Scale bar = 1 μm .

Figure 3. *A. scaber*. Forming valve, La Jolla, California. A tongue-like projection and ridges can be seen within the forming tube. Scale bar = 5 μm .

Figure 4. *A. scaber*. Tube of mature valve, Mission Bay, California. The tongue-like projection and ridges can be seen within the completed tube. Scale bar = 5 μm .

Figure 5. *A. scaber*. Mature frustule, Mission Bay, California. Scale bar = 20 μm .

8. Nanostructural Inventiveness

At the nanolevel, there is an impressive range of stress-relieving structures and what may be inventive solutions to problems whose nature is still unknown to us. While in some cases the link between structure and function is eminently clear (Examples 1–3), in many others it is completely obscure (Examples 4–6):

- (1) A prime example of the relation between form and function is the appearance in the later diatom phylogeny of the raphe slit. Active locomotion may certainly be an advantageous acquisition, but the structural discontinuity in the valve created by the raphe slit is a major blunder from the engineering perspective. In the central portion of the valve, the terminations of the raphe slit must therefore be surrounded by thick silica structures (Plate 2, Fig. 4, central oval and Plate 11, Fig. 4). At the apices of the valve, the raphe terminations form a notorious focus of stresses and they are therefore strengthened by ridges called helictoglossa (Plate 13, Fig. 1, arrow). In *Bacillaria paxillifer* O.F. Müller arch-shaped bars spanning the raphe slit (Plate 13, Fig. 1) have been added to compensate at least partially for the catastrophic structural weakening caused by the raphe slit.
- (2) As another illustration of constructal principles acting on the nanoscale level, consider the hexagonal chambers in the valve of a species of *Coscinodiscus*, which are strengthened at the wall intersections by thick silica deposits (Plate 13, Fig. 2). This is rather exceptional: hexagonal chambers are common in centric diatoms but they do not always show such strengthening at the corners.
- (3) The purpose of most diatom cell wall structures is enigmatic but some structures are likely to have a supportive function. An example of this is seen in those *Aulacodiscus* species that sport huge peripheral tubes, as in *Aulacodiscus scaber* Ralfs in Pritchard (Plate 12, Figs. 3–5). Here the tube has internal ridges and a structure resembling a “rolled tongue,” which apparently provides strength to buttress the large tube preventing damage to it in its usual habitat, the highly turbulent marine surf zone.
- (4) Why the internal and the external structures of the valve of some diatoms such as *D. geminata* (Plate 10, Figs. 1 and 2 and Plate 2, Fig. 1) should be so markedly different is wholly unclear.
- (5) Highly decorative designs grace the surface of many diatom valves and their resting stages (Plate 13, Figs. 2, 3 and 5). These are faithfully reproduced with every mitosis. Perhaps these nanodesigns and projections affect the hydrodynamics of a cell as it wafts about in the plankton conferring some adaptive purpose? No one yet knows.
- (6) Rigid “geometric” control on the nanoscale is illustrated by the regular grating-like spacing of the perforations in the valve of *Pleurosigma* species, where the intersection angle (crossing lines drawn in Plate 13, Fig. 4) is remarkably constant within a species. Once more, what the purpose of such strict control could be is difficult to fathom.

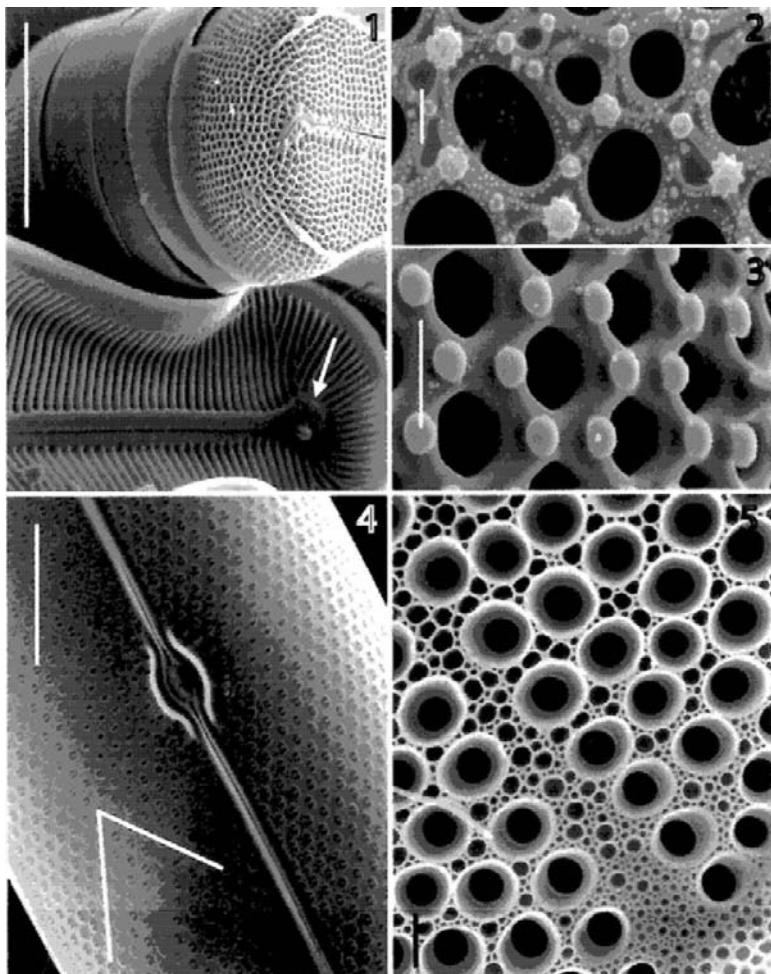
**Plate 13:**

Figure 1. *D. geminata*. Cache la Poudre River, Colorado. View of whole frustule and inner view of another valve. Arrow indicates solid silica plate carrying the reinforcing helictoglossa. Scale bar = 20 μm .

Figure 2. *Triceratium moreirae* Fernandes and de Souza-Mosimann. External view, Bremer Bay, Western Australia. Scale bar = 1 μm .

Figure 3. Resting spore of *Stephanopyxis turris* (Greville et Arnott) Ralfs in Pritchard. Bremer Bay, Western Australia. Scale bar = 5 μm .

Figure 4. *Pleurosigma acus* Mann. Bird Rock, La Jolla, California. Internal view. Lines indicate crossing-angle of striae. Scale bar = 5 μm .

Figure 5. *A. oregonus*. Cabrillo Beach, California. External view of forming valve surface in region near ray that leads to a tube, showing "bubble pattern." Scale bar = 1 μm .

9. Controlling Mechanisms

Two different categories of controlling cellular mechanisms have been proposed: pattern assembly by self-organizing processes or organic templates, and the presence of blueprint systems (spacer vesicles, endoplasmatic reticulum) along the plasmalemma, as in *Coscinodiscus* (see Schmid, 1994). These are not mutually exclusive, however, and it is most probable that additional mechanisms come into play.

Some diatom structures resemble an aggregate of bubbles, as the example in a forming valve of *A. oregonus* of Plate 13, Fig. 5. The network pattern in certain diatom species has been compared (e.g., Bach and Burkhardt, 1984) to foam. The boundaries of such reticulate patterns are called Voronoi tessellations and are regarded as an expression of the physical phenomena involved: growth, competition between physical spheres of influence, diminishing effect with increasing distance. Consequently, these are universal patterns caused by universal effects and thus they are widely distributed, being observed in the large-scale structure of the universe (empty bubbles surrounded by “walls” of galaxies), in computer simulations of growing tumors and in the “cells” of cellular phone networks. For such patterns in diatoms it could be postulated, therefore, that they are governed by physical rather than by exclusively biological mechanisms. Diatoms could thus be an example of “constructal design” (Bejan, 2000, 2005), “where miniaturization, global performance, compactness and complexity rule the design” (Ordonez et al., 2003). The “constructal flow” being optimized may be the diffusion-limited precipitation of silica (Gordon and Drum, 1994).

However, our knowledge of why diatoms have the structures and substructures they do is still very limited. Therefore what constructal functions are optimized, and whether different functions are optimized for different species, is a topic for future engineering research on diatoms. Parsimony is often an important issue, not because of the energy requirements involved (production of silica is not energy-expensive), but because the availability of Si in the environment may be a limiting factor. Diatom valve morphogenesis proceeds by precipitation of silica from the raphe or midring outward, often resulting in branching patterns of the amorphous silica (Schmid, 1984; Gordon and Drum, 1994). In fact, regular hexagonal patterns in some diatoms are replaced by branching patterns when silica is limiting (Fryxell and Hasle, 1977; Gordon and Drum, 1994). Under such conditions, where there are heavy demands on a limited supply, the rules of “minimal surfaces” geometry (see Karcher and Polthier, 2006) offer a potent evolutionary advantage. A “minimal surface” is the most parsimonious solution to the constructal requirements involved.

Therefore, the “constructal paradigm analogue” does not imply that the diatom’s frustule formation is exclusively controlled by physical factors (as opposed to biological factors such as natural selection). Of course, as in the streamlined design of diatoms that adhere to surfaces in fast running water (Gordon et al., 1996), optimum design is achieved through evolution. Thus, while the silica, once precipitated, does not flow in any sense in an individual diatom,

the pattern does “flow” when one considers the chain of generations over evolutionary time. The mechanical strength of diatoms, which are heavily predated, is another function that may be optimized through evolution (Hamm et al., 2003). Yet another example is the development of long bristles in such planktonic diatoms as *Chaetoceros*, by which the sinking rate is greatly reduced. It has long been known that depending on the water temperature, the aspect of *Chaetoceros* species (especially the length of the “bristles”) may be so different that the extremes may be taken for totally different species, populations from cold waters having smaller cells and longer bristles (Hendey, 1937). Finding a relation between form and function in diatoms is often highly vexing, however. The human observer gropes in darkness when considering the possible functional benefits when a *Triceratium* species switches from a triangular to a quadrangular shape, or deciding what biological consequences might result from the number of sectors in *A. splendens*.

This form versus function dilemma becomes acute when we consider the extremophilic diatoms we discussed earlier. A reviewer of the original manuscript rightly remarked that “it would be instructive to see how the specific constructions described help diatoms survive in extreme environments.” We fully agree, but we’re afraid that the answer is still obscure. The fascinating nanostructures we show here were all observed in definitely nonextremophilic diatoms. The definitely extremophilic diatoms we reviewed earlier, such as the species limited to acidic bogs, do not show any specific structures absent in mesophilic diatoms. Many peat-bog species are weakly silicified, but that is merely an expression of scarcity of Si in the habitat. This would suggest that “architectural trickery” offers no way out when diatoms are faced with extreme conditions such as hypersalinity or hyperacidity – the organism has to resort to its physiological repertoire. This is evident, for instance, in the switch from a freshwater to a hypersaline ecology we mentioned for *G. wormleyi*: based on their LM and SEM morphology it’s completely impossible to tell these populations apart. The same applies to species observed in both hot springs and temperate waters.

On the other hand, environmental factors may directly influence frustule structure by altering the conditions for silica precipitation in the SDV, such as the pH (Vrieling et al., 1999) or salinity (Gordon and Brodland, 1990; Vrieling et al., 2007). In summary, the application of the concept of constructal design to diatoms poses challenges to understand what ecological, evolutionary, or merely physicochemical functions have been selected to be optimized. Perhaps some of the extraordinary diversity of diatom shells is due to the choice of different optimization functions by different species? Which design parameters, such as perhaps the chemical conditions for silica precipitation in the SDV, are directly affected by mutation, and which, like the detailed pattern of a valve, are indirect consequences of these parameters, are yet to be worked out. Diatoms thus present an opportunity to directly explore the relationship between the genotype and the phenotype (Drum and Gordon, 2003), and direct manipulation of this relationship might be possible via genetic manipulation and artificial selection such as with a compustat (Gordon, 1996).

10. Epilogue

We have suggested that diatoms live in a “constructal” environment. Despite their great interspecific morphological variety, their intraspecific morphological variability is rigidly controlled. Nature acts as a stern architect and geometer on the micro- and nanoscale here, but the deep causal mechanisms and functions being optimized are far from clear as yet. In their paper comparing diatoms and pollen, Schmid et al. (1996) sum up the situation nicely and it would be most appropriate to end with a sobering quote from that paper, written by leading specialists in the field:

Pattern creation is one of the great unexplained enigmas. We do not know how the wide range of structural diversity is manifested or how the species-specific information is transformed into a 3-D intricate architecture. We are completely ignorant of how the sequential events responsible for wall deposition are started, controlled and stopped.

Although since the first such studies (Schultze, 1863a, b) progress has been made on modeling pattern formation by precipitating silica, for instance (Gordon and Drum, 1994; Parkinson et al., 1999; Pappas, 2005a) and its biochemical basis (Frigeri et al., 2006), we have achieved only the broadest strokes, in which we cannot yet have any confidence, let alone approach the exquisite detail and individuality of each species.

11. Acknowledgments

Richard Gordon would like to thank Natalie K. Björklund for suggesting that diatoms are extremophiles and worthy of inclusion in this volume. Mary Ann Tiffany thanks Prof. Stuart Hurlbert for the use of the SEM. We thank Joseph Seckbach for inviting us to contribute and finally, we are grateful to the reviewers for their constructive comments.

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THE MARGIN OF THE SEA:

Survival at the Top of the Tides

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With deep respect and gratitude I dedicate this chapter to George Russell – consummate scholar and mentor.

1. Introduction

From the upper reaches of the intertidal zone to the beginnings of terrestrial vegetation is a region of shoreline that is often sparsely inhabited by algae, and typically includes conspicuous expanses of bare rock. Inspection of the habitat reveals scattered or even abundant lichens, and often extremely patchy to extensive populations of macroscopic algae. The physiological ecology of photosynthetic algae in this part of the intertidal zone comprises the primary theme of this chapter. The organisms discussed here typically have extensive populations above mean high water neap tide (see Lüning, 1990; Lobban and Harrison, 1994; Little and Kitching, 1996, for introduction to tides and zonation). In general, these organisms are found exposed on bare rock and not in the rock pools where greater species richness occurs and less stringent environmental conditions are imposed. In terms of physiological constraints, the high intertidal and adjacent supratidal zone is among the most stressful encountered by organisms in general (Tomanek and Helmuth, 2002), and by marine macroalgae in particular (Davison and Pearson, 1996).

First, the rigors of the environment are explored, and then the various adaptive strategies of algae to survive and thrive in this habitat are discussed. Davison and Pearson (1996) reviewed stress tolerance in intertidal seaweeds as a whole; however, here the focus is on the upper intertidal zone and on disruptive stresses that cause damage or limit growth.

Physiological responses and survival strategies have been explored in organisms in a diversity of seaweeds including red, brown and green algae (Table 1). In addition to purely physiological studies, extensive research has been carried out on ecological strategies involving features of life history. This high intertidal zone is already a highly stressful one for its inhabitants; however, climate change, in particular the increase in ultraviolet radiation (UV), is impacting these organisms (e.g. Hanelt et al., 1997; Robinson et al., 2003). Because of the relative ease of access, the stressful nature of the environment, and the relatively few species involved, this part of the shore and its inhabitants have become model systems for research in physiology and ecology.

Table 1. Summary of genera of marine macroalgae used as model systems for the upper intertidal zone with respect to environmental tolerances.

Taxonomic group	Nature of tolerance	References
Chlorophyta		
<i>Blidingia</i>	Osmotic	Karsten and Kirst (1989) and Karsten et al. (1992)
<i>Prasiola</i>	Osmotic	Smith and Berry (1986), Jacob et al. (1991, 1992a, b), Bock et al. (1996) and Smith and Gremmen (2001)
	Desiccation	Davey (1989), Bock et al. (1996) and Smith and Gremmen (2001)
	Ultraviolet radiation	Jackson and Seppelt (1997), Lud et al. (2001a) and Holzinger et al. (2006)
	Temperature	Smith and Berry (1986), Davey (1989) and Jackson and Seppelt (1997)
<i>Ulothrix</i>	Osmotic	Karsten et al. (1991a, b, 1992)
Phaeophyceae		
<i>Fucus</i>	Temperature	Davison et al. (1989), Pearson and Davison (1993) and Li and Brawley (2004)
	Osmotic	Li and Brawley (2004)
	Desiccation	Schonbeck and Norton (1979)
	Ultraviolet radiation	Hanelt et al. (1997)
<i>Hesperophycus</i>	Desiccation	Oates and Murray (1983)
<i>Pelvetia</i>	Desiccation	Schonbeck and Norton (1979), Dring and Brown (1982), Rugg and Norton (1987), Harker et al. (1999) and Pfetzing et al. (2000)
	Temperature	Pfetzing et al. (2000)
<i>Silvetia</i>	Desiccation	Oates and Murray (1983)
Rhodophyta		
<i>Apophlaea</i>	Desiccation	Brown (1987)
	Ultraviolet radiation	Lamare et al. (2004)
<i>Bangia</i>	Ultraviolet radiation	Karsten and West (2000) and Boedeker and Karsten (2005)
<i>Gloiopeltis</i>	Desiccation	Ji and Tanaka (2002)
<i>Hildenbrandia</i>	Desiccation, osmotic, temperature	Kim and Garbary (2006)
<i>Porphyra</i>	Desiccation	Wiencke and Läuchli (1983), Levitt and Bolton (1991), Lipkin et al. (1993) and Ji and Tanaka (2002)
	Ultraviolet radiation	Hoyer et al. (2001), Peinado et al. (2004) and Korbee et al. (2005)
	Osmotic	Smith et al. (1986)

2. The Environment

The relatively benign environment of shallow subtidal to mid-intertidal zones of marine environments is a region of high biodiversity. As one moves up the intertidal zone, the environment becomes increasingly stressful as a consequence of increasing variations in temperature, wave action and salinity. There is a consequential loss of biodiversity and general increase in bare space as one approaches the high intertidal zone. Similarly, when one approaches the shore from the

terrestrial side, the increase in salt and the impact of desiccation gradually decreases the vegetation and soil integrity to produce the bare substratum of rocky shores. Organisms growing here are clearly able to survive, and important ecological questions surround the importance of sublethal stresses and the various physiological strategies that organisms have for survival and how these impact interspecific competition (Davison and Pearson, 1996). Algal genera that have been used as primary model systems for studying the upper intertidal zone are listed in Table 1 along with the primary aspects investigated.

2.1. TEMPERATURE

Because of the importance of elevated temperature in desiccation rate and sub-zero temperatures in relation to freezing tolerance, temperature may be the single most important environmental variable in determining survival in the upper reaches of the intertidal zone. At the bottom of the intertidal zone temperature largely reflects seawater temperature and is relatively stable over diurnal cycles. The greater the time out of the water during low tide the greater the extent to which organisms are exposed to air temperature and the number and rapidity of events associated with tissue freezing (Davison et al., 1989; Pearson and Davison, 1993) or tissue dehydration. With increasing tidal elevation, elevated air temperatures have greater impact although thalli have some protection from evaporative cooling, and thalli under multiple layers of overlapping algal fronds would remain cool. As the vegetation thins and solar radiation reaches rocky substratum there is considerable heating of the substratum that can exceed air temperature. The higher in the intertidal zone the greater the extent to which this occurs. Thus while water temperature may be a temperate 10–15°C, and air temperature on the order of 25°C, the algae may experience even greater temperatures as a consequence of substratum heating. At a temperate site in California when air temperature varied from 18°C to 25°C, the actual temperature range that *Porphyra fucicola* experienced was from 13°C (i.e. seawater temperature) to 33°C, all within a 6-h period (Biebl, 1970). When air temperatures were higher, thalli of *P. fucicola* would be exposed to even greater temperature fluctuations. Even without substratum heating, Lipkin et al. (1993) used 35°C in experiments with *Porphyra* because this reflected maximal temperatures during the growing season.

Extreme temperatures are also experienced by intertidal algae as a consequence of low temperature. While seawater typically reaches only –1.5°C, air temperatures in temperate and polar regions may reach –20°C or even lower. Organisms in the mid-intertidal zone might be protected from the most extreme low temperatures as a consequence of ice cover; however, at the top of the shore organisms may be exposed to the full brunt of these lower temperatures. Some temperate intertidal algae can survive freezing at –20 to –40°C and cultivation nets of *Porphyra* are typically air dried and stored at –20°C for several months before use.

Davison et al. (1989) showed that the extent of freezing tolerance in intertidal algae was related to their position on the shore with the high intertidal *Fucus spiralis* being unaffected by freezing for 3 h at -20°C, whereas a low intertidal zone fucoid showed a reduction of 97% for the same treatment. In a comparative study of intertidal fucoids Pearson and Davison (1993) showed that *F. spiralis* was much more tolerant to rapid freezing than species lower on the shore, and that full photosynthesis returned more rapidly following thawing. Thus freezing tolerance is associated with shore zonation (Chapman, 1995). Freezing intolerance was accompanied by a large efflux of amino acids, and freezing tolerance is considered an adaptation of the plasmalemma (Davison et al., 1989). In Antarctic green algae; however, high concentrations of dimethylsulphoniopropionate (DMSP) were suggested to function as a cryoprotectant (Karsten et al., 1992).

Membrane proteins called lipocalins have been identified in a diversity of land plants where they are associated with cold tolerance. Charron et al. (2005) demonstrated the presence of lipocalins in *Porphyra yezoensis* and suggested that these proteins may provide the basis for the desiccation tolerance of this high intertidal species.

The role of heat-shock proteins in marine algae has received limited study. Li and Brawley (2004) showed a 30% increase in survival of embryos of *Fucus vesiculosus* (mid-intertidal) and *F. spiralis* (high intertidal) following acclimation of parent thalli to sublethal conditions. This was attributed to the production of heat-shock proteins. *Chondrus crispus* (low intertidal) also produces heat-shock proteins among a wide variety of stress response genes (Collen et al., 2006). To date, none of the algae from the extreme high intertidal zone have been examined for production of heat-shock proteins.

2.2. SALINITY

From the bottom to the top of the intertidal zone the most conspicuous change in salinity to which organisms are exposed is the increase in variance of salinity. At the bottom of the shore algae are only out of seawater for short periods during low water, and wave action limits the extent to which thalli are exposed to reduced salinity. At higher positions in the intertidal zone organisms are more exposed to low-salinity rainwater for greater periods during precipitation. In the high intertidal and splash zone further exposures to rainwater occur as well as increased exposures to higher salinity water as a consequence of evaporation. Shallow rock pools in the splash zone may vary from low to high salinity depending upon rain and wave frequency. Thus organisms in the high intertidal zone must be tolerant of salinities at both extremes, and be able to survive or resist both water loss and ion loss during exposures to high and low salinities, respectively. Physiological studies have been carried out on numerous intertidal seaweeds. Intertidal seaweeds can tolerate short-term exposures to reduced salinity. Physiological adaptations are similar to those required for desiccation, since both stresses function to reduce water potential (Smith and Berry, 1986; Lobban

and Harrison, 1994). Similarly, freezing induces equivalent physiological constraints in that cellular water becomes inaccessible, and then adds the potential damage of ice crystal formation (Davison et al., 1989).

Different classes of low molecular weight carbohydrates have been identified as being important in osmoregulation in different algal groups (Kirst, 1990). In green algae osmotic regulation is typically associated with various ions and various organic solutes that include proline, sucrose and DMSP (Karsten et al., 1991b, 1992). In Phaeophyceae, mannitol is found in osmotically significant concentrations in many taxa (Reed et al., 1985), while volemitol can be abundant in *Pelvetia canaliculata* (Pfetzing et al., 2000). In the red algae *Porphyra* and *Bangia*, floridosides may play a similar role, although floridoside concentrations are often independent of salinity in the surrounding medium (Karsten et al., 1993; Karsten, 1999).

Physiological responses to salinity were examined in *Blidingia minima* (Karsten and Kirst, 1989). This species showed a remarkably stable photosynthetic rate over salinities from 7‰ to 50‰ and only declined at 70‰. A continual decline in tissue water and an increase in K⁺ and sucrose concentrations corresponded with the salinity gradient. Thus this species is clearly highly adapted to salinity extremes. The Antarctic filamentous green algae *Ulothrix implexa* and *Urospora subflaccida* showed little reduction in cell viability from salinities of 7‰ to 60‰, and the latter species showed major decline in cell viability only at 102‰. These responses were mirrored in specific growth rates and photosynthetic oxygen production (Karsten et al., 1991a, b).

Hildenbrandia rubra and *Prasiola crispa* may be the most tolerant marine algae to salinity stress in that *H. rubra* can recover its full photosynthetic response after 96 h at 4‰ (Kim and Garbary, 2006) while the latter can survive treatments from 0.35‰ to 175‰ (Jacob et al., 1991, 1992a, b). Adaptations for low salinities in *P. crispa* included the absence of vacuoles in exposures between 0.35‰ and 35‰. Growth and photosynthesis were almost unaffected at low salinities, but were strongly inhibited by extreme hyperosmotic conditions. In these experiments intracellular levels of inorganic ions (Na⁺, K⁺, NH₄⁺, Cl⁻, PO₄³⁻) were unaffected by decreased hypoosmotic conditions, but reached a maximum at 70‰. At extreme hyperosmotic levels the organic solutes sucrose and sorbitol were greatest.

2.3. DESICCATION TOLERANCE AND AVOIDANCE

Despite some exceptions and specialized modifications associated with cell walls and the intercellular matrix, the survival of marine algae following desiccation is considered to be based on tolerance of dehydration rather than avoidance. Populations of *P. canaliculata* may be emersed for up to 8 days between spring tides and thus require extreme tolerance in what is morphologically a highly complex organisms (Pfetzing et al., 2000). This is exceeded by *Porphyra* from Israel that can recover photosynthesis following 20 days of continuous exposure to air (Lipkin et al., 1993).

Although desiccation rates are largely a function of surface/volume ratio, a few studies have shown that higher intertidal organisms have a greater ability to resist desiccation. Some of the most extreme desiccation tolerant species have adaptations that slow desiccation and allow for more rapid rehydration. For example, *Prasiola crispa* showed slower water loss than for a *Fucus* species under similar humidity (Jacob et al., 1992b). This was attributed to lipophilic materials on the thallus surface and pectic compounds in the cell walls. Oates and Murray (1983) compared desiccation in *Herperophycus* relative to *Silvetia* (as *Pelvetia*) and found that the rate of water loss was against expectation based on surface/volume relationships.

Despite the above results, tolerance of desiccation and recovery of physiological processes following dehydration are the primary mechanisms of survival of the high intertidal algae. Extreme desiccation disrupts membranes and energy transfer during photosynthesis. This was evident in *Porphyra* where comparisons between the high intertidal *P. perforata* and the subtidal *P. nereocystis* showed greater phycobilin fluorescence in both osmotically challenged and air-dried thalli of *P. nereocystis* (Smith et al., 1986). In *P. linearis* fronds only began to show reductions in photosynthesis after a relative water content (RWC) of 0.60 had been reached, and even at a RWC of 0.2 photosynthesis was at 20% of maximum (Lipkin et al., 1993).

Overall, there does not appear to be a relationship between position in the intertidal zone, water content and resistance to desiccation (Dromgoole, 1980). The primary factor explaining desiccation rate is the surface/volume ratio of the plant. Ji and Tanaka (2002) found no relationship between photosynthetic rate following desiccation and tidal height. Smith and Berry (1986) however, reported that the recovery of photosynthetic rate following desiccation or high temperature was highly correlated with height in the intertidal zone. Even within a single species (*Porphyra perforata*) high intertidal fronds were more tolerant of desiccation.

Although adaptations to desiccation are thought to be similar to adaptations to salinity stresses, different physiological phenomena have been examined. In *Prasiola crispa*, Bock et al. (1996) showed changes in phosphate metabolism during exposures to a medium of the non-ionic osmoticum, polyethylene glycol. There were initial changes in cytoplasmic inorganic phosphate (increased) and polyphosphate (decreased) concentrations, followed by an increase in extracellular inorganic phosphate. Within 4 h of return to normal media, control levels of photosynthesis occurred. This work should be repeated on other algae to determine if this is a generalized response, or if it is unique to *Prasiola*.

2.4. SOLAR RADIATION

The only major environmental factors that show less variation at the top rather than the bottom of the intertidal zone are photosynthetically active radiation (PAR) and

UV. Organisms at the top of the shore are clearly more exposed to the damaging effects of excess light from the perspective of photoinhibition and damage from UV radiation. The possibility of light limitation increases down the intertidal zone; however, the potential for damage to photosystems and DNA decreases because of dramatically less exposure times and the increasing possibility of recovery during tidal submergence. Protection mechanisms against reactive oxygen species are clearly important but have not been well studied (see Dring, 2005, for review).

Prasiola species have been extensively studied from the perspective of the impacts of UV on growth, survival and physiology (Jacob, 1992; Jackson and Seppelt, 1997; Karsten et al., 2005; Holzinger et al., 2006). Like many other marine organisms, production of mycosporine-like amino acids (MAAs) is inducible in *Prasiola crispa* as a response to increased UV radiation. This is critical because high doses of UV will cause DNA damage and affect photosynthetic performance (Lud et al., 2001a).

Prasiola crispa has become a model system for the study of UV effects. Using pulse amplitude modulated (PAM) fluorescence Holzinger et al. (2006) showed that exposure to 6 h of PAR + UV had little effect on photosynthesis; however, 24-h exposure reduced photosystem II efficiency (Fv/Fm) to about 30% of control levels. There was also a major decline to 63% of control levels in relative electron transport rate (rETR) over a range of photon fluence exposures. The controls in this experiment showed photoinhibition at the higher levels of PAR tested; however, this was not apparent in the UV treated thalli. These changes were accompanied by changes in ultrastructure that included occasional dilations to thylakoids, a three to four times increase in 0.4–0.8 µm diameter plastoglobuli and slight damage to mitochondria. It would be of interest if the ultrastructural changes were associated with the physiological changes in photosynthesis. It remains to be demonstrated if the plastoglobuli are merely a damage response or if they are an adaptation providing protection.

Similar experiments were conducted by Lud et al. (2001a) on *Prasiola crispa* var. *antarctica*. Photosynthetic performance was not impaired by higher levels of UV exposure (2.0 mW m⁻² vs. 0.2 mW m⁻² UVB), although there was apparent damage to DNA. The UV tolerance shown by *Prasiola* species is also present in the high intertidal *Fucus distichus* that Hanelt et al. (1997) considered almost impervious to UV. This differs markedly from the greater impact of UV on subtidal red algae that do not form MAAs (Karsten et al., 1998).

The Holzinger et al. (2006) experiments are highly informative; however, they need to be extended to determine impact and recovery over more realistic time periods reflective of the polar and high latitude summers. Survival, despite the pathological changes, would emphasize the tolerance of this species.

Porphyra species have high concentrations of MAAs and accumulation of these amino acids reflects the overall exposure to UV radiation (Hoyer et al., 2001; Peinado et al., 2004; Korbee et al., 2005). The related genus, *Bangia*, also produces MAAs; however, it may not be able to adjust concentrations based on environmental conditions (Boedeker and Karsten, 2005).

3. Key Taxa of Rhodophyta, Chlorophyta and Phaeophyceae

3.1. FILAMENTS

There are species of red and green filamentous algae that grow in the high intertidal zone, but this habitat seems devoid of brown algal filaments. The primary taxa of green algae are *Ulothrix* and *Urospora* whereas the primary genus of red algae is *Bangia*. These genera are cosmopolitan. The strong seasonality and the ease with which species can be grown in culture makes them excellent models for physiological adaptations to environmental extremes (Table 1, e.g. Karsten and West, 2000).

3.2. FLAT BLADES AND TUBES

There are over 100 species of *Porphyra*. This is a cosmopolitan genus and different species occur from strictly subtidal habitats to the high intertidal zone. The range of habitats in related species of similar morphology makes this genus particularly attractive as models for adaptation to environmental stresses. Species from the high intertidal zone, e.g. *P. perforata*, *P. linearis* and *P. yezoensis* have remarkable tolerance to extremes of temperature and desiccation (e.g. Lipkin et al., 1993). Because of the extremely high surface to volume ratios, these species desiccate and rehydrate rapidly, and thus are useful models for physiological changes associated with water content.

Prasiola stipitata and its relatives are among the most conspicuously desiccation tolerant species of marine algae. Not only are species associated with the high intertidal zone, but they can also thrive in terrestrial communities in humid areas (e.g. Ireland, Rindi et al., 1999). These species have carved out a niche on exposed rocks high in the splash zone, and regularly undergo extreme desiccation. The monostromatic blades and their filamentous forms range from a few millimetres to several centimetres in height. In addition, the high nitrogen of bird droppings also characterized the habitat of *P. stipitata*. The latter is not a case of tolerance, but positive adaptation since populations can decline in adjacent areas even though space is apparently available for colonization. In *Porphyra*, MAAs are stimulated when thalli are exposed to high nitrogen (Korbee et al., 2005). If this is also the case in *Prasiola* it would help explain the apparent association of some species with avian feces.

Blidingia species have received little attention as physiological models. Exceptions include studies of salinity impacts on photosynthesis (Karsten and Kirst, 1989) and the impact of environmental factors on the production of DMSP (Karsten et al., 1992). The strong seasonality and wide distribution should provide the basis for further experimental work on environmental tolerances.

3.3. THICK LEATHERY FORMS

3.3.1. *Fucoids and Cartilaginous Red Algae*

Unlike the species discussed above that are annuals, *P. canaliculata* is a true perennial. Although there are species in western North America that appear similar to *P. canaliculata* (e.g. *Pelvetiopsis* and *Silvetia*), none occur at the same tidal heights, and none are as resistant to desiccation (Rugg and Norton, 1987; Pfetzing et al., 2000). Indeed, *P. canaliculata* may be a model organism for the high intertidal zone inasmuch as it cannot survive when transplanted to lower levels on the shore, and it decays when kept continuously immersed for six or more hours out of 12 h. The key to the tolerance of *P. canaliculata* may be the occurrence of a symbiosis with the ascomycete fungus *Mycophycias ascophylli*, the same fungus that plays such a prominent role in the biology of *Ascophyllum nodosum* (Garbary and Deckert, 2001).

Harker et al. (1999) examined photosynthetic reactions of *P. canaliculata* as a consequence of light and desiccation stress. They concluded that non-photochemical quenching was greater in *P. canaliculata* than in *Laminaria saccharina* (a subtidal species) in both fully hydrated and dehydrated thalli. The high level of non-photochemical quenching in *P. canaliculata* is a strong indicator of the physiological adaptation of the species to the high light levels on the upper shore. Pfetzing et al. (2000) showed that the alditols (mannitol and volemitol) were produced in high amounts by *P. canaliculata* and these were inferred to be stress metabolites based on dynamic changes in concentrations associated with temperature and emersion.

Henry et al. (1996) described a phenomenon in *Silvetia compressa* (as *Pelvetia fastigiata*) that might represent an adaptation to high salinity in developing zygotes. When exposed to 1.5 M sucrose, zygotes and developing embryos became highly plasmolyzed; however, there were specific positions (termed adhesions) where the plasma membrane remained attached to the cell wall. These attachment points were uniformly distributed in ungerminated zygotes, but were localized at the tip of developing rhizoids. The adhesion points were associated with F-actin staining in the cytoplasm. Adhesions occur in other plant systems where they can provide positional information for cytokinesis (e.g. Cleary, 2000). In the large cells of fucoid embryos this might minimize damage from plasmolysis by retaining positional information for directional growth following relaxation of plasmolysis. Accordingly, adhesions would be predicted to be less prominent in small-celled species (e.g. *Prasiola* spp.) or fucoids lower in the intertidal zone, and more prominent in fucoids higher on the shore (e.g. *P. canaliculata*).

Mazzarella parksii (= *Iridaea cornucopiae*), *Gloiopeletis furcata* and *Endocladia muricata* are three cartilaginous red algae from the high intertidal zone in the temperate North Pacific. The former occurs in extremely dense populations (see below) whereas the other species occur at lower tidal elevations and typically as scattered individuals or clumps rather than as continuous populations. Both *G. furcata* and *E. muricata* become conspicuously desiccated during low tides, whereas *M. parksii*, despite its higher shore elevation, does not normally dry to

the extent of becoming brittle. Little physiological work has been done on these species, although in a range of species from various tidal heights examined during desiccation, *G. furcata* had among the slowest rates of water loss and the most stable relative photosynthetic rate (Ji and Tanaka, 2002).

3.3.2. Crustose Red Algae

Hildenbrandia and *Apophlaea* are non-calcified crustose red algae that occur in the high intertidal zone. The former genus is cosmopolitan and has species that occur from the high intertidal zone (e.g. *H. rubra*) to the subtidal zone, whereas the latter has two species endemic to New Zealand (*A. sinclairii* and *A. lyallii*), both of which occur in the high intertidal zone. These crusts vary from extremely thin to several millimetres thick, and only *Apophlaea* has erect axes.

Both *Hildenbrandia* and *Apophlaea* must be highly adapted to the rigors of this habitat, and this was demonstrated experimentally. Over a 13-day period, winter collections of *H. rubra* from a mid-intertidal rock pool survived -17°C to 27°C, extreme desiccation and hyposaline conditions (4‰). Following each treatment, plants resumed control levels of photosynthesis within minutes. *Hildenbrandia* is extremely slow growing, and this contrasts with the rapidity of recovery following stresses imposed by Kim and Garbary (2006). Unlike algae from lower in the intertidal zone, *Apophlaea* showed almost complete recovery of photosynthesis following desiccation and there was little difference between loss of 10% of tissue water and 95% of tissue water (Brown, 1987). Brown also obtained similar results for the high intertidal red alga *Bostrychia arbuscula*. In the six species that Brown examined there was a clear response gradient to desiccation that correlated with height in the intertidal zone.

4. Survival Strategies

4.1. LIFE HISTORIES

4.1.1. Asexual Reproduction

Two distinct strategies of asexual reproduction are common in algae of the high intertidal zone: clonal growth and formation of asexual spores. Although these reproductive strategies are not limited to the high intertidal habitat, they are a prominent feature of these organisms. Most of the algae discussed here except the fucoids have an asexual spore bearing stage in which mitotically produced spores are formed in large numbers. This provides a rapid means of producing large populations during those short windows when the environment may be suitable. Well-studied examples of this are *P. stipitata* and *P. meridionalis* (e.g. Anderson and Foster, 1999; Rindi et al., 1999).

M. parksii is a high intertidal red alga common to the Pacific coast of North America in wave-exposed habitats. The alga is perennial and clonal, forming extensive patches with up to 20 fronds cm⁻² to mostly 2–4 cm in height. Although

the high density of fronds constrains photosynthesis via self-shading, decreased water loss (up to 43% at end of low tide) protects fronds from much higher desiccation than when densities are low (Scrosati and DeWreede, 1998).

4.1.2. Seasonality as an Escape Mechanism

Although the high intertidal zone presents a highly demanding environment year round, there are seasonal cycles of temperature, precipitation and solar radiation (including day length) that allow for seasonal growth of particular species. In cool temperate climates (e.g. western North America from Alaska to California) the high intertidal zone during winter often has tremendous growths of filamentous or finely bladed algae, including *Rosenvingiella*, *Ulothrix*, *Urospora*, *Blidingia* and *Bangia*. Many coastal habitats have long periods of cloud cover and precipitation accompanied by moderate temperatures. In the upper intertidal zone of Nova Scotia many rocky shores have a dense cover of *Bangia* and *Ulothrix* from winter to late spring (Garbary, unpublished). With the onset of summer most of these populations disappear or become highly reduced, except in shaded microhabitats. The tendency of these species to produce extensive populations in winter and spring has been attributed to both ameliorated environmental conditions and to a reduction in grazing pressure (e.g. Cubit, 1984; Anderson and Foster, 1999).

4.1.3. The High Intertidal Zone as an Escape from Herbivores

An explanation generally used for intertidal zonation has been that the upper distribution limits of species are limited by environmental constraints, and that the lower distribution limits of species are regulated by biological interactions of grazing and competition (Lüning, 1990). This generalization has been shown to be somewhat simplistic for the intertidal zone as a whole, as well as for the high intertidal zone (reviews by Foster, 1990, 1992). In addition, algal abundances can be regulated by site-specific processes that include distribution and abundance of grazers whose impact may be on the macroalgae or the microalgae (e.g. Anderson and Foster, 1999; Mak and Williams, 1999). These grazers are typically mollusks, but may include insect larvae and even mites. For example, herbivorous chironomid larvae in California regulated the high intertidal community of ephemeral green algae (Robles and Cubit, 1981). Also in California, populations of the high intertidal *P. meridionalis* were regulated seasonally not only by environmental factors, but also by herbivory of mollusks and mites (Anderson and Foster, 1999).

In addition to being an extreme environment for the algae, the high intertidal zone is also an extreme environment for potential herbivores. Thus, part of the reason these algae can grow in this environment is they may experience reduced herbivory. Chapman (1989) demonstrated that recruitment of *F. spiralis* was not dependent upon the consumer animals even though presence of grazers enhanced the cover of ephemeral algae including *Bangia*, *Ulothrix* and *Urospora* during the late winter and early spring. In experimental studies slightly lower in the intertidal zone Farrell (1991) showed that algal colonization was preceded by barnacle colonization. This was a secondary effect, in that the presence of barnacles

reduced foraging activity by limpets, and thereby gave the algae (*Silvetia*, *Fucus*, *Endocladia*) an opportunity to grow. Additional direct and indirect effects of herbivores were observed in algal succession in the high intertidal of Vancouver Island (Kim, 1997).

In conclusion, while environmental factors may be of greater significance in the high intertidal zone than lower on the shore, biological phenomena associated with competition and herbivory are also critical in determining the distribution, abundance and succession of species in this zone.

4.2. SYMBIOSES – ALGAL FUNGAL INTERACTIONS

4.2.1. *Prasiola borealis* and *Turgidosculum complicatum*

Two associations of the green algal genus *Prasiola* involve symbioses with fungi and have been described as lichens. These associations are geographically disjunct and are found in the high intertidal zone of northwestern North America and Antarctica where this mycophycobiosis is known as *P. borealis* and the lichen *T. complicatum* (= *Mastodia tessellata*). Although there has been some question as to whether this symbiosis was a fungal parasitism of *Prasiola crispa* or a true lichen, Lud et al. (2001b) showed a morphogenetic influence by the fungus on the alga, and that the association is established at the few-celled stage of the alga. Photosynthesis of the lichen has been studied by several authors (Huiskes et al., 1997; Smith and Gremmen, 2001). The latter found that photosynthesis declined at full salinity and that CO₂ assimilation was negatively affected by NH₄NO₃. When hydrated to a least 30% thalli underwent full photosynthesis, and the authors predicted that near maximal photosynthesis was possible for most of the year in the sub-Antarctic region (Smith and Gremmen, 2001).

As noted previously, *Prasiola* species are highly tolerant of UV exposure (Holzinger et al., 2006). The role of the fungal partner in the *Prasiola*-lichen association may be to heighten the UV tolerance of the association. This potential basis for a mutualism may be less significant in northwestern North America where solar radiation is reduced in the winter by cloud cover when *Prasiola* species in general are more conspicuous. In Antarctica, however, where UV radiation has increased as a result of the depletion of upper atmosphere ozone, this may be an important adaptation. It would be of interest to determine if lichenized *Prasiola* species are becoming more prevalent and able to survive greater exposures to UV than their non-lichenized forms.

4.2.2. *Apophlaea* and *Pelvetia*

A. sinclairii is an endemic red alga from the high intertidal zone of New Zealand in which perennial leathery crusts produce short, terete axes to 30 mm high. This alga is associated with the ascomycete fungus *Mycophycias apophlaeae* (previously *Mycosphaerella apophlaeae*) (Hawkes, 1983). Although no experimental studies have been published on the interactions between these species, this associ-

ation is similar to *A. nodosum* and *P. canaliculata* with their systemic populations of *M. ascophylli* (see Garbary and Deckert, 2001, for review). Thus the *Apophlaea/Mycophycias* association is also likely a mutualistic symbiosis. Pfetzing et al. (2000) suggest that the high volemitol production in *P. canaliculata* may be stimulated by the fungal symbiont. It would be of interest to know if volemitol is also produced in the *Apophlaea* symbioses.

Lamare et al. (2004) showed that *Apophlaea* had the highest concentration of MAAs of the marine organisms they examined, and it was the species from highest in the intertidal zone. It has not yet been established whether production or accumulation of MAAs is associated with the fungal partner, but we hypothesize that desiccation tolerance in *Apophlaea* is associated with its mycobiont similar to juvenile stages of *Ascophyllum* (Garbary and Deckert, 2001). The perennial crusts of *Apophlaea* would be a useful model system for studies of induction of MAAs and the role of heat-shock proteins in annual cycles of physiological adaptation.

Apophlaea is closely related to *Hildenbrandia*, a cosmopolitan red algal crust found from the high intertidal to subtidal habitats. Although some authors have reported fungal hyphae in *Hildenbrandia* species, these structures may be an artifact of sporangial cell walls. Regardless, these supposed fungal growths do not appear to form a systemic infection throughout the host (Pueschel, 1982).

5. The High Intertidal as a Model for Extraterrestrial Environments

Extraterrestrial environments are unlikely to be suitable for terrestrial organisms from most earth habitats, whether these habitats are aquatic or non-aquatic. Even the most earth-like of planets may preclude terrestrial organisms because of constraints of physical or chemical conditions. Thus, if there is the potential for terrestrial organisms to survive on exposed surfaces elsewhere, these species must be preadapted for survival in extreme earth habitats. Among the physiological adaptations that one would predict for survival in extraterrestrial environments are the following: tolerance of extremes of temperature, ability to tolerate extreme desiccation and intermittent water availability, as well as tolerance to extremes of solar radiation. Such organisms would likely be autotrophic, although not necessarily photosynthetic. These species are likely to occur in habitats with low biodiversity and few trophic levels, where there are few required interactions with other species for growth and reproduction. If the organisms are composite (i.e. lichens), then the symbionts would likely be tightly bound so that there is no stage at which the partners are required to be independent. They should also be capable of vegetative and asexual reproduction to maximize growth opportunities, and to allow rapid recovery after extensive mortality. This combination of features is met in many lichens and the algae that are discussed here, that is those that inhabit the upper reaches of the intertidal and supratidal zones of marine shores.

The combination of extremes of environmental factors that include temperature, solar radiation, and hyper- and hyposalinity make the high intertidal and supralittoral fringe among the most stringent (Tomanek and Helmuth, 2002). These coastal habitats have fluctuating conditions that make opportunities for growth and reproduction intermittent. These stresses result in the production of reactive oxygen species (Dring, 2005) for which metabolic protection mechanisms remain to be explored, but for which high intertidal organisms should provide ideal model systems. This combination of conditions may be a useful analogue for approximating the harshness of extraterrestrial conditions. Although relatively few algal species can survive in these conditions, these species are well adapted to this habitat.

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SEaweeds on the Abrasion Platforms of the Intertidal Zone of Eastern Mediterranean Shores

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1. Introduction

Over millions of years of evolution, marine macroalgae (commonly referred to as seaweeds) have remained within a narrow and restricted niche, compared to the extensive area covered by oceans and seas. This narrow fringe is the intertidal zone, in which seaweeds are intermittently exposed to harsh conditions such as high irradiance, desiccation and high temperatures. What were the adaptive strategies and physiological needs of these plants to thrive and complete their life cycles over millions of years in these harsh environments? Seaweed's first records date at least 300 million years, and within this period of time they went through several episodes of environmental change. Today, marine macroalgae comprise about 20,000 species of which a large number can be found within the intertidal zone. During evolution macroalgae diverged into three major categories or divisions: green (*Chlorophyta*), brown (*Phaeophyta*) and red (*Rhodophyta*) seaweeds. The present Mediterranean flora has a history of about five million years. After the isolation of the Mediterranean from the Atlantic, biota surviving the late cooling Miocene re-colonized the vacant basin and established the early Pliocene biota. Then, the Mediterranean Sea lost its coral reefs and its tropical character in general (Luning, 1990). The dramatic climate changes (glacial periods), which took place in this area in the Pleistocene, may have allowed a number of cold-temperate species to invade the area and to form disjunctive populations in cooler parts of the Mediterranean after the glaciations (Hoek and Breeman, 1990). Empty niche space and the climate changes in the late Pliocene and the Pleistocene may have promoted speciation and origin of endemic species. Today, the Mediterranean coasts are inhabited by a rich seaweed flora, including endemic, tropical, warm and cold-temperate species (Orfanidis, 1992).

2. The Intertidal Platforms – Tidal Cycles and Wave Action

The rocky shores of the eastern Mediterranean are distinctive abrasion platforms made of calcareous sandstones and limestones (Zahavi, 2006). These surfaces are largely influenced by tidal fluctuations, becoming submerged or exposed periodically. They range from few to about 30 m wide. The edges are usually higher than the rest of the platform surface, forming rims which enclose shallow tidal pools and potholes. These edges are the result of biological accretion of marine worms and snails, including *Vermetus triquetrus* and *Dendropoma petraeum*. Both species have a hard calcified skeleton, and they counter erosion by reinforcing the platforms. Tidal fluctuations in the eastern Mediterranean are modest (ca. 30 cm), yet they play a significant role when seaweeds on the platforms become exposed to air, particularly since dry conditions are common to the eastern Mediterranean. Extremely low sea levels occur during winter and early spring, when low humidity winds blow from the east (locally called 'Hamsin'). During calm seas and low tides, the algae may remain exposed for many hours at a time, affecting the dynamics of the seaweed community on these rocky surfaces. Prolonged exposures will temporarily (i.e. weeks) damage dominant and opportunistic species such as *Ulva spp.* and *Jania rubens*.

Thus, along the intertidal zone seaweeds are subject to the effect of two contrasting environments – the air and the sea. Several typical niches can be distinguished in this zone (Fig. 1).

The intertidal zone is both rich in algal biomass and variety of species. In the eastern Mediterranean the height of the waves is as important as the oscillations

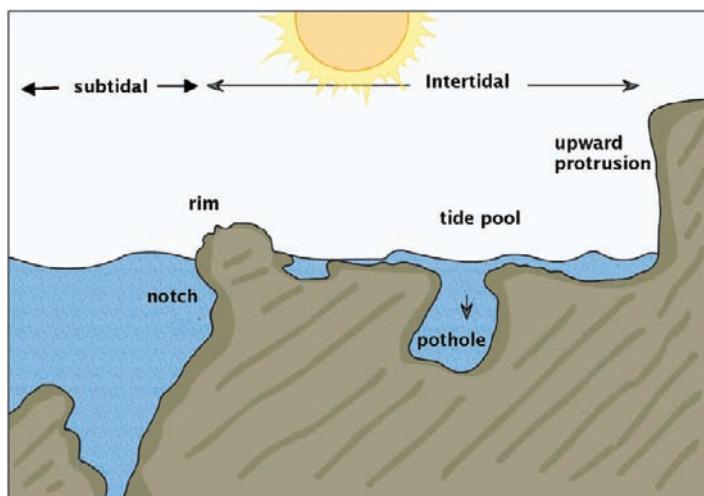


Figure 1. A vertical profile of an intertidal platform in the eastern Mediterranean showing the typical niches created in which seaweeds can be found.

of the tides, therefore, seaweeds in the intertidal zone are generally found between the tidal water lines. Algal communities found above the tidal zone are rarely covered by seawater but rather sprayed by waves. It is difficult to distinguish between the individual effects of various environmental parameters on littoral algal communities. Sunlight provides the light and temperature required to for sustaining photosynthesis, but also causes dehydration and raises intracellular salt levels. In turn, the concentration and speciation of inorganic carbon (C_i) are affected by temperature, pH and the presence of vegetation and animals in the area. While determining the level of competition for resources in nature is not trivial, it seems that the resource most in demand in the intertidal zone is substrate.

Rocky beaches are generally richer in algal species than sandy beaches where the substrate is highly unstable. Rocky shores are, nevertheless, comparatively rare in the eastern Mediterranean due to the significant effect of sand runoff from the nearby Nile River. Seaweeds need stable substrates to attach, which are coincidentally located in exposed sites with abundant nutrient supply. The development of macroalgae also hinders the development of many other marine communities. Seaweeds therefore comprise a major factor in determining the composition and profusion of communities. In the eastern Mediterranean most macroalgae grow to small sizes; thus one clear trend is the smaller size of seaweeds and other organisms in the eastern Mediterranean in relation to counterpart species in the western basin. This ‘nanism’ syndrome within the eastern Mediterranean applies also to fish and shellfish, although comprehensive studies into this matter have not yet been carried out. One explanation is that the relatively high water temperature prompts earlier sexual maturity in marine organisms before they reach full size. Some studies consider this a form of adaptation to the oligotrophic conditions common in this area (Sonin et al., 1996; Einav, 2004).

Winds in the eastern Mediterranean blow year round from the west and the northwest. They carry energy from up to 500 km away (Goldsmith and Golik, 1978) creating waves, turbulence and foam along the shore line. Overall, seaweeds can benefit indirectly by waves as these erode the abrasion platforms, or directly, as turbulence supplies minerals and dissolved CO_2 . A study carried out offshore (Hayonim Island, c.a. 35 km south of Haifa), compared algal communities and showed that those sides of the Island more exposed to the effect of waves had both larger standing stocks and algal species richness than less exposed areas (Einav et al., 1996, 1998).

3. Air Exposure and Desiccation

Air exposure in seaweeds promotes water loss and dehydration leading to increasing solute concentration. In contrast to most terrestrial plants, marine plants are not well equipped with mechanisms or anatomical features that prevent water loss from their tissues. When exposed to air, seaweeds loose significant amount of water in an exponential fashion. The rate of water loss depends on local environmental

conditions such as temperature, relative air humidity, wind velocity and topography. Most seaweeds are equally vulnerable to desiccation; however, morphology and other features may contribute to delay dehydration. Photosynthesis of most algae may initially not be affected from exposure to air, but here too responses vary. As dehydration sets in, the algal ability to absorb CO_2 from the air or from the thin seawater film on the thallus surface decreases, as do many intracellular enzymatic processes. One crucial enzyme, carbonic anhydrase, which catalyzes the interconversion of HCO_3^- (abundant in seawater) to CO_2 (the ultimate substrate for photosynthesis), is located on the cell's surface and is impaired by dehydration (Beer and Eshel, 1983a). If water loss continues, other physiological functions of the alga will be damaged. During low tides in spring algae may be exposed to prolonged desiccation leading to massive bleaching and partial destruction of the communities (Fig. 2).

Various species of algae excel in their ability to maintain positive photosynthetic levels, even after losing 90% of their water content (Friedlander et al., 1991; Lipkin et al., 1993). Examples of high tolerance to air are found in *Gelidiella pannosa* (Einav, 2004) or the leaf-like *Porphyra* and *Ulva* that grow in dense communities in the upper intertidal zone (Fig. 3). Survival strategies in these species may involve a combination of 're-sorting' of the layers with wave action and tide change and/or shielding of the main bulk of the plant by the upper layers. In this way, the thalli underneath are protected from desiccation and photodynamic damage. This phenomenon is called overlapping. The adaptability of intertidal algae to air exposure is composed of two strategies. One is the ability to maintain positive photosynthesis during exposure, and the other is the ability to recover



Figure 2. Bleaching of *Ulva* in the upper intertidal after prolonged periods of desiccation.

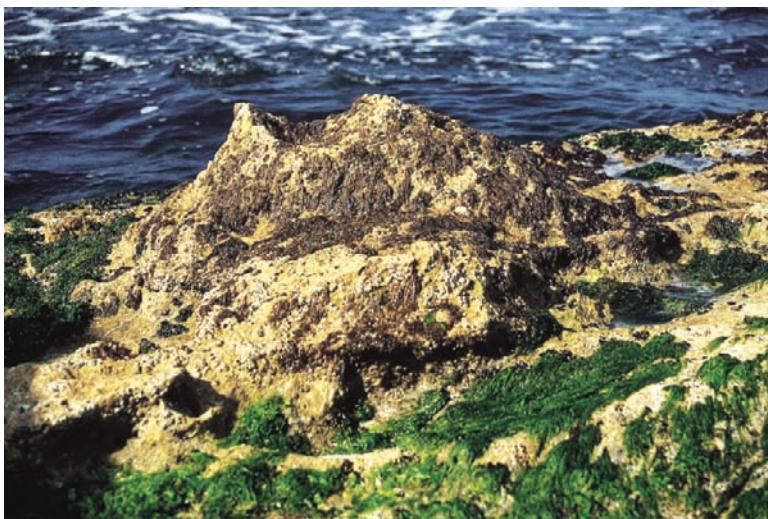


Figure 3. Zonation of seaweeds in the upper intertidal showing *Porphyra* and *Ulva* when exposed to low tide.

quickly upon immersion in seawater. Clearly, algal resistance to dehydration is one of the key factors affecting seaweed community patterns in the intertidal zone.

The advantage of air exposure to the photosynthesis of some seaweeds is due to their capacity to use air-born CO₂ directly, conferring clear advantage over those unable to do so. Two conditions must be met to make this CO₂ usable: (i) the seawater film on the thallus surface during emersion needs to be thick enough to ameliorate water loss yet (ii) not too thick as to avoid CO₂ passage from the air into the cells. In some species, rates of photosynthesis measured in the air may be 3–5 fold higher than rates measured when submerged. This can be maintained as long as other environmental parameters such as increased temperature, salinity or dehydration do not significantly harm the photosynthetic capabilities of exposed algae (Einav and Beer, 1993; Einav et al., 1995).

4. Irradiance

Light plays a critical role in sustaining photosynthesis and growth, as seaweeds transform it into chemical energy needed for metabolic processes. Light penetrating into seawater swiftly loses energy; therefore, light may be a limiting resource in many marine photosynthetic organisms. However, algae that develop on the abrasion platforms are not limited by sunlight (Fig. 4). Indeed, many Mediterranean species reach maximum photosynthesis at intensities 1–10% of



Figure 4. A rocky shore in Hayonim Island exposed at low tide showing upward protrusion, tide pools and potholes bearing abundant seaweed populations during spring when light is already plentiful.

full sunlight (Einav and Beer, 1993). Further increases in irradiance will lead to photochemical inhibition of photosynthesis and growth. With depth in the subtidal zone the irradiance decreases and so does the algal community. This is even more pronounced due to the fact that water turbulence and sediment agitation creates littoral waters with relatively high turbidity (Goldsmith and Sofer, 1983). Approaching the lower limit of the photic zone (not accurately measured, however believed to be at approximately 100 m), calcifying algae prevail, such as *Corallina elongata* and *Alsidium corallinum*, and in even more shaded areas – *Lobophora variegata*, *Peyssonnelia squamaria* and *Bryopsis plumosa*. Also, lack of sunlight in intertidal cavities is a significantly limiting environmental factor. The darkest areas are populated by mostly sponges, colonial hydras and bryozoa, displacing the algae. Thus, light intensity is a controlling factor in seaweed community development in cavities and at increasing depths, but not in the intertidal zone, where algae are saturated with light.

5. Temperature

As for most biochemical processes, algal photosynthesis and respiration are strongly affected by temperature (Lobban and Harrison, 1994). Indeed, seawater temperature is another key environmental factor determining variations in algal communities throughout the year. Numerous studies have examined the relationships between temperature, algal growth and the structure of the algal communities. During winter, several species, such as *Acanthophora nayadiformis* and *Hypnea musciformis*, disappear from the coast and are visibly replaced by species of the order Ulvales, which then dominate large parts of the intertidal zone (Einav and Beer, 1993). Generally, with decreasing temperature both photosynthetic as well as respiratory levels drop, thus directly inhibiting growth. This enables winter species or other opportunistic species to invade the area and take over.

Whereas seawater temperature is rather homogenous and changes gradually with seasons, air temperature may shift swiftly over a 10–15°C range during a 24-h period. During low tide, or as a result of low wave activity, temperature changes are apparent in potholes. In some isolated potholes and rocky pools increasing temperature lowers gas solubility resulting in low CO₂ and O₂ contents. This stands in contrast to the higher demand for CO₂ as the temperature rises. Several species are distinguished according to their ability to withstand high temperatures for extended periods of time.

6. Salinity

The eastern Mediterranean basin is both warmer (17–29°C) and saltier (39‰) than the western basin in the proximity of the Atlantic Ocean (12–27°C and 36‰, respectively), creating low chlorophyll levels in open waters (Kress and Herut, 1998). In microenvironments within the tidal zone, such as in tidal pools or potholes, seaweeds may face increasing salt levels during low tides. Upon exposure to air and the onset of water evaporation salinity levels in the immediate algal environment rise, thus affecting the algal intracellular salt balance. Salinity may also temporarily decrease as a result of intense rain or proximity to freshwater sources in rivers; however, these situations are rare to find in the eastern Mediterranean. High salinity causes changes in osmotic levels and affects several of the algal biochemical and physiological processes such as water exchange, cell membrane functions and impaired ability to produce spores (Russell, 1987; Israel et al., 1999a). Seaweeds have different adaptations to varying salinity with responses ranging from a lowering of photosynthetic activity to its complete cessation. Algae that develop in elevated pits (potholes located far beyond sea levels) may over time develop a better resistance to rising salinity levels, but this is a slow process, lengthier than the extent of intertidal oscillation cycles (Einav, 2004). Exposures of days or weeks to a hypersaline environment may induce morphological changes in algal morphology and life cycle. Organisms that migrate from the Red Sea find niches

in the Mediterranean Sea which has lower salt content than average salinities of the Red Sea (39‰ vs. 42‰; Einav, 2004). One example is *Hypnea cornuta* that frequently inhabits rock pools where salinity is high.

7. Inorganic Carbon, Nitrogen and Phosphorous

The Mediterranean is essentially an oligotrophic sea with key nutrients such as nitrogen (0.05–6 ppm total N) and phosphorous (0.03 ppm total P in average) existing at low concentrations, thus generally limiting algal growth (Barak et al., 2005). The N:P ratios vary throughout the year from 1:1 to 1:16. Water flow from the Atlantic Ocean into the Mediterranean is limited and relatively poor in nutrients. A large part of the nutrients in the eastern basin originates from rivers and continental runoff. Recently, the contribution of the Nile and other minor freshwater rivers has diminished considerably.

In order to maintain positive photosynthesis and growth (i.e. positive balance of CO_2 uptake vs. CO_2 respired), marine macroalgae require sufficient levels of light and Ci. While light is plentiful, Ci must reach the chloroplasts in the form of CO_2 to be effective for photosynthesis. The seawater medium has a strong buffer capacity in which a fixed level of acidity is maintained (i.e. pH 8.2). Under these conditions HCO_3^- is present at a concentration about 200 times that of CO_2 (ca. 2.0 mM vs. 15 μM). The well recognized carbon concentrating mechanisms allow seaweeds to utilize HCO_3^- in a very efficient manner. These mechanisms seem to be also widespread in Mediterranean species including those present in the intertidal area, such as *Ulva (Enteromorpha) compressa*, *Ulva rigida*, *Gracilaria conferta* and *Gelidiopsis* (Beer and Eshel, 1983b; Israel and Friedlander, 1998; Israel et al., 1999b). Therefore, in general, marine algae do not suffer from carbon limitations in the HCO_3^- rich medium that normal seawater provides, even under exposed or semi-exposed conditions normal in the intertidal zone. Further constraints on carbon acquisition of seaweeds deal with the unstirred boundary layer created at the thallus surface which impedes diffusion of Ci into the cells.

It must be remembered that photosynthesis of marine macroalgae would be severely limited if it were dependent only on the diffusional flux of CO_2 from the medium to the site of fixation via Rubisco. The reasons for CO_2 limitations in seawater involve (i) rather low concentrations of CO_2 [ca. 30% lower (v/v) than in the air (depending on temperature) and ca. 15% lower than in freshwater (depending on salinity)], (ii) low diffusion rates in liquid media (four orders of magnitude slower diffusion than in air), and (iii) slow uncatalyzed rates of formation from HCO_3^- (the dehydration half-time can be up to 0.5 min). The pH in the diffusion boundary layer is positively related to the photosynthetic activity of the plant, and the latter is both species-specific and a function of external parameters such as temperature, irradiance and nutrient status (cf. Drechsler et al., 1994; Axelsson et al., 1995). In the ubiquitous, fast-growing green alga *Ulva lactuca* the environ-

mental conditions set the preferred mechanism of HCO_3^- utilization; thus, the typical carbonic anhydrase-mediated dehydration of HCO_3^- at average seawater conditions (i.e. 25°C and pH 8.2) is switched to mainly direct HCO_3^- uptake at higher external pH (Axelsson et al., 1995).

8. Grazing

It is widely accepted that grazers such as fish, snails and a variety of shellfish in the subtidal are more active than in the intertidal zone. In general, vertical distribution of seaweeds within the intertidal shows that, whereas the upper boundaries of algal species are determined by environmental/physiological limitations, the lower ones are determined by herbivory, especially by fish. *Ulva (Enteromorpha) compressa* and *Ulva olivascens*, usually found in the uppermost zones of the intertidal, are scarce at depths. Apparently, these species have no physiological constraints to growth deeper in the subtidal zone; yet they become less dense due to heavy herbivory. They may be found in the subtidal zone at the peak of their growth season before the fish arrive. For example, the omnivorous species *Siganus rivulatus* and *Siganus luridus*, both immigrants from the Red Sea, feed mainly on these seaweeds (Lundberg, 1989). They prefer specific species of algae and do not eat all species equally. Fish activity may be related to the disappearance of some species from the intertidal, such as *Caulerpa prolifera* and *Halymenia sp.* (Einav, 1998). Others, such as the calcified *C. elongata* are not particularly attractive to species of *Siganus*. The profusion of calcifying species in the subtidal zone may be the result of massive herbivore activity on other, softer, algae.

9. Seaweeds in Globally Changing Marine Environments

In the marine environment global changes will include higher total Ci concentrations in seawater due to a continuous rise in atmospheric CO_2 (predicted to double within the next century; Conway and Tans, 1996), a ‘greenhouse effect’ created by specific gases, particularly CO_2 , trapping heat which might increase the seawater temperature by an average of 3°C, and an increase of solar flux of UV radiation in its two damaging forms, UVA (320–400 nm) and UVB (290–320 nm) (Crutzen, 1992). Consequently, in approximately 50 to 100 years time, the oceans and seas of the world could likely become more acidic, richer in inorganic carbon and slightly warmer than they are today. These global changes will certainly place further environmental constraints also on seaweed communities from the eastern Mediterranean.

Although restricted to intertidal areas, benthic macroalgae play an important role in marine primary production. Also, they serve as food source for herbivores and detritivores, nursery areas for juvenile fish and crustaceans, and food stuff and production of natural products for humans. In addition, marine

vegetation interacts with its environment and other marine organisms by buffering against large changes in nutrient concentrations. Despite the clear importance of marine macroalgae in coastal systems effects of rising Ci in seawater and UV radiation in nutrient rich areas are fundamentally missing, particularly for seaweeds (Franklin and Forster, 1997; Israel and Hophy, 2002). Both UVA and UVB wavelengths can penetrate to ecologically significant depths in seawater with the absorption of UV radiation largely dependent on the concentration of chlorophyll and dissolved organic matter (Jerlov, 1950; Smith and Baker, 1979, 1989; Wood, 1987). It has been conventionally assumed that UVB radiation produces the greatest damage to living organisms (although one 'positive' effect known so far is the increased availability of Fe in marine plants; Palenik et al., 1991); however, there is increasing, yet inconsistent, evidence that UVA by itself or combined with UVB may be less harmful than expected.

An increasing number of studies have shown that macroalgae exposed to deleterious UV radiation may suffer damage of the photosynthetic apparatus and, under severe UV stress, DNA damage (Franklin and Forster, 1997). Even under 'normal' conditions solar UVB is known to have inhibitory effects on photosynthetic performance and nutrient uptake (Larkum and Wood, 1993). It further seems that the response to UVB is species-specific and that many organisms have evolved strategies such as photorepair or the presence of screening compounds for coping with deleterious effects of UVB radiation (Franklin and Forster, 1997). Many intertidal macroalgae show less inhibition of photosynthesis by UVB than their subtidal counterparts (Wood, 1987; Gomez and Figueroa, 1998), clearly because differences exist between macroalgae living deep in shaded environments and those regularly exposed to surface or near surface levels of radiation. Thus, production of UV-blocking agents (such as mycosporine-like amino acids – MAAs) in algae exposed to excessive UV levels are more commonly found for intertidal species (Gröniger et al., 1999; Sinha et al., 2000) and confer an important strategy to lessen UV photodamage. Thallus morphology can also influence the susceptibility of marine macroalgae to UV radiation, with thicker thalli being less sensitive than thinner thalli, although exceptions such as the leaf-like red alga *Porphyra* do exist (Gröniger et al., 1999).

Global warming due to increasing levels of atmospheric CO₂ and other 'greenhouse gases' forecast rising sea levels as well. Thus, seawater temperature levels and sea level trends were reported to be correlated. Sea level has risen more than 120 m since the peak of the last ice age, about 18,000 years ago. The bulk of that rise occurred about 6,000 years ago. From 3,000 years ago to the start of the 19th century, sea levels have risen at a constant rate of 0.1–0.2 mm y⁻¹; since 1900 the level has risen at 1–3 mm y⁻¹; and since 1992 satellite altimetry from TOPEX/Poseidon indicates a rate of about 3 mm y⁻¹. This rapid rising should be regarded as a warning sign of the effect of global warming on sea levels. The impact of rising sea level is important because the likely scenario involves the abrasion platforms becoming permanently submerged. Consequently, the dynamics and composition of algal

communities on today's intertidal zone will be different when shifting temperatures, light intensities and Ci create a different competitive environment for both intertidal and subtidal seaweeds.

10. Summary

In the eastern Mediterranean, intertidal algal communities are abundant with high standing stocks developing on abrasion platforms during high growth seasons (spring and fall). These platforms are, however, periodically exposed to air during low tides. Although tidal fluctuations are limited (ca. 30 cm), seaweeds become exposed to severe conditions of temperature, irradiance and dehydration. These particularly harsh conditions allow for the growth of many species not found in the deeper waters of the benthic zone.

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STATUS OF MANGROVE ECOSYSTEM:

Exploring the Potential Role of Cyanobacteria in Restoration and Afforestation

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1. Introduction

‘Mangrove’ is an overall term to indicate a tropical or subtropical community of highly adapted trees and shrub species growing in intertidal estuarine and secluded marine areas. Mangroves act as physical barrier to mitigate the effects of coastal disasters like tsunami, hurricanes, and waves. Mangroves create unique niche that hosts rich agglomeration of species diversity. The submerged part of mangrove roots, trunks, and branches serve as islands of habitat that may attract rich epiflora and faunal communities including bacteria, fungi, macroalgae, and invertebrates. Despite low nutrient levels, mangroves grow efficiently in this environment (Sengupta and Chaudhuri, 1991; Alongi et al., 1993; Vazquez et al., 2000; Bashan and Holguin, 2002) through efficient recycling of available nutrients by the activity of microorganisms (Alongi et al., 1993; Kathiresan, 2000; Holguin et al., 2001; Bashan and Holguin, 2002). Even though mangrove ecosystem is one of the valuable ecosystems, this is the most threatened one at present (Farnsworth and Ellison, 1997; Kathiresan, 2000; Adeel and Pomeroy, 2002). Besides making awareness amongst society, policy-makers can also constitute by-laws for the conservation and restoration of mangrove ecosystem.

Bacteria have significant role in the recycling of nitrogen in mangrove environments. Cyanobacteria, a group of photosynthetic prokaryotes, are vital component of the microbiota ranging from unicellular colonial to filamentous contribute a source of nitrogen in every mangrove ecosystems (Kathiresan and Bingham, 2001). This is one of the ignored groups where only a very few studies have been conducted. The studies on cyanobacteria associated with mangroves are very important not only because of their abundance, but also of their high capability for nitrogen fixation, which are natural candidates for future reforestation and rehabilitation of destroyed mangroves (Bashan et al., 1998). Hence, this chapter is aimed at depicting the present status of mangroves, their importance, and to analyze the pioneer articles on cyanobacteria inhabiting in mangrove ecosystems.

2. Mangrove Ecosystem – A Bountiful Resource

Mangroves have immense ecological value and they play important role in protecting and stabilizing coastlines (Field, 1999; Lewis, 1990; Rajiv, 1999). They improve the quality of coastal waters by exporting large amounts of carbon

Table 1. Uses of mangroves at many levels.

Ecological uses	Economical uses	Social uses
Erosion control	Pearl culture	Education
Protection from damage/tsunami	Apiculture	Ecotourism
Indicator of climate change	Lac culture	Food
Habitat provision	Silviculture	Local employment
Water quality management	Shrimp and crab industries	Agriculture
	Charcoal production	Traditional medicine
	Timber production	
	Firewood	

and nitrogen, yielding commercial forest products, and support coastal fisheries (Kannan, 1990; Vidy, 2000; Holguin et al., 2001; Alongi and McKinnon, 2005; Romigh et al., 2006).

This ecosystem serves the mankind in many ways (Table 1). The benefits obtained from mangrove ecosystem can be classified into three types viz., economical, social, and ecological. The economical and social uses exclusively cater to human population directly. However, the ecological uses tend to maintain the balance in the ecosystem. For the sustainable exploitation, the governing bodies should consider both the livelihood of dependents and perpetuation of the ecosystem. This can be achieved by involving the dependents and adopting scientific advancements.

In a way, most of the benefits derived from mangrove forest contribute to the degradation too. All such uses can be made into sustainable outcome from mangrove forest, if the management considers the balance between the exploitation and conservation of the system. For example, leaving the juvenile plants to grow and/or planting new seedlings periodically can conserve mangrove area though the plants are cut off for the timber production.

3. Present Status of Mangroves

Food and Agriculture Organization (FAO) has given the details of global estimate of mangroves (Fig. 1). FAO denoted the reduction of about 5 million hectares in global area of mangrove forest. According to the survey, the most comprehensive data on the state of the world's mangrove forests revealed that the mangrove area worldwide had fallen below 15 million hectares at the end of 2000 down from an estimated 19.8 million hectares in 1980 (FAO, 2003).

The deforestation is because of many reasons especially anthropogenic effects rather than natural calamity (Kairo et al., 2001). This human infringement of habitat destruction includes exploitation of land for urbanization, agriculture, aquaculture and mining, and clearing forest for fire wood and timber (Farnsworth and Ellison, 1997; Kairo et al., 2001; Adeel and Pomeroy, 2002).

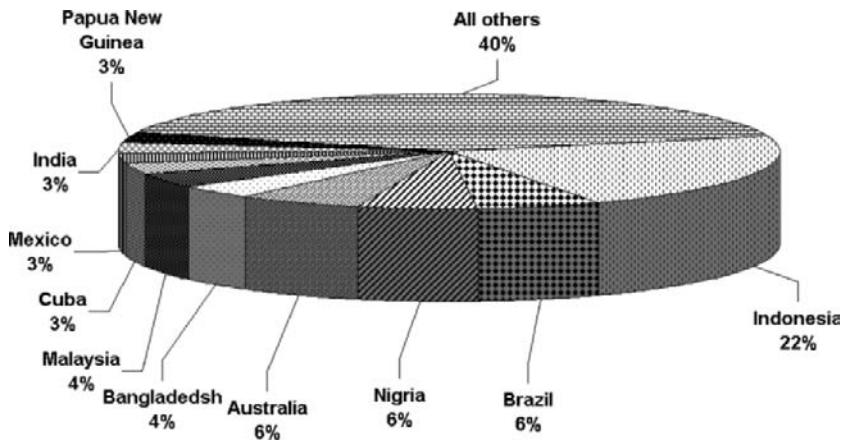


Figure 1. Global estimate of mangrove forest. Source: FAO (2003).

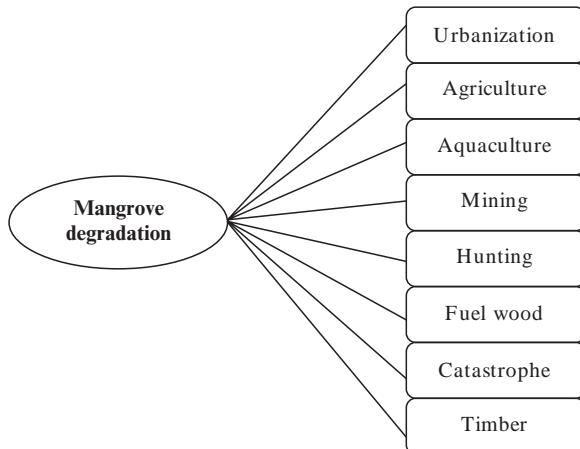


Figure 2. Reasons for mangrove degradation.

The diverted use of freshwater for irrigation and land recovery has minimized the freshwater input that lead to rapid destruction of mangrove forests. Further, dam construction has also affected the freshwater inflow (Kathiresan and Bingham, 2001). The major reasons for mangrove degradation are generally categorized as below based on their modes (Fig. 2). There is a critical need to ameliorate the above said problems for the betterment of mangrove ecosystems. The public awareness should be spread all over the world. This will promote the

understanding of the incredible mangrove ecosystem and make them to involve in the conservation programs.

4. Conservation and Restoration of Mangroves

The conservation and restoration of threatened mangrove ecosystem were already insisted (Field, 1999; Kairo et al., 2001; Toledo et al., 2001). Conservation of mangrove is an important commitment for all the countries that possess mangrove ecosystem. Awareness spreading globally on the value of such ecosystem is increasing. Hence, governments of these countries need to conserve by legislating new laws. Adger et al., (2005) stated the enhancement of social-ecological resilience as an immediate necessity for coastal disaster management. Restoration of an ecosystem is the act of bringing an ecosystem back to, as nearly as possible, its original condition (Field, 1998). Milano (1999) has dealt with the success of restoration and enhancement program (over 121.5 hectares) in Florida and ultimately suggested such program for global consideration by adopting different approaches.

4.1. NURSERY DEVELOPMENT

The establishment of nursery of coastal plants especially well-adapted mangroves will help in such process of resilience to mitigate the effect of coastal disasters. Nursery approach is one of the best traditional methods of conserving mangroves. Experiments and field trials were performed on this approach and the feasibility of this approach was already recognized (Field, 1999; Rajiv, 1999; Kairo et al., 2001; Toledo et al., 2001; Clarke and Johns, 2002). There were many considerations to succeed in this approach with proper nursery technique (Toledo et al., 2001; Clarke and Johns, 2002). Modern biotechnological approach is considered a necessity for development of nursery.

4.2. ECOLOGICAL ENGINEERING

Ecological engineering is a developing field of science which resolves problems pertaining to any of the ecosystem. Mesocosm is a closed system that contains all the biotic and abiotic components of interest, and mimics environmental conditions in the laboratory. Mesocosm study of mangrove forest was carried out to understand ecology and species composition (Finn, 1996; Finn et al., 1999). It was proved that physical alteration in hydrology of mangrove ecosystem a highly successful approach for such restoration (Lewis, 2005). Hence, for restoration program ecological engineering should also be considered for its success.

4.3. BIOTECHNOLOGICAL APPROACH

Biotechnological approach includes production of genetically improved trees, application of plant growth promoting microorganisms, propagation of mangroves by micropagation or vegetative propagation or seed propagation (Bashan et al., 1998; Field, 1999; Bashan et al., 2000). Many of these methods should be taken for detailed research in the view toward future conservation and restoration of mangrove forest and it requires standardization for every mangrove species. Further, though during the last decade of the twentieth century, attempts have been initiated to apply microorganisms for the growth of mangroves (Bashan et al., 1998; Bashan et al., 2000; Rojas et al., 2001) it is not fully established.

5. Inhabitation of Cyanobacteria on Mangroves

The presence of cyanobacteria in several mangrove ecosystems was explored in many countries (Santra et al., 1988; Kannan and Vasantha, 1992; Mani, 1992; Hoffmann, 1999; Nogueira and Ferreira-correia, 2001; Kyaruzi and Muruke, 2003). Mangroves are occupied by diverse cyanobacterial communities, which reside on leaf, root litter, live roots, and often form extensive mats on the surrounding sediments; many of these communities are capable of fixing atmospheric nitrogen (Hoffmann, 1999). The genera *Oscillatoria*, *Lyngbya*, *Phormidium*, and *Microcoleus* are widespread in these habitats, as heterocystous genera, like *Scytonema*, in some areas (Hoffmann, 1999). The taxonomic survey of the Cyanobacteria in a red mangrove forest of the Brazil estuaries revealed total of 15 taxa in 8 families, as follows: *Synechococcaceae* (2 taxa), *Chroococcaceae* (1 taxa), *Hyellaceae* (1 taxa), *Xenococcaceae* (1 taxa), *Oscillatoriaceae* (1 taxa), *Scytonemataceae* (2 taxa), *Phormidiaceae* (5 taxa), and *Pseudanabaenaceae* (2 taxa) (Nogueira and Ferreira-correia, 2001). A total of ten genera of cyanobacteria were recorded in mangrove ecosystem adjacent to Zanzibar, these consist of: (i) the heterocystous cyanobacteria genera viz., *Anabaena* and *Rivularia* and (ii) non-heterocystous genera viz., *Aphanocapsa*, *Merismopedia*, *Lyngbya*, *Microcoleus*, *Oscillatoria*, *Phormidium*, *Schizothrix*, and *Spirulina* (Kyaruzi and Muruke, 2003).

The colonization of different species prefers various parts of the tree. In many cases, the aerial roots, especially pneumatophores, give refuge to specific cyanobacterial populations that may show sharp vertical zonation (Kathiresan and Bingham, 2001). Non-heterocystous, particularly filamentous cyanobacteria resembling like *Lyngbya* sp. and *Oscillatoria* sp. colonized the bottom part near to the sediment, the filamentous cyanobacteria resembling *Microcoleus* sp. colonized the central zone, and by coccoidal cyanobacteria resembling *Aphanathece* sp. mixed with undefined filamentous cyanobacteria colonized the upper part (Toledo et al., 1995b). *Lyngbya* sp., *Polysiphonia* sp., and *Oscillatoria* sp. are common epiphytic cyanobacteria of submerged root system of *Rhizophora* sp., (Krishnamurthy and Jayaseelan, 1983). The pneumatophores of mangroves in West Bengal, India,

are colonized by a number of cyanobacteria viz., species of *Calothrix*, *Anabaena*, *Lyngbya*, *Hydrocoleum*, and *Schizothrix* (Santra et al., 1988).

The planktonic cyanobacteria found in West Bengal, India, were belong to the species of *Trichodesmium*, *Synechococcus*, *Aphanothece*, *Gloeocapsa*, *Gloeothece*, *Merismopedia*, *Oscillatoria*, *Johannesbaptistia*, *Microcystis*, and *Stigonema* (Table 2) (Santra et al., 1988). Three species of planktonic cyanobacteria have been recorded in Pichavaram mangroves, *Anabaena* sp., *Oscillatoria* sp., and *Trichodesmium* sp., (Kannan and Vasantha, 1992; Mani, 1992). Eight

Table 2. Cyanobacteria associated with mangroves.

Site/category	Cyanobacterial species	Location	References
Pneumatophore	<i>Calothrix</i> sp.	West Bengal, India	Santra et al. (1988)
	<i>Anabaena</i> sp.		
	<i>Lyngbya</i> sp.	Mexico	Toledo et al. (1995a)
	<i>Hydrocoleum</i> sp.		
	<i>Schizothrix</i> sp.		
	<i>Microcoleus</i> sp.		
	<i>Lyngbya</i> sp.		
	<i>Plectonema</i> sp.		
	<i>Anabaena</i> sp.		
Epiphytic	<i>Dermocarpa</i> sp.	West Bengal, India	Santra et al. (1988)
	<i>Xenococcus</i> sp.		
	<i>Chamaesiphon</i> sp.		
	<i>Stichosiphon</i> sp.		
Planktonic	<i>Trichodesmium</i> sp.	West Bengal, India	Santra et al. (1988)
	<i>Synechococcus</i> sp.		
	<i>Aphanothece</i> sp.		
	<i>Gloeocapsa</i> sp.		
	<i>Gloeothece</i> sp.		
	<i>Merismopedia</i> sp.		
	<i>Oscillatoria</i> sp.		
	<i>Johannesbaptistia</i> sp.		
	<i>Microcystis</i> sp.		
	<i>Stigonema</i> sp.		
	<i>Pseudanabaena</i> sp.	West Bengal, India	Banerjee and Santra (2001)
	<i>Anabaena</i> sp.		
	<i>Coelosphaerium</i> sp.		
	<i>Lyngbya</i> sp.		
	<i>Merismopedia</i> sp.		
	<i>Microcystis</i> sp.		
	<i>Oscillatoria</i> sp.		
	<i>Spirulina</i> sp.		
	<i>Trichodesmium</i> sp.	Pichavaram, Tamilnadu	Mani (1992) and Kannan and Vasantha (1992)
	<i>Anabaena</i> sp.		
	<i>Oscillatoria</i> sp.		

planktonic cyanobacterial species were isolated from Sundarban mangrove estuary in India (Mani, 1992). The population density of phytoplankton is decreased in monsoon and increased in pre-monsoon periods, but the percentage of cyanobacterial population has increased during monsoon and decreased in pre-monsoon periods (Mani, 1992; Banerjee and Santra, 2001). The change in population density of cyanobacteria is due to the variations in environmental factors such as temperature, salinity, light, etc. (Banerjee and Santra, 2001).

A study on mangrove-associated cyanobacteria in Muthupet estuary region in India has recorded the presence of 17 cyanobacterial species (Selvakumar and Sundararaman, 2001). The said study was aimed at to reveal the unique associates of cyanobacteria on mangrove trees. Cyanobacterial species viz., *Aphanocapsa koordersi*, *Johannesbaptista pellucida*, *Oscillatoria vizagapatensis*, and *O. tenuis*, colonized on *Avicennia marina*; cyanobacterial species viz., *Porphyrosiphon natarsii*, *Phormidium* sp., *Oscillatoria calcuttensis*, and *Schizothrix telephorides* were found only on *Aegiceras corniculatum*; cyanobacterial species viz., *Aphanocapsa bullosa* and *A. littoralis* were specific to *Excoecaria agallocha* and the cyanobacterial species *Oscillatoria claricentrosa* was found only on *Sueada martima* (Selvakumar and Sundararaman, 2001). The species specificity may be attributed to the root exudates and also the environment concern. The root exudates may play a key role in forming a community structure by selectively stimulate and enrich certain groups of bacteria (Burgmann et al., 2005). Hence, the knowledge of species specificity of cyanobacteria is most important while applying it as 'biofertilizer' for the restoration of mangrove ecosystem even at the nursery stage.

6. Ability to Fix Atmospheric Nitrogen

In different environments, nitrogen availability can limit the growth and ecosystem productivity (Feller et al., 2003). Biological nitrogen fixation, or diazotrophy, the fixation of atmospheric nitrogen gas (N_2) into biologically available ammonia (NH_3), is important in making nitrogen available in many ecosystems (Omoregie et al., 2004).

Nitrogen fixation represents a new source of nitrogen to mangrove forests, but the contribution of this process to the nitrogen budget of mangrove wetlands remains poorly understood (Pelegri and Twilley, 1998). Rate of nitrogen fixation in mangrove wetlands varies with species, community types of nitrogen-fixers, concentration of organic carbon substrates, and ambient conditions such as inundation, temperature and light (Gotto and Taylor, 1976; Zuberer and Silver, 1978; Potts, 1979; Van der Valk and Attiwill, 1984). Cyanobacteria, common in mangroves may play a pivotal role in fixing atmospheric nitrogen (Gotto and Taylor, 1976; Van der Valk and Attiwill, 1984). The amount of nitrogen fixed by cyanobacterial mat is comparatively high (Howarth et al., 1988). Nitrogen fixation in a mangrove ecosystem in South Australia could supply about 40% of the annual nitrogen requirement, estimated to be $13\text{ g N m}^{-2}\text{ year}^{-1}$ for *Avicennia* trees (Van der Valk and Attiwill, 1984). Mangrove in Florida, biological nitrogen fixation could supply up to

60% of the nitrogen requirement (Zuberer and Silver, 1978) and nitrogen-fixation rates associated with different locations of coastal environments was elaborately given in Table 3. (Howarth et al., 1988).

In mangrove ecosystems, high rates of nitrogen fixation have been associated with dead and decomposing leaves (Goto and Taylor, 1976; Zuberer and Silver, 1978, 1979; Van der Valk and Attiwill, 1984; Hicks and Silvester , 1985; Mann and Steinke, 1989), pneumatophores (Zuberer and Silver, 1978; Potts, 1979; Hicks and Silvester, 1985; Toledo et al., 1995a), the rhizosphere soil

Table 3. Nitrogen-fixation rates in different locations of coastal environment. (Howarth et al., 1988).

Nature of location	Location	Nitrogen-fixation rate (ARA) g N m ⁻² year ⁻¹
Planktonic nitrogen fixation in estuaries	S.W. Bothnian Sea (Baltic)	0.06
	Aland Sea (Baltic)	0.07
	Asko area (Baltic)	0.80
	Stockholm archipelago, Sweden	0.013–1.8
	Harvey estuary, Australia	1.2
Sediments and well-developed cyanobacterial mats in Estuaries and coastal seas	Vostok Bay, Japan	0.002
	Upper Cook Inlet, Alaska	0.01
	Narragansett Bay, Rhode Island	0.03
	Kamishak Bay, Alaska	0.03
	Norton Sound, Alaska	0.03
	Beaufort Sea	0.03
	Elso Lagoon, Beaufort Sea	0.07
	Shelikoff Strait, Alaska	0.08
	Rhode River estuary, Maryland	0.13
	Lune estuary, England	0.14
	Waccasassa estuary, Florida	0.37
	Bank End, England	0.43
	Kanchoe Bay, Hawaii	0.60
	Flax Pond mud flat, New York	0.65
	Barataria Basin, Louisiana	1.56
	Great Barrier Reef, Australia	1.32
Cyanobacterial mats of mangrove forests and coastal seas	Sippewissett marsh, Massachusetts	1.42
	Enewetak Atoll, Marshall Islands	2–40
	Sippewissett marsh, Massachusetts	2.28
	Texas Gulf Coast	4.00
	Colne Point marsh, England	5.99
	Gulf of Elat	7.59
	Hiddensee Island, Baltic	7.60
	Aldabra Atoll, Indian Ocean	12.20
	Flax Pond salt marsh, New York	13.44
	Bank End, England	20.06
	Enewetak Atoll, Marshall Islands	65.70
	Kaneohe Bay, Hawaii	76.00

(Zuberer and Silver, 1978), tree bark (Uchino et al., 1984), and cyanobacterial mats covering the surface of the sediment (Toledo et al., 1995a).

Most nitrogen fixation by planktons is by cyanobacteria rather than by heterotrophic bacteria, and rates of fixation are reasonably correlated with the biomass of nitrogen-fixing cyanobacteria (Howarth et al., 1988). The compounds like polysaccharides secreted by plant roots may influence the diazotrophs to fix nitrogen (Burgmann et al., 2005). A positive correlation was found between acetylene-reduction rates and the availability of organic matter (Holguin et al., 2001). It is also possible that heterotrophic bacteria are involved in nitrogen fixation, fueled by phototrophs (Paerl, 1990; Omorogie et al., 2004).

The rate of nitrogen fixation varies with different seasons. Nitrogen fixation associated with *Avicennia germinans* aerial roots in a Mexican mangrove showed that rates were up to ten times higher during the summer than during autumn and winter. The light intensity and water temperature are main factors influencing nitrogen fixation (Toledo et al., 1995a). Nitrogenase activity associated with pneumatophores was light dependent and was probably attributable to one or more species of cyanobacteria present as an epiphyte. (Hicks and Silvester, 1985).

The application of cyanobacteria, *Microcoleus* spp. was alone studied for the growth of mangroves (Toledo et al., 1995b; Bashan et al., 1998). The filamentous cyanobacterium *Microcoleus* sp. was isolated and inoculated on to young mangrove seedlings. Nitrogen fixation (acetylene reduction) is gradually increased with time and reached its peak 5 days after inoculation. Later, it decreased sharply. Cyanobacterial filaments colonized the roots by gradual production of biofilm (Toledo et al., 1995b). In another study, filamentous cyanobacteria *Microcoleus chthonoplastes* was inoculated on to mangrove seedlings and found that the total nitrogen and ^{15}N incorporation levels increased (Bashan et al., 1998). Hence, this chapter insists the scientific endeavor to explore the potential usage of cyanobacteria for the restoration of degraded mangrove forests.

7. Potential Bacteria Dwelling In Mangroves

In addition to cyanobacterial population, it is obligatory to discuss about bacteria influencing the mangrove ecosystem directly. They contribute inevitably for the recycling of nutrients in the mangrove ecosystem (Holguin et al., 2001). Many potential bacteria were isolated from mangrove ecosystem such as nitrogen-fixers, Phosphate solubilizers, photosynthetic anoxygenic sulfur bacteria, Methanogenic and methane oxidizing bacteria, which involve in efficient nutrient recycling (Uchino et al., 1984; Saxena et al., 1988; Ramamurthy et al., 1990; Vazquez et al., 2000; Holguin et al., 2001; Heyer et al., 2002; Rueda-Puente et al., 2003). Bacteria isolated from mangroves can also act as oil-degraders and biopolymer producers (Burns et al., 1999; Rawte and Mavinkurve, 2002). The dinitrogen-fixing bacteria in the mangrove rhizoplan are able to use root exudates and/or sloughed cell debris as energy sources for dinitrogen fixation (Zuberer and Silver, 1978). Some

of the potential bacteria were experimented to exploit for the growth of mangroves (Bashan et al., 2000; Rojas et al., 2001; Bashan and Holguin, 2002; Rueda-Puente et al., 2003). Hence, it is obviously presumed that exploring the bacterial diversity of mangroves will reveal many mind boggling biotechnological potentials in addition to other microbial flora.

8. Conclusion

In this chapter reports on cyanobacteria inhabiting mangroves are analyzed thoroughly, though the literature are available scarcely. Cyanobacteria have been explored for their ability to fix nitrogen, exclusively. Even though nitrogen-fixing activity of cyanobacteria on mangrove is well studied, the nature of relationship between them is still to be explored. By seeing ostensibly, it is clear that the interaction is non-pathogenic but there is no explicit studies has been taken to reveal the relationship between cyanobacteria and mangroves. There is paucity in revealing the role of cyanobacteria other than nitrogen fixation like plant growth promoting factors with respect to mangrove community. Exploring the unveiled abilities of cyanobacteria will add more value for the exploitation.

After the threat elicited by tsunami in Southeast Asia, the global awareness of the importance of mangrove forests has spread rapidly. Hence, the worldwide scientific and social endeavors have been promoted on the afforestation. The establishment of nursery is essential in reforestation programs, provides good quality of seedlings at the right quantity in time (Melana et al., 2000; Kairo et al., 2001). Since, the availability of seedlings is limited to seasons in many mangrove species, it is important to maintain a nursery to make the seedlings available throughout the year (Rajiv, 1999). Already cyanobacteria were exploited in agriculture as biofertilizer, especially on rice fields. The ex situ conservation of mangroves by rearing nursery is a new concept for future afforestation programs. Thus, the suggestion proposed for the exploitation of epiphytic cyanobacteria along with other beneficial bacteria as biofertilizer with apposite field during the development of nursery (Fig. 3).

9. Summary

Mangroves are woody trees and shrubs that grow at the interface between land and sea in tropical and subtropical area. Mangroves purvey with enormous and multi-dimensional benefits. The global status of mangrove forests shows tremendous decline in area and species diversity within 20 years. The observation states that the anthropogenic effects have greater influence on these forests. Hence, the conservation and restoration is the timely commitment of social liability. Microbiology of mangrove forests has pivotal consideration in conservation and restoration,

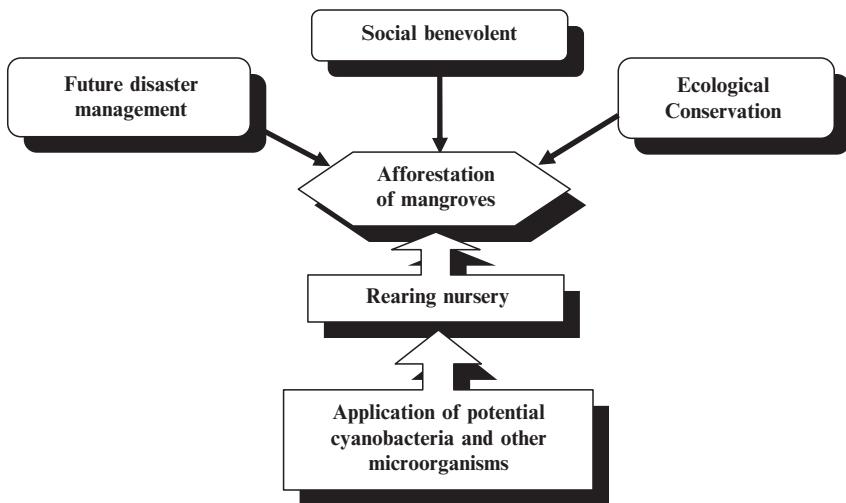


Figure 3. Strategies for mangrove afforestation.

especially, epiphytic phototropic, nitrogen-fixing and other cyanobacteria. The delineation of cyanobacterial role was discussed in the chapter and the suggestion was proposed on the exploitation of cyanobacteria for future nursery development and restoration program.

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INTERTIDAL SANDY BEACHES AS A HABITAT WHERE PLASTID ACQUISITION PROCESSES ARE ONGOING

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Edge of the sea is a strange and beautiful place.

— R. Carson, in *The Edge of the Sea* (1955)

1. Introduction

An intertidal sandy beach is a constantly changing habitat, and, in that sense, it could be regarded as an extreme environment. It alternates between a seabed and a land with every tidal transition, and this alternation changes physical conditions such as beach morphology, water level, nutrients, oxygen level, salinity, temperature, light intensity, etc. Sand is an unstable substratum. Tides and waves constantly move sands on the submerged shore face. Even a single rainfall during the low tide changes the physical conditions, and a one-night storm could change even the landscape of the shore resulting in a catastrophe for its microbial communities.

The intertidal sandy shore often appears not to be vegetated. Yet, there is an unexpectedly rich microalgal community under the beach. Many ecological studies have revealed that shallow sand/mud seabeds, including intertidal beaches are “secret gardens” that are rich in microalgal communities (for review, MacIntyre et al., 1996). Well-known members of these gardens are cyanobacteria and diatoms. There are also a variety of flagellate algae, such as dinoflagellates, cryptophytes, euglenophytes, chlorophytes, and prasinophytes. Nevertheless, our understanding of the flora and fauna of such communities is still fragmentary compared to our understanding of those of coastal waters. Spatial and temporal heterogeneities of the environment make extensive investigations of microbial communities in these extreme environments difficult. The intertidal sandy shore remains an unexplored frontier for modern phycology and protistology.

Our focus here is to introduce a unicellular flagellate that we discovered from an intertidal sandy shore in Japan (Okamoto and Inouye, 2005; Okamoto and Inouye, 2006). The organism, *Hatena arenicola*, possesses a green algal symbiont, and most likely represents an early intermediate stage of the plastid acquisition process via secondary endosymbiosis. As the majority of the extant algae are thought to have acquired the plastid via a similar process (McFadden,

2001; Bhattacharya et al., 2004; Falkowski et al., 2004), the symbiosis revealed in *H. arenicola* would help our understanding of plant evolution.

In this chapter, we focus on selected features of the intertidal sandy shore as an extreme habitat, and then, we take up the endosymbiosis of *H. arenicola* and comparative examples that are also known from similar habitats. The intertidal beach is an important habitat to which phycologists should pay special attention.

2. Variation of Intertidal Sandy Beaches

Geomorphological features and detailed classifications of the intertidal sandy beach have been reviewed (e.g., Short, 1999a). Brown and McLachlan (1990) covered broad topics about a sandy beach ecosystem that are helpful in understanding how physical features affect the biota, although their main subject is the meiobenthic community. Elliott et al., (1998) and Asmus et al., (2004) include useful links to a wide range of references for further reading. Here we point out some important features that affect the microalgal distribution.

2.1. VARIATION OF SANDY BEACHES

Light and water are essential for algal growth. Because the euphotic zone is at most several millimeters deep, algae grow on the shore face where the water is more or less continually present. Such a condition is found at the sheltered beaches (Short, 1999b), where the waves brake before they reach the shore face. Sheltered beaches have a shallow slope composed of finer sand grains. The smaller the particle size is, the larger total volume of the interstitial space is, and the more effective capillary action becomes. This allows larger volume of the water stay in the interstitial space and keeps the shore face wet even during the low tide. This wet area at low tide is called the *seepage face* (Brown and McLachlan, 1990). Fig. 1 illustrates a shore profile. The seepage face is developed where ground water level decouples from the sea level during the low tide due to the capillary action (Masselink and Turner, 1999).

The extreme of the sheltered beach is a tidal flat, where the water rarely moves. Less water exchange result in an accumulation of organic matter and a less aeration, which enhances bacterial growth and makes the environment reductive. Under such a condition, a bacterial loop dominates the ecosystem and algal growth is limited to the surface layer.

The opposite extreme is the exposed beach. The exposed beach has a steeper slope with coarse sand grains. Waves directly brake at the shore face. Coarse particles create less capillary action, so that the water directly penetrates in and out, but does not stay. Consequently, the shore face is dry during the low tide, so the algal distribution is restricted to the littoral zone at the lowest of intertidal range, where waves constantly bring water (Brown and McLachlan, 1990).

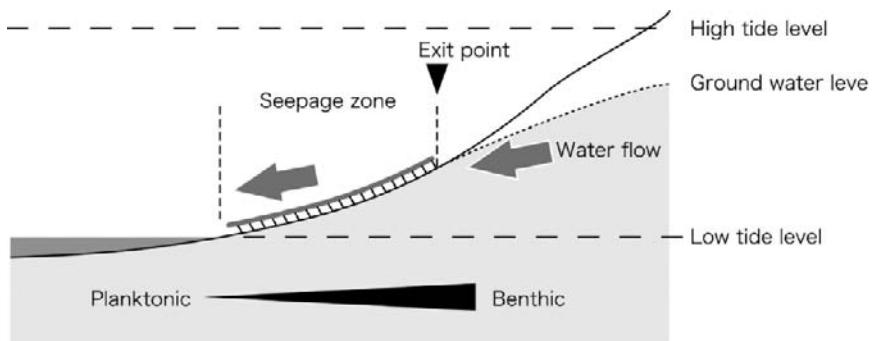


Figure 1. Profile of a sandy shore.

On a continuum between those two extreme, we can find a moderately sheltered beach. Unlike the tidal flat, the physical mixture during high tide and the continuous flow of the ground water near or on the shore face at low tide keep the environment aerated. This surface water often has a dense algal population. Isonoura beach in Japan, where we found *H. arenicola* falls into this category.

2.2. HETEROGENEITY WITHIN A SANDY BEACH

A sandy shore is a place where physico-chemical conditions vary drastically within a small distance, which means the algae with very different requirements can occur right next to each other. One of the most influential conditions is the decline of light intensity along the depth gradient. The euphotic zone is restricted to the surface layer of less than 5 mm deep in most cases (Brown and McLachlan, 1990), which is strikingly different from the open water where the boundary is located more than 100 m deep in the water column.

The oxic-anoxic state also varies along the depth. The oxic-anoxic condition is much more dependent on the grain size. The boundary is located within a several millimeters depth (at the tidal flat) or at a more than 1 m depth (at the exposed beach) (Brown and McLachlan, 1990). The oxic-anoxic state affects the type of the nitrogen source available (NO_2 , NO_3 , or NH_4), and therefore restricts the distribution of some algae (e.g., Kingston, 1999 and references therein).

The vertical gradation of physio-chemical conditions together with the effect of the waves induces the vertical migration of microalgae (e.g., Kingston, 2002; Saburova and Polikarpov, 2003; see references therein). Vertical migration of intertidal benthic microalgae has been known for 100 years for a wide range of algae such as cyanobacteria, diatoms, dinoflagellates, and euglenophytes. They often form a visible bloom on the sand surface. The typical pattern is to migrate upwards and stay in the surface photic layer during the daytime at the low tide, then to

migrate downwards during the daytime at the high tide and throughout the night to avoid wave disturbance and to take up nutrients depleted in the surface layer.

3. Nature's Ongoing Experiments at the Intertidal Sandy Beach

As outlined above, the intertidal sandy shore is a dynamic and complex environment. Although there seem to be rich microbenthos communities including a variety of algae inhabiting sandy beaches, our knowledge of these communities is insufficient and many undescribed species remain. One of them is *H. arenicola*, a unicellular flagellate we discovered from a sandy shore in Japan, was one of these. This organism is most likely currently undergoing plastid acquisition via endosymbiosis.

3.1. SYMBIOTIC ORIGIN OF PLASTIDS

Eukaryotes comprise a half dozen supergroups and algae distribute polyphyletically across these different supergroups (Keeling et al., 2005 and references therein). The algal diversity and polyphyly are explained by multiple endosymbioses (Bhattacharya et al., 2004 and references therein). It is assumed that all the plastids have originated from an ancestral cyanobacterium taken up by a heterotrophic eukaryote. This symbiosis is referred to as the "primary endosymbiosis." Glaucophytes, rhodophytes, and Viridiplantae (green algae and land plants) are considered to have been derived from a single primary endosymbiosis. Subsequently, the "secondary endosymbioses" in which these photosynthetic algae are engulfed by different heterotrophic eukaryotes took place. Through the secondary endosymbioses, four algal groups (heterokontophytes, haptophytes, cryptophytes, and dinoflagellates) plus a parasitic group (Apicomplexa) acquired red algal plastids, and two algal groups (euglenophytes and chlorarachniophytes) acquired green algal plastids. Thus understanding the plastid acquisition process via endosymbiosis is essential to understanding algal evolution.

One approach to understanding this aspect of algal evolution is to investigate the organisms that are undergoing the process in real time. Recently we reported a potential candidate, *H. arenicola* from Isonoura beach in Japan (Okamoto and Inouye, 2005; Okamoto and Inouye, 2006).

3.2. *H. ARENICOLA* FROM A SANDY BEACH

Isonoura beach is a moderately sheltered sandy shore and the tidal range is around 2 m at spring tide. It has a well-developed seepage face. Microalgae often form noticeable patchy blooms around the upper edge of the seepage face (the exit point). The most abundant bloom-forming algae are diatoms, cryptophytes (some *Chroomonas* species), prasinophytes (*Pyramimonas* and *Tetraselmis* algae), and euglenophytes. As the blooms are visible only for several hours after the surface is above sea level, they are likely migrating vertically.

H. arenicola is distributed at the upper zone of the seepage face as are many other microalgae. Although this organism is constantly present except during the winter, it is not abundant and we have never seen a visible bloom of this alga since we discovered it in 2000.

H. arenicola appeared to be a new green algal taxon when first encountered. A chlorophyll *a/b*- containing plastid-like structure and a red conspicuous eye-spot is always at the cell apex. However, the division of the “plastid” is not coupled with cell division and the structure is inherited by only one of two daughter cells. Therefore, the structure is not a “plastid” but a temporary symbiont.

Molecular phylogenetic analysis showed that the symbiont is a *Nephroselmis* (Prasinophyceae, Chlorophyta). Electron microscopy revealed that the symbiont retains the cytoplasm as well as the plastid, though the cell morphology is greatly different from that of the ordinary free-living *Nephroselmis* cell (Fig. 2; Okamoto and Inouye, 2005; Okamoto and Inouye, 2006). The plastid of the symbiont is selectively enlarged up to more than ten-fold. In contrast, the cytoplasm is greatly



Figure 2. *Hatena arenicola*.

reduced. It only retains the nucleus, a mitochondrion that is often degraded and occasionally a vestigial Golgi body-like structure. Other structures such as the cell covering, the cytoskeleton and the endomembrane system are absent. Such a reduced cytoplasm seems to be insufficient to sustain the enlarged plastid, so the host may compensate for some of the symbiont's lost metabolism.

We occasionally found symbiont-lacking cells in the environment. Interestingly, the symbiont-lacking cell has a complex feeding apparatus at the cell apex and used it for taking up algal cells (Fig. 2). As the feeding apparatus is absent in the symbiont-bearing cell, it would be decomposed after the uptake of *Nephroselmis* symbiont. In this sense, the symbiotic association in *H. arenicola* causes a drastic change of the morphology of both the host and the symbiont.

This association between the host and symbiont in *H. arenicola* provides us with a new view on this type of symbiosis. The symbiosis is not a mere enslavement of the symbiont; rather it is a process that unites two alien cells into a single organism, that is both morphologically and functionally different.

3.3. EYESPOT: A POSSIBLE FUNCTIONAL ASSOCIATION

The eyespot region is one of the places where the host–symbiont association is significantly established. Four different membranes, i.e., the host's plasma membrane, a symbiont-enveloping membrane of an unknown origin, and the symbiont's outer and inner plastid membrane are closely layered together, then the eyespot granules are attached to the inner most side of the membrane complex.

Although we do not have enough evidence, it is still worth asking whether the membrane-eyespot complex is functioning for the host's phototaxis. The algal eyespot is part of photo-sensing machinery (Foster and Smyth, 1980; Melkonian, 1984; Gualtieri, 2001). It shades a photoreceptor in a near-by membrane, helping to detect the light. The arrangement of the membrane-eyespot complex seems reasonable for this purpose. In a preliminary observation, *H. arenicola* cells with the symbiont show a negative response to laterally projected light. This particular photoresponse would be appropriate for this cell's type of motility. That is, *H. arenicola* cell does not swim but crawls two-dimensionally on the substratum using two flagella, so only the lateral incident illumination matters for the movement of *H. arenicola*. We also observed that *H. arenicola* could not survive under a 14–10 h light–dark cycle with incident illumination of ca. 10 mol photons m⁻² sec⁻¹. Under this light condition, the symbiont becomes swollen and the host cell dies. On the other hand, under the complete dark condition, the symbiont become pale and was eventually lost. To maintain a stable partnership, *H. arenicola* needs some phototactic ability to choose the proper light conditions, weak enough to avoid symbiont's outgrowth but strong enough to maintain its photosynthesis. This is all the more critical at the intertidal sandy shore where the light intensity drastically declines within several millimeters, and light-induced vertical migration is advantageous. Therefore, it would be quite intriguing to determine, in the

future, if the symbiont's eyespot can contribute to, or even make changes in the host's phototaxis.

3.4. HALF PLANT AND HALF PREDATOR MODEL

Based on the observation of the nonsynchronized cell divisions and ultrastructural features, we proposed a hypothetical life cycle, in which *H. arenicola* possibly switches its trophic strategy between heterotroph and phototroph (Okamoto and Inouye, 2005; Okamoto and Inouye, 2006). The symbiosis between these organisms is unique in the suggestion that the plastid acquisition is a more mutual process than expected. It was assumed that the plastid acquisition is a process of "enslaving" an endosymbiont to utilize it as an organelle (Douglas et al., 2001; Cavalier-Smith, 2002; Cavalier-Smith, 2003). This view is compatible with the observation that the plastid structure and genome in any extant plant is greatly reduced and the host controls the much of its function. However, that is the result of evolution and does not really tell us about trials and errors through which the host and the symbiont went during the integration process. That is the reason we need to investigate such symbioses at intermediate steps of the plastid acquisition.

3.5. EVOLUTIONARY IMPLICATIONS AND OTHER EXAMPLES

The process of endosymbiosis is essential to understanding plant evolution. In addition to the case of *H. arenicola*, comparable endosymbioses have been reported from some dinoflagellates. The plastid of most photosynthetic dinoflagellates is of red algal origin acquired via secondary endosymbiosis. Some dinoflagellates are known to replace the original plastid through an additional endosymbiosis with a cryptophyte, diatom, prasinophyte, or haptophyte symbiont (Schnepp and Elbrächter, 1999; Morden and Sherwood, 2002; Hackett et al., 2004). The extent of associations vary, so that these relationships are thought to represent different intermediate stages of integration.

Fig. 3 shows the hypothesized integration process and corresponding examples. First, a phagotrophic flagellate would start to retain a certain prey in the cytoplasm and use it as a temporary symbiont (Stage I; *Amphidinium latum*, *Amphidinium poecilochroum*, and *Gymnodinium acidotum*). At this stage, the symbiont retains the plastid as well as the nucleus, mitochondrion, endoplasmic reticulum, etc. The host–symbiont association is still temporary and the symbiont would be either digested or lost upon the host's cell division. The host would repeatedly take up the partner, while feeding on the other prey cells. *H. arenicola* represents Stage I, in that (i) the host and symbiont have an intimate association, but (ii) their cell cycles are not coupled.

The next stage (Stage II, *Durinskia baltica* (formerly *Peridinium balticum*) and *Kryptoperidinium foliaceum* (formerly *Peridinium foliaceum*)) is synchronization of

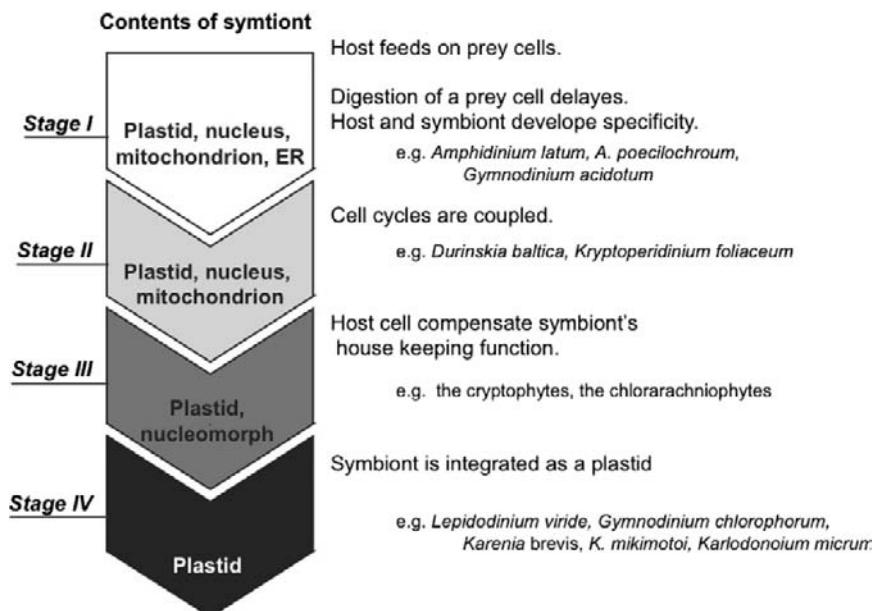


Figure 3. Hypothetical intermediate steps of plastid integration.

the cell cycles of the host and the symbiont. This ensures an even inheritance of the daughter symbionts to the daughter host cells, and allows the host to behave as an alga. Then the symbiont continues to lose most of the cell content such as ER and a mitochondrion. The following stage (Stage III) is complete reduction of the symbiont's cytoplasm. The nucleus persists to the last. We can still see the remnant of the nucleus as the nucleomorph in some algae in the cryptophytes and chlorarachniophytes (Douglas et al., 2001; Gilson and McFadden, 2002). Finally, the nucleomorph disappears, and the process of integration of the plastid is complete (Stage IV; *Lepidodinium viride*, *Gymnodinium chlorophorum*, *Karenia brevis*, *Karenia mikimotoi*, *Karlodonium micrum*).

At some point of the process, gene transfer from the symbiont to the host's nucleus would happen (e.g., Gilson and McFadden, 2002; Archibald, 2005). This is accompanied with by the invention of protein import machinery from the host to the symbiont. The host gradually compensates the symbiont's metabolism. This eventually leads to the reduction of the symbiont's genome and organelles and the loss of its autonomy.

Interestingly, some of the examples mentioned above are reported from the intertidal sandy beach habitat. *A. latum* (Horiguchi and Pienaar, 1992) and *A. poecilochroum* (Larsen, 1988) are from the intertidal sandy beach and represent early stage I. Both have a symbiont of cryptophyte origin but their partnership is not exclusive. *A. latum* was observed to retain two different cryptophytes species in one cell. Other examples are *Gymnodinium quadrilobatum* (Horiguchi and Pienaar, 1994) and *Galeidinium rugatum* (Tamura et al., 2005). They have a

diatom symbiont and would represent stage II, although many of the studies about this stage have been performed on *D. baltica* and *K. foliaceum*, especially studies on the cell division process.

It is unclear why the intertidal sandy shore can incubate symbioses that would represent the plastid acquisition process. Of course, examples of plastid acquisition are not restricted to sandy beaches, but also occur in open waters. However, sandy beach habitats seem to be one of most important habitats wherein evolution of plastid acquisition via secondary endosymbioses are ongoing. Because the habitat has not been explored enough, our understanding is far less than needed to reveal the hidden diversity of the sandy shore. The sandy shore is dynamic and heterogeneous at many levels. This extreme condition could be a key for hunting for organisms like *H. arenicola* that are in the process of plastid acquisition. To fully understand this habitat, we need to perform extensive research across different types of beaches with careful consideration of the variation of physio-chemical conditions including those discussed above.

4. Conclusion

The intertidal sandy shore is often close to human activity, yet it is an unexplored frontier. Its dynamic and heterogenetic nature allows various organisms to accumulate to a dense community within a small space. However, the very nature and patchy distribution of microalgae, responding to the microenvironments make it difficult to draw a clear picture of the diversity of interstitial microbes. The endosymbiosis of *H. arenicola* and some dinoflagellates are good examples of the hidden treasures in these habitats for studies of plant evolution.

New algal species as well as undescribed heterotrophic protists, that could be candidates for emerging symbioses, are still constantly being described (e.g., Larsen and Patterson, 1990; Lee and Patterson, 2000; Yoshimatsu et al., 2000; Tamura et al., 2005). In addition to *H. arenicola*, we have recognized at least a new lineage of heterokontophyta, a new *Nephroelminis* species, and a marine *Malomonas* species. We have also found a dinoflagellate, *Amphidinium* sp. from the same location that is possibly undergoing the Stage I endosymbiosis with cryptophyte symbiont.

Many sandy shores are always close to us and no specialized vehicles or equipment are needed to explore them. And, these habitats certainly seem to another treasure, one that opens up new possibilities for our understanding of the evolution of algae and related organisms.

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HYDROCHEMICAL KEY TO THE GENESIS OF CALCAREOUS NONLAMINATED AND LAMINATED CYANOBACTERIAL MICROBIALITES

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1. Introduction

Theoretical geochemical considerations (Kempe and Degens, 1985) and field work at sites of active growth of in situ calcified cyanobacterial mats and biofilms (e.g., Kempe et al., 1991; Kempe and Kazmierczak, 1993; Kazmierczak and Kempe, 2006) convinced us that in the past the ocean must have been more alkaline than at present and that it was of higher CaCO_3 supersaturation ($\text{SI}_{\text{Calcite}} > 0.8$) (Kempe and Kazmierczak, 1990a, 1994; Kazmierczak et al., 2004). Two processes contributed to the higher alkalinity: (1) the slowly declining high primary alkalinity established in the Hadean ocean by binding large amounts of degassed and cometary CO_2 through silicate weathering as CO_3^{2-} and HCO_3^- in ocean waters (“Soda Ocean”) and (2) the effect of the export of excess alkalinity from sulfate reduction processes in stagnant marine basins (“Alkalinity Pump”) (Kempe, 1990; Kempe and Kazmierczak, 1994). The latter alkalinity source started to be effective only after enough sulfate became available in the ocean (probably during the last 1.0–0.8 Ga – for evaluation of sulfate level in the Precambrian ocean see e.g., Buick, 1992; Grotzinger and Kasting, 1993; Eriksson et al., 2005). It could be the single most important factor for short-term modifications of Phanerozoic ocean chemistry. Sudden, in geological terms, export of alkalinity from overturning anaerobic basins could cause high pH and Ca^{2+} -stress upon the marine biota (Kempe and Kazmierczak, 1994; Brennan et al. 2004; Kazmierczak and Kempe, 2004b), and is associated with negative $\delta^{13}\text{C}$ excursions in carbonate sequences at the Precambrian/Cambrian transition (e.g., Knoll et al., 1986; Magaritz, 1989; Magaritz et al., 1991), where biocalcification started in several phyla almost simultaneously (e.g., Lowenstam and Margulis, 1980; Lowenstam and Weiner, 1989).

The precipitation of alkaline earth carbonate minerals (calcite, aragonite, and dolomite) from solutions is one of the fundamental geological processes. Weathering in continental soils and in marine or continental hydrothermal environments releases Ca and Mg from silicate minerals and consumes free CO_2 to produce alkalinity

(the total anionic charge due to weak acids, i.e., $\text{Alk}_{\text{carb}} = 2^*\text{CO}_3^{2-} + \text{HCO}_3^-$). Since weathering is – on a geological timescale – a fast process, the oceans quickly accumulate enough Ca, Mg, and Alk to reach saturation of the common carbonate minerals. Constant removal must therefore have taken place throughout the entire history of this planet. Today, biocalcification is the process which removes almost all of the Ca from seawater producing about 5 Gt of calcite and aragonite each year of which 40% is redissolved (Milliman, 1993). Almost all modern marine calcification is conducted enzymatically. This biocalcification arose shortly before the Precambrian/Cambrian boundary. Prior to this date, precipitation of carbonates must have proceeded entirely nonenzymatically and extracellularly. The products consist of vast carbonate sequences, some of them containing textures which clearly mark them as biologically mediated precipitates (microbialites in general, or thrombolites and stromatolites according to the absence or presence of lamination).

In order to reconstruct the environmental conditions, under which these deposits arose, one cannot refer to present-day ocean chemistry because of the importance of the ubiquitous biocalcification in Phanerozoic times. Rather we have to look for extreme environments, which may serve as models for ancient oceanic conditions. These models may also shed light on the reoccurrence of calcareous microbialite formation within the marine Phanerozoic rock record.

We discovered three sites where *in situ* calcified microbial structures form at present. These are highly alkaline Lake Van, Turkey, exemplified later (Kempe et al., 1991; Kazmierczak et al., 2004; López-García et al., 2005) and the crater lake on Satonda Island, Indonesia, filled with altered seawater (Kazmierczak and Kempe, 1990, 1992, 2004a; Kempe and Kazmierczak, 1990b, 1993), and the recently described caldera lakes on Niuafo'ou Island, Tonga (Kazmierczak and Kempe, 2006). In addition, we searched the literature concerning environments in which recent stromatolitic structures occur and which had an adequate coverage of hydrochemical data. These, apart from the lakes Van and Satonda accounted later, included: Walker Lake, Lake Tanganyika, and Andros Island (Kempe and Kazmierczak, 1990a). In view of these considerations, we think that the statement of Krauskopf (1979, p. 261), apparently reflecting the general opinion of the geologic community, that “... deposits of desert basins provide a complicated and rewarding geochemical study, but the subject is hardly of sufficiently general interest . . .” is overdue for revision.

2. Fundamentals of Abiogenic Calcium Carbonate Precipitation

Abiogenic precipitation of minerals can proceed only in supersaturated solutions. Therefore, all environments in which nonenzymatic calcite or aragonite precipitation occurs must be supersaturated. Supersaturation, expressed as Saturation Index (SI), is best measured by comparing the ion activity product of the free calcium $[\text{Ca}^{2+}]$ and the free carbonate ion $[\text{CO}_3^{2-}]$ in solution with the measured solubility constants at that temperature:

$$SI = \log([Ca^{2+}][CO_3^{2-}]/K_{calcite, aragonite}). \quad (1)$$

The logarithm is used, so that the SI is negative at undersaturation, zero at saturation, and positive at supersaturation. This notation has advantages over using the simple quotient (saturation, also known as Omega) as done in the older literature. Calculating the index is, in spite of its simple formula, not trivial and involves iterating techniques to obtain the activities of the free Ca^{2+} and CO_3^{2-} ions from their initial totals as derived from the chemical analysis. Today, computer programs aid in the detailed evaluations. We used PHREEQE (Parkhurst et al., 1990; Schulz and Kölling, 1992) for this study to conduct the calculations.

In most thermodynamic systems, precipitation of mineral substance starts when saturation is reached (e.g., gypsum starts to crystallize from evaporating seawater at saturation). Alkaline earth carbonate minerals are a cumbersome exception. They do not precipitate at saturation, but can sustain high supersaturation. A survey of the entire North Sea (122 stations) in summer 1986 showed that the surface waters had an average $SI_{calcite}$ of 0.567 ± 0.096 (Pegler and Kempe, 1988) (Table 1). The waters of the North Sea show widely different characteristics as to salinity, alkalinity, and total CO_2 , the average SI_{Cc} therefore gives a good idea about average present-day ocean SI_{Cc} . Areas of intense plankton blooms yielded SI_{Cc} values of up to 0.90 without signs of inorganic calcification.

In calculating the supersaturation for natural calcite precipitating systems, it has been found that the SI must reach values >0.8 before precipitation commences (Kempe and Kazmierczak, 1990a; Zaihua et al., 1995; Merz-Preß and Riding, 1999; Zeebe and Wolf-Gladrow, 2001; Kazmierczak and Kempe, 2006). Deposition experiments by Svensson (1992) show that in fact measurable precipitation ceases much before the thermodynamic saturation is reached. In many of his experiments, using solutions prepared from natural limestone, the SI_{Cc} does not fall below 0.8 (Fig. 1), whereas solutions prepared with artificial calcite can sustain calcite deposition at somewhat lower SI values. A similar behavior is also observed in dissolution experiments where the rate of dissolution of natural but not of artificial calcite switches to second-order kinetics once a saturation of $>70\%$ is

Table 1. Calcite supersaturation of surface seawater, example north sea, survey of 2.05.1986, 122 stations (Pegler and Kempe, 1988).

Parameter	Average	Standard deviation	Maximal	Minimal
$SI_{calcite}$	0.567	± 0.096	0.904	0.375
pH	8.273	± 0.106	8.76	8.07
Temperature, °C	8.557	± 1.35	12.66	6.26
Salinity, %	33.873	± 1.928	35.244	27.000
Total alkalinity, meq/kg	2.319	± 0.060	2.437	2.144
Total CO_2 , mmol/kg	2.068	± 0.062	2.155	1.865

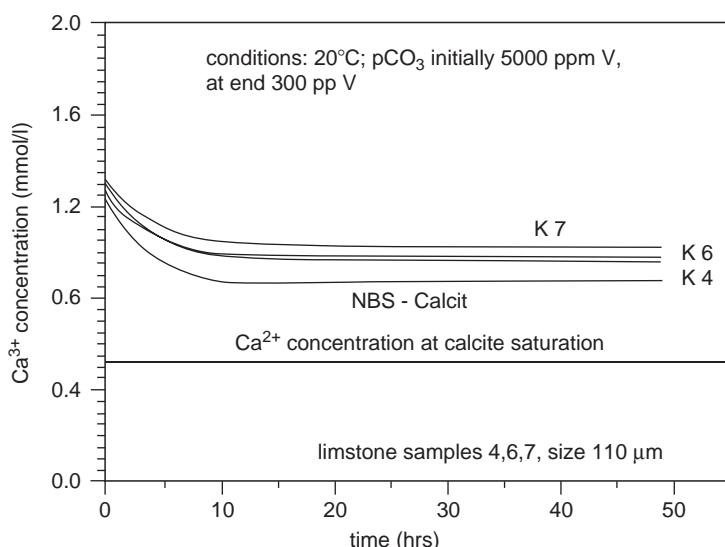


Figure 1. Calcium carbonate deposition experiments (decrease of Ca^{2+} concentration plotted against time) illustrating the inhibition of calcite precipitation far above the thermodynamic saturation (adapted from Svensson, 1992, fig. 8). In this experiment, solutions of natural limestones and artificial calcite (NBS) were equilibrated at a PCO_2 of 5,000 ppmv and then degassed to 300 ppmv. Deposition of calcite ceased after about 10 h at SI_{Cc} of 0.82 for limestone No. 6 and of 0.58 for the NBS-calcite. In all experiments, NBS-calcite can precipitate at lower SI_{Cc} than natural limestones. This inhibition, also seen in the dissolution experiments, which display a slower rate near saturation ($\text{saturation} > 0.7$), is due to the increasing area covered with Ca^{2+} and HCO_3^- ions at adsorption sites (such as PO_4^{3-} or Mg^{2+}) on the crystal surfaces as the system approaches saturation. This rate limitation is described by a Fowler–Frumkin Isotherm (Svensson, 1992).

reached. Contaminating NBS-calcite with KH_2PO_4 induces the same change in kinetics for artificial calcite as for natural limestone. The most probable reason for this inhibition is the presence of adsorption sites (PO_4^{3-} , Mg^{2+}) on the crystal surface to which Ca^{2+} and HCO_3^- ions can adsorb, “blinding” the calcite against further interaction with the solution (Svensson, 1992).

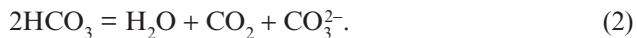
Supersaturation can arise from a combination of factors which govern the parameters in Eq. 1: addition of calcium to a solution is, for example, one of the possibilities. Another one is an increase of the total alkalinity of the solution. Yet another one is an increase of the carbonate ion portion of the total alkalinity by degassing CO_2 (the most common way cave sinters are formed). One should note that high supersaturation can arise even in very Ca^{2+} -poor solutions if the total carbonate alkalinity is high enough.

Abiogenic precipitation can proceed without or with the interaction of biota. An example for the former is flowstone formation in caves. There, unsaturated water charged with a high CO_2 pressure (PCO_2) enters abruptly an environment of a low

PCO_2 (the cave). The resulting CO_2 loss drives the solution from the unsaturated into the highly supersaturated state, causing – with time – the formation of intricate speleothems (Fig. 1). Calcium carbonate precipitation proceeds also where highly alkaline waters (which carry only small amounts of Ca^{2+}) mix with seepage waters rich in Ca^{2+} . We found calcite forming in such a manner on the floor of Lake Van. Groundwater seeps slowly into the highly alkaline lake waters forming bushy forms of very soft material, similar to the structures growing in chemical gardens. The forms apparently grow fast but cannot be preserved in toto because of their soft consistency. Analyses of the content of organic carbon showed that they apparently grow without organic interaction (Kempe et al., 1991). Similarly, whittings can form in mid-water during the mixing of alkaline lake waters with river waters rich in Ca^{2+} . In the case of whittings, it is however possible that biological interaction is involved (Thompson and Ferris, 1990; Robbins and Blackwelder, 1992).

Paleontologically, carbonates which form by mediation of microbiota are certainly most interesting. Biological mediation can proceed here mainly via two pathways: through the extraction of CO_2 and/or HCO_3^- during photosynthesis and through the addition of alkalinity during anaerobic sulfate reduction (for review, see Altermann et al., 2006).

Extraction of CO_2 can lead to the increase of the carbonate ion and hence pH at constant alkalinity:



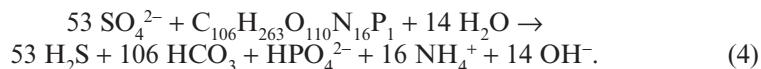
Alkalinity is shifted only after CaCO_3 precipitation starts. CaCO_3 precipitation in turn liberates CO_2 , counterbalancing the negative effect of photosynthesis on pH and decreasing the overall alkalinity:



Measurements of PCO_2 during ongoing calcification in reefs or during coccolithophorid blooms do in fact show this liberation of CO_2 . Nevertheless, photosynthetic CO_2 uptake does not lead to extracellular calcification in the present ocean. Three reasons account for this circumstance: first of all, the amount of CO_2 which can be removed is small due to overall low nutrient concentrations in surface waters; second, drawdown of the aquatic PCO_2 leads to the invasion of CO_2 from the air counterbalancing the carbonate ion production; and third, the ocean is not sufficiently supersaturated to start inorganic CaCO_3 precipitation because intracellular calcification keeps it at a relatively low state of supersaturation. Even in places where large nutrient inputs occur, for example, at the mouth of the Elbe river, and where occasionally pH values rise up to above 8.5, no extracellular calcite precipitation has been reported as yet (Kempe and Pegler, 1991).

Therefore, extraction of CO_2 by photosynthesis can trigger calcification only in environments which have a much higher primary supersaturation than that present in marine waters. This is apparently the case at the sites discussed later.

Microbial communities gaining energy by sulfate reduction have to substitute the charge of the consumed sulfate ion by releasing bicarbonate or carbonate ions, thereby increasing the alkalinity and supersaturation:



In this process, organic matter (i.e., of a Redfield C/N/P composition; Stumm and Morgan, 1970) is decomposed by reducing sulfate to sulfide, and a charge-equivalent amount of alkalinity is produced. This process occurs in anaerobic basins (e.g., in the Black Sea where the alkalinity has reached values of 4.5, compared with 2.1 meq/kg in the open ocean, e.g., Goyet et al., 1991), in anaerobic sediments (where pore waters can lose all of the contained sulfate), and in the lower parts of microbial mats growing in sulfate-rich environments.

Because the present ocean is almost fully oxygenated, sulfate reduction as a modifier of alkalinity is not very well understood, but may well have played a major role in modifying marine alkalinity during times of extensive anaerobic bottom waters (Kempe, 1990; Kempe and Kazmierczak, 1994).

3. Examples of Modern Environments Sustaining Formation of In situ Calcified Cyanobacterial Microbialities

3.1. LAKE VAN

3.1.1. Hydrochemistry

Lake Van, situated in eastern Anatolia, Turkey, is the largest soda lake (Kempe, 1977) and the fourth largest closed lake (after the Caspian Sea, Lake Aral and Issy Kul) on Earth by volume (576 km³, maximal depth 451 m). Soda lakes are common phenomena in endorheic drainage basins occurring in volcanic regions in the back of active plate boundaries (Kempe and Degens, 1985). Weathering of fresh volcanic silicate rocks produces solutions with a surplus of bicarbonate over Ca plus Mg. Upon evaporating such solutions, the alkaline earth carbonates precipitate first, leaving Na and K carbonates in solution. Upon further evaporation in terminal lakes, highly alkaline waters arise (Garrels and Mackenzie, 1967). In short, the preconditions to obtain alkaline solutions are:

$$\begin{aligned} & ([\text{Mg}^{2+}] + [\text{Ca}^{2+}]) < ([\text{CO}_3^{2-}] + [\text{HCO}_3^-]) \text{ or} \\ & ([\text{Na}^+] + [\text{K}^+]) > ([\text{Cl}^-] + [\text{SO}_4^{2-}]). \end{aligned} \quad (5)$$

In Lake Van (Table 2), alkalinity amounts to >150 meq/l, second only to the Cl⁻ concentration, the pH reaches 9.75, with <10 meq/l of alkaline earth ions (Tables 2 and 3). The Ca concentration amounts to only 0.2 meq/l, and the concentration of the free Ca ion is as low as 2.6–2.2 10⁻⁵ meq/l. The supersaturation of calcite

Table 2. Water composition and carbonate mineral saturations for water samples taken at various depths from Lake Van/Turkey, a sublacustrine spring at resadiye (southern shore of Lake Van), Satonda Crater Lake/Indonesia, and seawater for comparison.

	Temp., °C	Conductance, mS/cm	pH	Alkalinity, meq/l	Cl ⁻ , mg/l	SO ₄ ²⁻ , mg/l	Na ⁺ , mg/l	K ⁺ , mg/l	Mg ²⁺ , mg/l	Ca ²⁺ , mg/l
Seawater	25	—	8.21	2.32	19,353	2,712	10,768	399.1	1,291	412.3
Lake Van										
9 m	17.7	26,000	9.75	151.2	5,680	2,340	7,780	423	108.5	4.08
400 m	3.2	26,700	9.69	155.7	5,890	2,440	7,980	434	110.3	3.52
Spring Q1	11.0	1.065	6.91	7.45	86.4	50.1	136.7	12.12	24.5	52.9
Satonda Salinity										
5 m	30.9	31.4	8.59	4.15	17,155	1,779	9,645	449.6	1,035	183.6
40 m	29.5	37.5	7.21	7.75	19,953	1,956	11,144	529.8	1,240	232.1
65 m	29.4	41.8	6.92	53.9	22,443	96.1	12,673	607.6	1,442	240.1

Note: Concentrations are given in mg/l

Table 3. Water composition and carbonate mineral saturations for water samples taken at various depths from Lake Van/Turkey, a sublacustrine spring at resadiye (southern shore of Lake Van), Satonda Crater Lake/Indonesia, and seawater for comparison.

	CO ₃ ²⁻ , meq/l	Alkalinity, meq/l	Cl ⁻ , meq/l	SO ₄ ²⁻ , meq/l	Sum anions	Na ⁺ , meq/l	K ⁺ , meq/l	Mg ²⁺ , meq/l	Ca ²⁺ , meq/l	Sum cations
Seawater	0.071	2.32	545.9	56.46	604.68	468.4	10.21	106.2	20.57	605.38
Lake Van										
9 m	48	151.2	160.3	48.80	360.3	338.2	10.83	8.93	0.204	358.2
400 m	58	155.7	166.0	50.74	372.4	346.9	11.10	9.08	0.176	367.2
Spring Q1	0.0028	7.45	2.44	0.52	10.41	5.95	0.31	1.01	1.32	8.59
Satonda										
5 m		4.15	483.84	37.24	525.23	419.41	11.5	85.16	8.96	525.03
40 m		7.75	562.73	40.72	611.20	484.58	13.55	101.96	11.58	611.67
60 m		53.9	632.96	2.0	688.86	551.09	15.54	118.36	11.98	696.97

Note: Concentrations are given in meq/l

reaches 1.13 and that of aragonite 0.98 at the surface of the lake. Due to intense winter cooling, the lake mixes partially every year. Therefore, differences in composition between surface and bottom waters are small. Nutrients increase with depth, and oxygen decreases (Kempe and Reimer, 1990). This is indicative of remineralization of sinking organic matter, which leads to an increase of PCO_2 and to a reduction of supersaturation with depth (Table 4). This reduction is, however, so small that degassing the bottom waters would not cause it to surpass the surface supersaturation (Table 4). Even deep mixing of the lake would therefore not upset the state of the supersaturation significantly. In summer, the warm surface layer is diluted with river water, lowering its alkalinity and pH slightly compared with the bulk of the lake water (Tables 2 and 3). Nevertheless, the seasonal warming causes the supersaturation to increase slightly compared with the water at depth (Table 4), causing CaCO_3 to precipitate lake wide (summer whitings). At river mouths and at places where groundwater seeps into the lake (compare the composition of a sublacustrine spring near Resadiye, at the southern shore of the lake in Tables 2–4, Spring, Q1), waters rich in Ca^{2+} mix with the alkaline lake waters, also causing CaCO_3 precipitation locally (river whitings and bush-like “chemical gardens” at the shallow lake bottom).

3.1.2. Microbialites

Echosounding surveys and observations by scuba diving have revealed an abundance of large calcareous microbialites along the shore of the Tatvan Bay and south of Adilçevaz (see Kempe et al., 1991, Fig. 1). The microbialites occur as dense groups of variously sized, branched towers and columns up to 40 m high, and extending down to a depth of >100 m. The limited occurrence apparently is determined by coastal aquifers delivering Ca^{2+} -rich groundwater into the alkaline lake water.

The studied microbialitic columns display two different types: (1) those with a dark green, hard, knobby, and microgranular aragonitic surface (Fig. 2A and C) occurring at water depths >10–12 m and (2) those with a yellow-greenish, soft, mixed calcitic, and aragonitic surface, densely overgrown with slimy noncalcified microorganisms occurring at depths <10–12 m.

The hard aragonitic surfaces are overgrown discontinuously with a very thin (often <50 μm) film of coccoid cyanobacteria (Fig. 2D and G) which, according to currently made molecular diversity studies based on amplification of bacterial 16S rRNA genes (López-García et al., 2005), revealed the presence of representatives of the groups Pleurocapsales *sensu* (Rippka et al., 1981) (*Stanieria cyanosphaera*, *Dermocarpa* sp.) and Nostocales (*Nostoc* sp.). Gessner (1957) originally identified them as members of *Entophysalis granulosa*. The cyanobacterial mat is colonized by a dense population of peritrichid ciliates (*Carchesium* sp.) and benthic diatoms (most common are representatives of the genera *Rhopalodia*, *Nitzschia*, and *Surirella* (Fig. 2E and G).

The shallow-water surfaces consist of loosely distributed subglobular, spherulitic calcite bodies, 50–100 μm in diameter, with addition of rare aragonitic

Table 4. Mineral saturation indices ($\log(IAP/K_{\text{mineral}})$) of some carbonate and sulfate minerals, CO_2 -pressure ($p\text{PCO}_2 = -\log \text{PCO}_2$), ionic strength and free Ca^{2+} (mol per liter) concentration in seawater, Lake Van and Satonda Crater Lake.

	Calcite	Aragonite	Dolomite	Na_2CO_3	Trona	Gypsum	$p\text{PCO}_2$	$\text{PCO}_2, \text{ppmv}$	Ionic strength	Free Ca^{2+}
Seawater	0.698	0.555	2.28	-6.80	-5.45	-0.68	3.39	407	0.638	$8.98 * 10^{-3}$
Lake Van										
9 m	+1.132	+0.982	+4.09	-4.10	-2.72	-2.94	3.50	316	0.364	$2.64 * 10^{-5}$
400 m	+1.044	+0.883	+3.74	-3.90	-2.52	-2.93	3.27	537	0.389	$2.41 * 10^{-5}$
400 m degas.	+1.066	+0.905	+3.79	-3.85	-2.47	-2.96	3.46	347	0.391	$2.23 * 10^{-5}$
Spring Q1	-0.440	-0.594	-1.07	-	-	-	1.35644080	-	-	
Satonda										
5 m	+0.997	+0.857	+3.20	-6.32	-4.97	-1.15	3.57	270	0.561	$4.14 * 10^{-3}$
40 m	+0.140	-0.001	+1.45	-7.13	-5.79	-1.05	1.75	17,700	0.654	$5.25 * 10^{-3}$
65 m	+0.675	+0.535	+2.59	-6.48	-5.15	-2.41	0.63	2,36,000	0.737	$5.24 * 10^{-3}$
40 m degas.	+1.373	+1.232	+3.92	-5.89	-4.55	-1.05	3.46	347	0.653	$5.19 * 10^{-3}$
65 m degas.	+2.360	+2.219	+5.98	-4.75	-3.42	-2.47	3.46	347	0.722	$4.67 * 10^{-3}$

Note: Degas. = PCO_2 Degassed to 347 ppmv

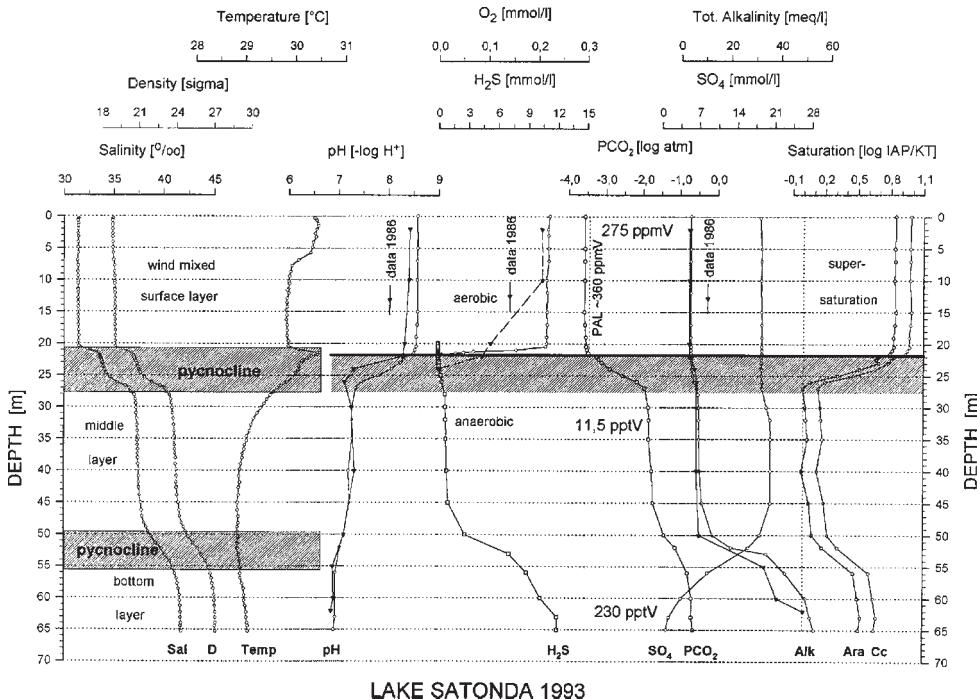


Figure 2. Hydrochemical profiles of the water column of Satonda Crater Lake as sampled during 1986 (black triangles), 1993 (open circles), and 1996 (black circles) expeditions. Two pycnoclines (at 21–27 m and 49–56 m) separate the lake into three layers: the middle and bottom layers with salinities above, and the surface layer with a salinity below that of ambient seawater (34.6 %). The middle and bottom layers are strongly reducing, and the upper layer is oxygenated and wind mixed. Alkalinity is produced in the lower layers due to sulfate reduction and exported to the upper layer (“alkalinity pump”) by diffusion and/or mixing events. Such an event has probably occurred between the two sampling periods as is evident from the differences in the 1986 and 1993 pH values and alkalinity curves in the upper pycnocline.

spherules 20–30 µm in diameter. Numerous diatoms of the genera *Chaetoceros*, *Rhopalodia*, and *Surirella*, and colonies of the mucilaginous *Amphipleura rutilans* overgrow the surface along with filamentous cyanobacteria from the genera *Oscillatoria* and *Phormidium*.

The hard, micritic aragonitic crust of the towers and their branches is from a few millimeters to 2–3 cm thick, encasing a highly porous, often tubular, axial zone built of irregular cystose sheets 2–5 mm thick (Fig. 2B). The sheets consist of subglobular aragonitic bodies, microstructurally identical with the aragonitic marginal crust. Most of the axial sheets are covered with finely laminated low-Mg calcite, often spherulitic in structure. Calcite spherulites, authigenic quartz and chalcedony, and a variety of authigenic silicates (zeolites) also often occur. The axial aragonite transforms in many places into subglobular and star-like aggregates of low-Mg calcite. The axial structures are usually coated with brown and/or black films of Fe oxide and Mn oxide.

The surfaces of the shallow columns are often decorated with snow-white, soft tips composed of apparently inorganically precipitated calcite spherules, identical with those occurring in the axial zone.

In thin sections, the marginal aragonite crust appears almost homogeneous (micritic) or indistinctly clotted (thrombolitic). It contains numerous small, empty voids (fenestrae), irregularly distributed (Fig. 2C). The close association of the microgranular aragonite with the living coccoid cyanobacterial mat (Fig. 2E and F) and the near-edge x-ray absorption fine structure (NEXAFS) spectra at the C and N K-edges of the aragonite nanograins (Benzerara et al., 2006) indicate that the precipitation of the aragonite has been induced and mediated by the mat metabolic activity. Also $\delta^{13}\text{C}$ analyses of the micrite support this conclusion. They show values 2.5 to almost 4% heavier than the $\delta^{13}\text{C}$ value of the lake water (average DIC, $\delta^{13}\text{C} = 2.70\text{\textperthousand}$, $n = 19$). This shift is typical for carbonates precipitated *in vivo* by cyanobacterial mats due to preferential uptake of ^{12}C from the photoassimilated CO_2 and/or HCO_3^- (e.g., Pentecost and Spiro, 1990; Merz, 1992; Sakata et al., 1997).

The living cyanobacterial mat grades into microgranular aragonite enclosing numerous remnants of cyanobacterial sheaths (capsules) and then, deeper, into pure aragonitic micrite (Fig. 2F). In the outermost 100–200 µm of the crust, the amount of organic cyanobacterial substance (mostly remnants of the outer mucilage sheaths, i.e., glycocalyx) remaining postmortem is distinctly larger (Fig. 2C) than that in the deeper layers. The texture of the Lake Van microbialites is similar, if not identical, to clotted fabric described as thrombolitic from many ancient calcareous deposits, which are usually interpreted as cyanobacterial products (cf. e.g., Aitken, 1967; Kennard and James, 1986; Burne and Moore, 1987).

3.1.3. Hydrochemical Control of the Microbialite Fabric

The Lake Van calcareous structures originate from biologically induced as well as inorganic precipitation. Because the percentage of inorganically precipitated CaCO_3 is, by volume, quite high, they can be classified as *microbial tufa*. The

hard, cyanobacterially precipitated outer crust is undoubtedly reinforcing their stability, allowing the pinnacles reach extraordinary sizes. The vertical growth is induced by seepage of low-density groundwater rising through the porous inside of the columns (Kempe et al., 1991). Scuba observations show that some of the columns discharge substantial wells, whereas others do not have any visible orifices. However, drilling through the crusts and sampling the water inside the columns shows that these are also filled with low-density groundwater, slowly percolating through the encasing crust.

The absence of lamination in the outer cyanobacterial crust is striking. Its clotted microfabric and the abundance of fenestrae is comparable with the so-called thrombolitic and birdseyes microfabric, characterizing many fossil marine carbonates (e.g., Bathurst, 1975; Kennard and James, 1986; Burne and Moore, 1987; Flügel, 2004). This lack of lamination can also be explained by groundwater input. When the groundwater oozes out through the crust from the interior of the column, it creates, due to the mixing of Ca^{2+} -rich groundwater and CO_3^{2-} -rich lake water, an environment even more highly supersaturated than the lake water itself, promoting *in vivo* permineralization of the coccoid cyanobacterial mats. At places of high discharge, however, that is, mostly at the tips of the columns and branches, precipitation of inorganic calcite proceeds. The transport of Ca^{2+} with the groundwater is therefore the controlling factor for the growth rate of Lake Van microbial tufas. Because of the continuous mode of Ca^{2+} supply and because of the lack of strong seasonal fluctuations, it is understandable that Lake Van microbial crusts do not develop lamination.

3.2. SATONDA CRATER LAKE

3.2.1. *Hydrochemistry*

Satonda Island is a small volcanic island to the north of the Tambora volcano on Sumbawa/Indonesia ($8^{\circ}7' \Sigma$, $117^{\circ}45' E$) (Kempe and Kazmierczak, 1990b, 1993). It contains two nested craters filled with a lake that has a surface area of 0.77 km^2 and a volume of 0.034 km^3 .

Until about 3,000 years ago (^{14}C dates obtained from peat underlying the quasi-marine sequence) the lake contained fresh water. Then the regionally rising sea level caused infiltration of seawater through the lowest part of the caldera wall at the western end of the lake. The seawater-filled lake was settled by a few marine bivalves and gastropods. Also serpulids started to grow small fringe reefs all the way down to at least 35 m depth.

Due to the wind-protected situation of the lake inside the up to 300-m-high caldera walls, the lake became soon stagnant at a depth of about 50 m (Fig. 3). Brines, produced by strong evaporation in small lagoons along the shore, flowed to the bottom of the lake, stabilizing an even more saline (up to 41%) and anaerobic bottom water. Sulfate-reducing conditions were soon reached, and the alkalinity started to rise. The bottom layer now has an alkalinity of up to 54 meq/l, and

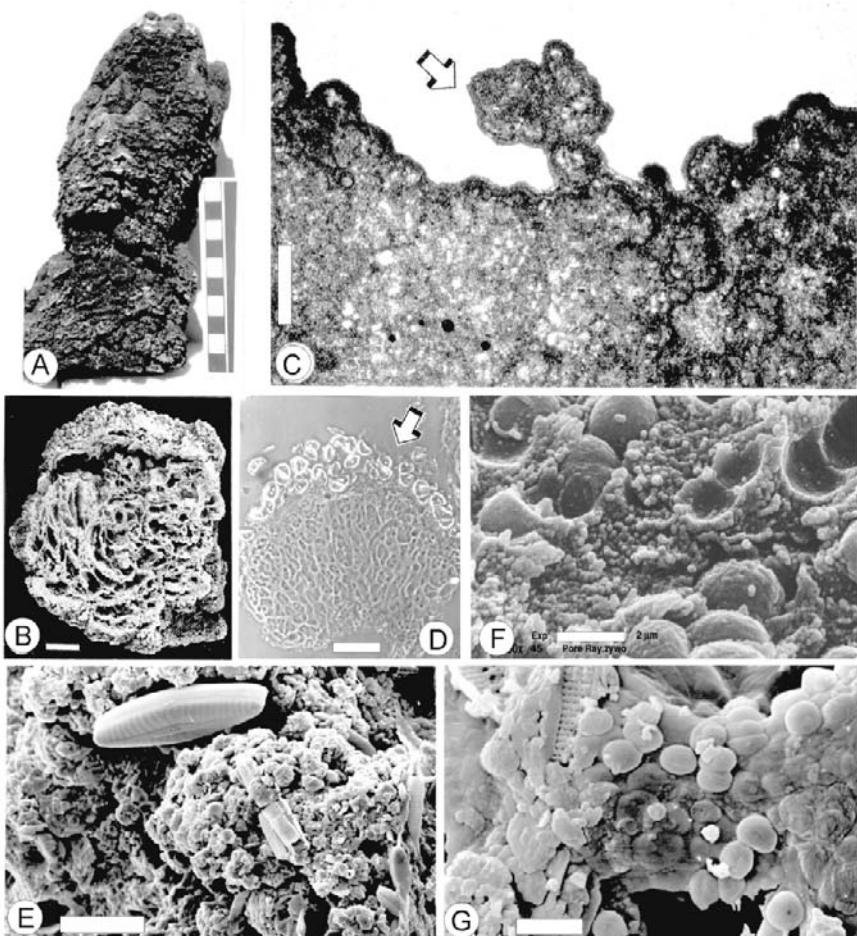


Figure 3. Nonlaminated cyanobacterial microbialites from Lake Van (Tatvan Bay), Turkey. (A), Lateral view of a side-branch of a ca. 4-m-high microbialitic column rising from a water depth of about 19 m; scale bar in centimeters. (B), Transverse section of the sidebranch illustrated in (A) to show the cystose interior of the branch and the thin external wall; scale bar equals 2 cm. (C), Magnification of the knobby surface of the branch shown in (A) produced by in vivo calcifying mat of coccoid cyanobacteria; scale bar equals 1 cm. (D), Vertical section through a fragment of living uncalcified coccoid (pleurocapsalean) mat from a living microbialite surface at about 14 m water depth. The thick common mucilage sheaths surrounding subglobular groups of cells and numerous diatoms (*Rhopalodia musculus*) growing on the mat surface (arrow in C) are well visible; transmitted light micrograph (Nomarski illumination); scale bar equals 50 μ m. (E), SEM view of the surface of a living pleurocapsalean mat covered with in vivo precipitated anhedral aragonite granules and corroded diatom frustules; water depth about 19 m; scale bar equals 5 μ m. (F), SEM image of cross-section close the surface of an air-dried living pleurocapsalean mat showing slightly shrunken coccoid cells embedded in masses of ultra small aragonite granules precipitated in vivo within the mucus sheaths; scale bar equals 10 μ m. (G), SEM picture of living surface of pleurocapsalean mat weakly encrusted with in vivo precipitated microgranular aragonite; sample taken from a large, tower-like microbialitic structure at water depth of about 19 m; scale bar equals 10 μ m.

almost all of the sulfate is lost (residual concentration: 2 meq/l) (Tables 2–4; Fig. 3). The PCO_2 in this layer reaches 0.24 atmospheres, keeping this water less supersaturated than the threshold of 0.8 SI_{Cc} at which calcification would occur. If the water is, however, allowed to degas (Table 4), then it would be very highly supersaturated with respect to all alkaline earth carbonate minerals and would spontaneously precipitate aragonite. The sulfate reduction is fueled by organic debris of terrestrial plants washed into the lake. It has a very low $\delta^{13}\text{C}$ value (a composite of leaves of the most common trees in the crater yielded a $\delta^{13}\text{C}$ of $-28.7\text{\textperthousand}$). In consequence, the released excess alkalinity has a very low $\delta^{13}\text{C}$ signature itself (up to $-19.5\text{\textperthousand}$ in the bottom layer). Part of the excess alkalinity is mixed up into the surface layer (i.e., in the fashion of an “alkalinity pump”; see Kempe, 1990; Kempe and Kazmierczak, 1994), and in consequence, the lake became too alkaline to sustain marine molluscs any longer, which all disappeared, except one cerithiid species (Kempe and Kazmierczak, 1993). Instead, reefs of calcareous red algae and in situ calcifying cyanobacteria began to grow.

A few hundred years ago, the lake became also stratified at mid-water depth (ca. 23 m). The reasons for the initiation of the secondary stratification may be climatic in origin. Today, the surface layer is less saline (30.9 %) than seawater (34.4 % in the sea surrounding the island), indicative of wetter conditions than those previously. The lower density of the surface water layer prevents deep mixing, and the middle section of the water column became anaerobic as well. Its alkalinity rose to 7.8 meq/l and its $\delta^{13}\text{C}$ value dropped to ca. $-10\text{\textperthousand}$.

When we first visited the lake in October 1986 at the end of the dry season, the alkalinity of the surface layer amounted to 3.60 meq/l, the pH was 8.43 (at 2 m depth) and the SI_{Cc} was 0.81. When we revisited the island in November 1993 for more extensive geochemical observations, the alkalinity had risen to 4.15 meq/l, the pH amounted to 8.59, and the SI_{Cc} to 1.00 (at 5 m depth, Tables 2–4). Part of this new alkalinity must have derived from a mixing event, which brought high-alkalinity water from the top of the anaerobic water body upward. This is supported by the observation that salinity and alkalinity at a depth of 21–23 m were lower in 1993 than in 1986. This mixing must have led to a substantial removal of CaCO_3 , because the Ca^{2+} concentration dropped from 10.78 meq/l in 1986 (at 2 m depth) to 8.96 meq/l in 1993 (5 m sample, Tables 2–4) (note that seawater has a Ca^{2+} concentration of 20.6 meq/l). This conclusion is substantiated by the fact, that the $\delta^{13}\text{C}$ dropped from $-8.44\text{\textperthousand}$ (at 10 m depth) in 1986 to $-4.9\text{\textperthousand}$ (at 15 m depth) in 1993, indicating the loss of heavy carbon in a calcification event. Furthermore, H_2S has apparently been introduced into the lower part of the epilimnion, causing dieback of calcareous red algae below ca. 15 m depth.

These data show that the seawater in Satonda Lake is not only much more highly supersaturated than seawater, but that the alkalinity pump is a powerful mechanism to create supersaturation events on an interannual timescale.

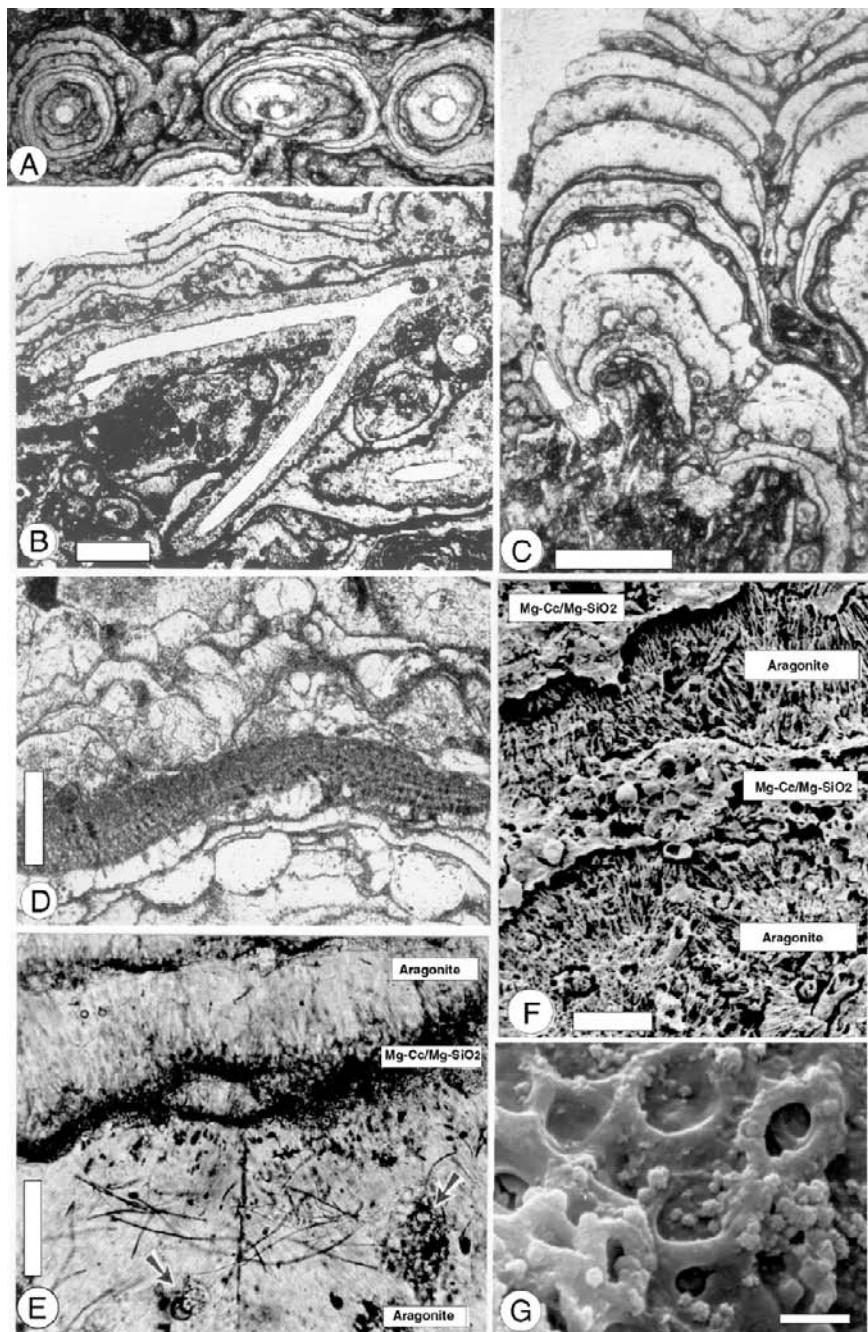
Apart from these interannual alterations in the water chemistry of the lake, we most probably also have significant seasonal alterations. In the rainy season, the lake level rises by about 1 m, diluting the upper 20 m by about 5% with rain

water. At the same time, temperatures decrease and remineralization of organic matter washed into the lake most likely increases the PCO₂ of the water. Modeling these alterations with regard to the carbonate mineral supersaturation (Kazmierczak and Kempe, 1990) suggests that the lake could experience strong enough annual alterations in supersaturation to cause calcification event toward the end of the dry season and therefore explain the observed lamination in cyanobacterial microbialites.

3.2.2. Microbialites

The striking feature of Satonda microbialites making them unique among all other modern in situ calcifying cyanobacterial mats is their association with marine organisms in a hydrochemically quasi-marine environment (see earlier). The marine character of the lake macrobiota is accentuated by the presence of such stenohaline organisms as corallinacean (*Lithoporella melobesioides*) and squamariacean (*Peyssonnelia squamaria*) red algae, suberitid demosponges, and nubecullinid foraminifers. The cyanobacterial mats are composed of thin layers of subglobular aggregates of monospecific coccoid cells arranged in a so-called pseudoparenchymatous pattern. According to current classification (Rippka et al., 1981), they can be assigned to the *Pleurocapsa* group. The living mat shows a capsular organization typical for these cyanobacteria and its surface is locally covered with anhedral granules of Mg-calcite (Fig. 4G). Permineralized cyanobacterial mats intergrow with the arcuate thalli of red algae and, together

Figure 4. Laminated cyanobacterial microbialites from the quasi-marine Motitoi crater lake on Satonda Island, Indonesia. (A and B), Transmitted light micrographs of transversal (A) and tangential (B) thin section of laminated microbialites (microstromatolites) composed of alternating aragonite (white) and Mg-calcite/Mg-silicate (dark) laminae. Cystose growth mode of the stromatolitic structure is visible in some places; sample taken from 50 cm water depth; scale bar equals 500 µm. (C), Transmitted light micrograph of vertical cross-section of laminated (microstromatolitic) microbialite grown on a bundle of filamentous thalli of siphonocladalean chlorophytes; samples taken from 20 cm water depth; scale bar equals 500 µm. (D), Transmitted light micrograph of cystose microbialitic structure composed similarly as the microstromatolites shown in (A–C) of alternating laminae of aragonite and Mg-calcite/Mg-silicate overgrowing from both sides an arcuate thallus of a squamariacean red alga (*P. squamaria*); water depth 6 m; scale bar equals 250 µm. (E), Transmitted light micrograph of magnified vertical thin section of a microstromatolite composed of alternating layers of sparry aragonite and microgranular (micritic) Mg-calcite/Mg-silicate; remnants of primary coccoid cyanobacteria (arrows) and of thread-like decomposing microbes (flexibacteria?) are distinctly visible within the lower aragonitic layer; subfossil specimen sampled from a microbialitic head exposed during the end of the dry season above the water level; scale bar equals 50 µm. (F), SEM micrograph of acid-etched (5% formic acid for 15 sec) polished platelet of a microstromatolite showing fibrous texture of the aragonite laminae and granular fabric of the Mg-calcitic/Mg-silicate laminae. Remnants of pleurocapsalean cyanobacteria are visible in the middle Mg-calcite/Mg-silicate lamina and fungal hyphae in the lower aragonite lamina; scale bar equals 10 µm. (G), SEM view of magnified fragment of the surface shown in (A) covered with patches of in vivo precipitated minute granules of Mg-calcite; scale bar equals 2 µm.



with agglomerations of nubecullinid foraminifers and serpulids, generate massive calcareous mounds and irregularly shaped crusts 0.5–1.20 m in thickness at rocky points along the lake shore. Living *in situ* calcifying cyanobacterial biofilms have been observed to occur from the lake surface down to the O₂–H₂S interface.

Concerning the depth of occurrence, biotic association, and internal structure, three groups of cyanobacterial microbialites can be generally recognized in the crater lake (comp. Kazmierczak and Kempe, 1990, 1992; Kempe and Kazmierczak, 1993). The first group is represented by crusts composed of tightly adhering laminated columns, 3–7 mm high and ca. 1–3 mm in diameter, growing on tufted thalli of siphonocladalean green algae (Fig. 4C). These evidently microstromatolitic microbialites are mostly occurring at very shallow water depths, usually not deeper than 20–30 cm, but as tiny individual columns they can be found at depths reaching almost the chemocline. The second group, which comprises the bulk of Satonda microbialites, is represented by dense agglomerations of elongated and irregularly twisted microlaminated bodies. Their central parts are occupied by one, rarely by two or three cylindrical tubes, cylindrical tube 90–220 µm in diameter, representing moulds of filaments of siphonocladalean green algae (*Cladophoropsis* sp.) around which the microbialitic (microstromatolitic) structures have been accreted (Fig. 4A and B). The bathymetric distribution of these microbialites is limited by the growth of *Cladophoropsis* not exceeding 10 m water depth. The third group is represented by laminated cystose and tubuloid microbialitic structures occurring in crypts and crevices between the foliaceous thalli of calcareous red algae (Fig. 4D) and agglomerations of nubecullinid foraminifers. The common feature of all these microbialitic structures is their distinctly bimimetic character and well-laminated internal structure. Observations in transmitted light show that the microbialites are composed of alternating dark and light laminae (Fig. 4A–D). The thinner dark laminae are composed of microgranular Mg-calcite, often with admixture of Mg-silicate and/or silica, and the thicker light ones of fibrous aragonite (Fig. 4E). Remains of capsules or molds of them, which can be found both in the Mg-calcitic and aragonitic laminae (Fig. 4E and F), indicate that the microbialitic structures are built of colonies of pleurocapsalean cyanobacteria which, during their growth, must have been exposed to varying external factors modifying temporally their calcification mode and consequently their final mineralogical and textural appearance.

3.2.3. Hydrochemical Control of the Microbialite Fabric

The alternation of Mg-calcitic and aragonitic laminae typical for the Satonda microbialites can be best explained as an effect of the periodical (seasonal) fluctuations in lake water chemistry. Two basically different modes of calcium carbonate permineralization of the coccoid cyanobacterial mats participate in the microbialite morphogenesis, both strictly controlled by the ambient hydrochemical factors. The precipitation of the microgranular (micritic) Mg-calcite laminae proceeds apparently at the living mat surface during increase of calcium carbonate

saturation (SI_{Cc} up to 1) in surface waters during the dry season. The formation of the aragonite laminae is, in contrast, initiated and mediated early postmortem by metabolic activity of heterotrophic microbes (flexibacteria and fungi – comp. Fig. 4E and F) decomposing the dead lower portion of the mat grown uncalcified during the wet season when the ambient $CaCO_3$ supersaturation is lower. The decomposition continues after the uncalcified part of the mat is sealed by an *in vivo* precipitated Mg-calcite lamina during the next dry season. The activity of the decomposing microbiota dominated by sulfate-reducing bacteria produces anaerobic or dysaerobic conditions in such a cryptic microenvironment (e.g., Skyring et al., 1983; Ward et al., 1984; Baumgartner et al., 2006). This results in an alkalinity increase (cf. Eq. 4) which, by increasing the supersaturation level, can easily trigger precipitation of early diagenetic aragonite. Calcification evoked by bacterial degradation (particularly by sulfate reducing and/or purple bacteria) of cyanobacterial mats below the zone of photosynthesis has been described as a significant factor in $CaCO_3$ formation in decaying cyanobacterial mats (e.g., Krumbein and Cohen, 1977; Lyons et al., 1984; Kühl et al., 2003; Kazmierczak et al., 2004; López-García et al., 2005; Ludwig et al., 2005; Baumgartner et al., 2006; Altermann et al., 2006). Whereas the precipitation of Mg-calcite (21–27% $MgCO_3$) at the living mat surface is easily explainable by the high Mg/Ca ratio (9:1) in the lake water (see Table 3), the origin of aragonite in the cryptic environment appears puzzling at first (Arp et al., 2003). A plausible explanation is perhaps that in the cryptic environment an even higher Mg/Ca ratio arises due to the liberation of large amounts of Mg^{2+} from decomposing cyanobacterial sheaths which are known to be significantly enriched in Mg^{2+} compared with ambient water (Gebelein and Hoffman, 1973).

4. Lessons for Reconstructing the Past

The elucidation of the significance of environmental chemical factors controlling *in situ* calcification of benthic cyanobacterial mats in the two settings presented above offers interesting clues for the paleoenvironmental reconstruction of ancient seas, which supported the growth of similar biostructures. Contrary to the widespread opinion (for review, see e.g., Fairchild, 1991; Ginsburg 1991; Riding, 1991, 2000) assuming that the bulk of ancient marine calcareous microbialites are products of trapping and binding of extraneous carbonate particles by cyanobacterial mats, the examples presented earlier have convinced us that *in situ* calcification was the main mechanism generating such microbialites in past seas. Since recent seas do not apparently support *in situ* calcification of benthic cyanobacterial mats, the immediate conclusion drawn from our studies is that the supersaturation with respect to carbonate minerals in the paleo-epicontinental seas was higher than that today. It is not possible to evaluate precisely the state of the carbonate system in the past environments. However, in the light of our investigations, it is almost beyond doubt that a supersaturation threshold of SI_{Cc} or

$SI_{Ara} > 0.8$ in the vicinity of the mat was necessary to induce *in vivo* mineral precipitation on and within the cyanobacterial sheaths (Kazmierczak et al., 2004). This high supersaturation was caused by alkalinity exported from anaerobic basins (“alkalinity pump” – Kempe, 1990; Kempe and Kazmierczak, 1994). Alkalinity therefore was presumably the most important factor controlling the saturation state in past environments.

The abundance of calcareous microbial pinnacles in Lake Van at sites of Ca^{2+} -rich groundwater outflows suggests a similar origin of certain microbial structures reported from past lacustrine and marine sediments. For example, some columnal or conical carbonate stromatolite-like bodies described from Precambrian deposits show tube-like or cystose axial zones, mostly named conophytons (e.g., Komar et al., 1965; Donaldson, 1976). Similar structures have also been noticed in Cambrian and Ordovician shallow-water carbonate deposits (Maslov, 1960; Galloway and St. Jean, 1961). Tubular calcareous buildups reminiscent of Lake Van microbialites have also been described from Miocene lacustrine algal reefs (Straccia et al., 1990). According to new observations (Hovland and Judd, 1988, for review), various calcareous “seabed pockmarks” of presumably microbial origin are attributed also to groundwater outflows. Interestingly, these fairly common structures in modern marine environments may occur quite far offshore. The microfabric of the Lake Van microbialites (see also Kazmierczak et al., 2004) has proved to be identical with the microfabric observed in fossil (Jurassic) marine micritic and peloidal limestones (Kazmierczak et al., 1996). This underscores the role of benthic coccoid cyanobacteria in the genesis of these very common types of ancient open marine carbonates (Keupp, 1977; Kazmierczak et al., 1996; Tribouillard, 1998).

The discovery of well-laminated microbialites on Satonda Island, which are interpreted as the products of fluctuations in the environmental carbonate supersaturation (Kempe and Kazmierczak, 1990a, b, 1993; Kazmierczak and Kempe, 1992; Arp et al., 2003; Kazmierczak et al., 2004), implies a similar mechanism for the origin of many calcareous stromatolites recorded from the past seas (Altermann et al., 2006, for review). Depending on duration and regularity of these fluctuations, a great variety of internal textures and microfabric could have been produced (for review, see e.g., Hofmann, 1973; Semikhatov et al., 1979). Analogs of common Palaeozoic enigmatic fossils, known as stromatoporoids and wetheredellids, identified in Satonda microbialites (Kazmierczak and Kempe, 1990, 1992, 2004a) are the best testimony for the geological importance of the search for modern settings sustaining *in situ* calcifying cyanobacterial mats.

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SOIL AND FRESHWATER MICRO-ALGAE AS A FOOD SOURCE FOR INVERTEBRATES IN EXTREME ENVIRONMENTS

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1. Introduction

Microscopic algae and cyanobacteria (the term micro-algae will be used in the text to cover both eukaryotic algae and prokaryotic cyanobacteria) are able to colonize almost all of the biotopes on earth. They are the most important primary producers in both sea and freshwater ecosystems. Their importance in terrestrial ecosystems increases further in extreme habitats because of the decreased competition of higher plants. For example, in the Antarctic, the role of algae as primary producers increases from the maritime to the continental areas where harsher conditions limit the development of mosses (Wynn-Williams, 1985). Algal mats and biological soil crusts are found worldwide in various extreme environments (Broady, 1979; Vincent, 1988; Cohen and Rosenberg, 1989; Belnap and Lange, 2001). As primary producers, micro-algae represent the bottom of the food webs, and serve as an important food source for a wide spectrum of animals.

The aim of this chapter is to summarize recent knowledge about the role of terrestrial and freshwater micro-algae as a food source for invertebrates, with particular attention to extreme habitats.

2. Methods of Study

Before starting to discuss invertebrate-micro-algae relationships, we will briefly review methods used for their study, so that the reader can better understand their potential and limitations (Lukešová, 2000). Basically we can pose three types of questions: what algae are consumed, how are they digested and what do they bring to invertebrates? Algal consumption can be judged from the comparative analyses of algae from biotopes inhabited by invertebrates and from the gut contents and/or faeces of invertebrates, using microscopy or cultivation techniques. Consumption of easily digestible species, however, can be greatly underestimated. So, if some particular species is present in the environment but absent in the gut we do not know if it is not being consumed, or if it is consumed and completely digested. This can be elucidated by laboratory food-preference tests (Lukešová and Tajovský, 1999; Frouz et al., 2004a). These studies can provide reliable data

on the level of consumption and digestion of particular species of algae. On the other hand, application of these results to the field is problematic. Single-species culture can be used to evaluate the nutrition value of individual algal species (Frouz et al., 2004b). A promising technique is comparing the isotope compositions (^{15}N or ^{13}C) of algae and invertebrates (Shtina et al., 1981; Nekrasova and Aleksandrova, 1982; Kozlovskaia and Shtina, 1987; Lewis et al., 2001; Scheu and Folger, 2004); however, this technique is still quite expensive and the results usually cannot be extrapolated to the individual algal species. Therefore, a combination of various laboratory and field approaches is highly recommended (Lukešová and Tajovský, 1999; Worland and Lukešová, 2000).

3. Invertebrates Using Algae as a Food

Algal feeders were found among all larger groups of invertebrates (see Table 1). In the freshwater environment, algae are the most important primary producers, thus it is not surprising that many protozoa, rotifers and planktonic crustaceans feed on them. Along with planktonic algae also algal periphyton forms an important food source for many benthic invertebrates (Lampert, 1987; Sterner and Hessen, 1994; Gulati and DeMott, 1997; Agrawal, 1998; Peterson et al., 1998; Kumar and Rao, 1999; Levine et al., 1999; Degans and De mecster, 2002; Rosi-Marshall and Wallace, 2002; Balayla and Moss, 2004).

In terrestrial habitats, vascular plants are the most important primary producers. However, plant litter is typically hard to digest and represents a poor source of nutrients. Terrestrial algae may be an essential supplementary food source for many soil-dwelling saprophagous invertebrates (Table 1), similar to soil bacteria or fungi. Saprophagous invertebrates play a principal role in both soil formation and nutrient cycling (Lavelle et al., 1997).

Algivores and non-selective omnivores appear among soil Protozoa, mainly in amoebae (Heal and Felton, 1970; Laminger, 1980; Couteaux and Darbyshire, 1998). The feeding of different collembolan species upon algae has been proven based both on the analyses of field materials (Zettel et al., 2002) and from laboratory experiments (Nekrasova, 1980; Lukešová, 1989; Scheu and Folger, 2004). As summarized by Small (1987) and Yeates et al. (1993) for nematodes, most genera of the order Chromadorida and omnivorous genera of Dorylaimida are reported as algal feeders. The feeding upon, and mostly selective consumption and digestion of, micro-algae were additionally documented in oribatid mites (Littlewood, 1969; Luxton, 1972; Seniczak, 1998; Hubert and Lukešová, 2001; Smrž and Norton, 2004), larvae of some soil-dwelling insects (Frouz and Lukešová, 1995), enchytraeids (Nekrasova and Domracheva, 1972; Shtina et al., 1981; Krištufek et al., 1997) and both millipedes and terrestrial isopods (Kozlovskaia and Shtina, 1987; Lukešová and Tajovský, 1999; Nováková et al., 2005). Viable algae were regularly isolated also from guts of earthworms (Pierce, 1978; Lukešová, 1989), and partial digestion of algae ingested with a substrate was confirmed using ^{15}N isotope (Nekrasova and Aleksandrova, 1982).

Table 1. Diversity of algal feeders in terrestrial and aquatic habitats.

		Terrestrial			Freshwater			
		In general		Extreme habitats	In general		Extreme habitats	
Protozoa	xx	Heal and Felton (1970); Nekrasova et al. (1976); Nekrasova (1987)	xx	Deserts Belnap (2001)	xx	Weisse (2002)	xx	Cryoconite Porazinska et al. (2004)
Rotatoria					xxx	Nogrady et al. (1993); Ricci and Balsamo (2000)	xx	Cryoconite Porazinska et al. (2004)
Tardigrada			x	Chihuahuan Desert Belnap (2001)			xx	Cryoconite Porazinska et al. (2004)
Nematoda	xx	Small (1987); Yeates et al. (1993); Bongers and Bongers (1998)	xx	Antarctica Spaull (1973); Newsham et al. (2004) Surtsey Schwabe (1973)				
Oligochaeta	xx	Nekrasova et al. (1976); Pierce (1978); Shtina et al. (1981); Nekrasova and Aleksandrova (1982); Lukešová (1989)						
Crustacea	xx	Terrestrial Isopoda Lukešová and Tajovský (1999); Nováková et al. (2005); Sustr et al. (2005)			xxx	Cladocera and Copepoda Peters and De Bernardi (1987); Benzie (2005)		
Acari	xx	Littlewood (1969); Nekrasova et al. (1976); Lukešová (1989, 2000); Seniczak (1998); Hubert and Lukešová (2001)	xx	Antarctica Burn (1986); Worland and Lukešová (2000) Chihuahuan Desert Belnap (2001)				

(Continued)

Table 1. Diversity of algal feeders in terrestrial and aquatic habitats—cont'd.

		Terrestrial				Freshwater			
		In general		Extreme habitats		In general		Extreme habitats	
Diplopoda	xx	Kozlovskaia et al. (1975); Kozlovskaia and Shtina (1987); Lukešová and Tajovský (1999)							
Collembola	xx	Nekrasova (1980); Nekrasova and Aleksandrova (1982); Lukešová (1989); Zettel et al. (2002); Scheu and Folger (2004)	xx	Arctic Hodgkinson et al. (1994) Antarctica Broady (1979); Davidson and Broady (1996); Worland and Lukešová (2000); Sinclair et al. (2003)					
Insecta	xx	Nekrasova et al. (1976); Smith (1989); Frouz and Lukešová (1995); Belnap (2001)			xx	Smith (1989); Berg (1995); Foote (1995)	xx	Glacial brook, hot spring Berg (1995) Hot spring Foote (1995)	
Mollusca			xx	Negev desert Shachach and Steinberger (1980)	xx	Pires et al., (2005)			

x occasional feeding (supplementary food source), xx important food source at least in some species, xxxx important food source in most species

4. Invertebrates Using Algae as a Food in Extreme Environments

As already shown, a broad variety of invertebrates can use algae as a food source (see Table 1), however, not all are able to live in extreme environments (Vincent and James, 1996). On the other hand, as a direct consequence of the lower diversity and biomass of vascular plants in harsh environments, the importance of algae as a food source increases in extreme environments. Further, their consumption by dominant invertebrate species plays a principal role in the energetics of ecosystems. Most attention has been focused on the possible role of algae and cyanobacteria as a food source for invertebrates in polar regions.

In the Arctic, collembolans are one of the dominant groups of invertebrates in the tundra where soil algae are important colonizers of bare soils. From the literature summarized by Nekrasova (1980), it is evident that collembola are often present on mass growths of algae on the soil surfaces and the algae may be a good food source for them. Analyses of the gut contents of field-collected *Onychiurus arcticus* (a springtail widely distributed throughout the northern parts of Palearctic region), as well as laboratory feeding experiments have shown that this animal, at least in part, feeds as a herbivore on bryophytes and algae. Some animals appear to have browsed, significantly, on those algae which grew on moist rock surfaces (Hodkinson et al., 1994).

The invertebrate fauna of the Antarctic is dominated by mites (Acari) and springtails (Collembola) and includes only few insect (Diptera) species along with nematodes, tardigrades and other microscopic organisms (Block, 1984). In some very harsh environments, such as those occurring in the McMurdo Dry Valleys of the Continental Antarctica, nematodes are the top predators in what are thought to be the simplest soil food webs encountered on Earth (Freckman and Virginia, 1997). Broady (1989) found only testate amoebae, ciliate protozoa, rotifers and tardigrades at Edward VII Peninsula (Marie Byrd Land) after studying biota at 23 nunataks (islands of rocks in glacier). Neither insects nor crustacean species have been recorded in extreme aquatic environments – lakes, ponds and streams – of the Ross Sea sector but most contain planktonic and/or benthic communities that are composed exclusively of microscopic organisms (Vincent and James, 1997).

Most detailed feeding studies have been performed on Antarctic springtails. *Cryptopygus antarcticus*, a common Antarctic species which dominates in moss communities on Signy Island (Tilbrook, 1977), consumed and mostly digested well a broad spectrum of algae and cyanobacteria. This has been demonstrated by both gut and faecal pellet analyses of field-collected animals (Broady, 1979; Burn, 1984). Selective feeding of 24 algae isolated from soils inhabited by *C. antarcticus* in Rothera Point and the complete or high level digestion of most species offered were also found in food-preference laboratory experiments (Worland and Lukešová, 2000). High feeding rates of *C. antarcticus* in the field in summer were confirmed by Burn (1982). Also, the analyses of the guts and faecal pellets of *Gomphiocephalus hodgsoni*, a small collembolan endemic to the Ross Sea regions of Antarctica (Salmon, 1962), confirmed that this animal uses a

broad spectrum of algae and cyanobacteria in its diet (Fitzsimons, 1971; Davidson and Broady, 1996). Algae also dominated within the guts of a springtail *Parisotoma octooculata* from Signy Island (Burn, 1984) and *Isotoma klovstadi* being extremely abundant in areas vegetated with the green alga *Prasiola crispa* on the scree slopes at the Cape Hallett, North Victoria Land (Sinclair et al., 2003). *P. crispa* generally seems to be an important food source for Antarctic collembolans.

Little information exists about the role of algae in the feeding of Antarctic oribatid mites. Mainly unicellular algae, but also thalose and filamentous algae, diatoms and cyanobacteria were found in the guts of *Alaskozetes antarcticus* (Acari: Oribatida), a common Antarctic freeze-avoiding micro-arthropod (Burn, 1986). *Alaskozetes antarcticus* very selectively fed on 24 algal species offered in food-preference tests. Big differences were also found in the digestion (from 0 to 100%) of particular species of algae. The most preferred species were usually also well digested (Worland and Lukešová, 2000).

Despite nematodes being dominant components of Antarctic soil food webs, little is known about their ability to consume algae. Direct observations suggest that *Mesodorylaimus*, a species of a nematode at Signy Island in the Maritime Antarctic, may feed upon coccoid algae and dead collembolans. *Coomansus gerlachei* has also been shown to feed upon algae, fungal hyphae and spores, arthropods, rotifers and tardigrades (Spaul, 1973). Newsham et al. (2004) studied the feeding preferences of the three most abundant nematode taxa at Signy Island, currently classified as microbivores. They found that only *Geomonhystera villosa* was able to feed on soil algae and fungi, and should be classified as a unicellular eukaryote feeder.

Much less information about the role of algae as a possible food source for invertebrates is available from other extreme environments such as hot deserts, newly exposed substrata, etc.

Biological soil crusts, often dominated by cyanobacteria and algae, are widespread over the world, and they provide habitats for soil invertebrates, especially in extreme environments such as cold and hot deserts (Belnap and Lange, 2001). Prostigmata mites, oribatid mites, tardigrades, isopods, snails, mole crickets, tenebrionid beetles, protozoans, termites and ants all have been recorded as feeding on cyanobacteria, lichens and mosses (reviewed by Belnap, 2001). One snail (the pulmonate snail *Sphincterochila zonata*) fed exclusively on crusts dominated by *Microcoleus* sp. that grew, following rain, upon the soil surface in the Negev desert (Shachak and Steinberger, 1980).

Schwabe (1973) found amoebae, infusoria, rotifers and nematodes (genus *Diplogaster*) in the surface layer, which consisted of algae of the genus *Chlamydomonas*, developing on rocks after post-volcanic activity on Surtsey (Iceland). The intestines of the nematodes were full of chlorophyll, and the nematodes remained in biofilms until all the algae were eaten.

As has already been mentioned, algae form the bottom of the food web in many freshwater ecosystems, which is for the most part true in extreme situations. Thermal springs and cryoconite holes representing opposite ends of the temper-

ature gradient in freshwater habitats are particularly noteworthy. Algae are the most important primary producers in thermal springs and, not surprisingly, many invertebrates dwelling in these habitats, from nematodes to insects, feed on micro-algae (Hoepli and Chu, 1932; Winterbourn, 1969; Richard and Rodger, 1973; Foote, 1995). Cryoconite holes are formed by the melting of glacier surfaces due to accumulations of dust or other fine debris which reduce albedo, and thus, increase the trapping of solar radiation (Takeuchi et al., 2001; Porazinska et al., 2004). These holes host algal mats which, in turn, promote future melting of the glacier due to the dark colouration. They are also a probable food source for the invertebrate community within these holes consisting of rotifers (*Philodina gregaria* and *Cephalodella catellina*), tardigrades (*Acutuncus antarcticus* and *Hypsibius* spp.) and ciliates (Porazinska et al., 2004).

5. Factors Affecting Ingestion and Digestion of Algae by Invertebrates

Although feeding on algae has been reported in most groups of invertebrates, little is currently known about the role of particular species. The ability to consume and digest any particular species depends on both the algal and the animal species. The properties of the algae and the behaviour of the animals are both affected by environmental factors.

5.1. ALGAE-DRIVEN FACTORS

The attractiveness and palatability of a particular species of micro-algae can differ according its nutritional value, cell wall properties, cell size, life cycle stage, culture age, toxicity, etc.

Algae, and especially cyanobacteria, are able to produce a broad spectrum of compounds which are essential in a soil animal's diet, such as polyunsaturated fatty acids (PUFAs) (Elhottová et al., 2002).

The structure of the cell walls can reduce the digestibility of those algae; especially the presence of the chemically resistant sporopollenin, derived from the oxidative polymerization of carotenoids (Puel et al., 1987), as well as other classes of resistant biopolymers (with different chemical structures and biosynthesis from sporopollenin) found in many green algae and/or the presence of gelatinous envelopes (sheaths). The confirmed occurrence of resistant biopolymers of algal walls is restricted to certain green algae, for example *Scenedesmus obliquus*, *Scenedesmus quadricauda*, *Ankistrodesmus braunii*, *Chlorella* sp., *Chlorella fusca* (Burczyk, 1987); *Botryococcus braunii* (Laureillard et al., 1986); lichen phyco-bionts *Coccomyxa*, *Elliptochloris*, *Myrmecia* (Honegger and Brunner, 1981; Brunner and Honegger, 1985); *Trebouxia*, *Pseudotrebouxia* (Konig and Pevelling, 1984); *Characium terrestre*, *Coelastrum microporum*, *Enallax coelastroides*, *Enallax* sp., *Scenedesmus* sp., *Scotiella chlorelloidea*, *Scotiellopsis rubescens*, *Spongiochloris spongiosa* (Xiong et al., 1997); the zygospores of *Chlamydomonas*

geitleri (žárský et al., 1985); loricas of the green flagellate *Dismorphococcus globosus* (Porcella and Walne, 1980); mature zygosporangia of *Coleochaete orbicularis* (Delwiche et al., 1989) and vegetative thalli of the sub-aerial green alga *Phycopeltis* (Good and Chapman, 1978).

Some algae develop specific morphological forms as a response to grazing. *Scenedesmus* forms longer chains of cells in response to grazing, these clonal forms being more difficult for crustacean filtrators to consume (Mayeli et al., 2004). The formation of these defence structures corresponds with the presence of daphnia, but may be also affected by other factors such as nutrient status (Kaler et al., 2000; Lurling and Van Donk, 2000). On the contrary, small algal cell size has been shown to be a limiting factor for consumption by *Aporcellaimellus* nematode populations, which developed only on large spherical cells, which were suitable for the animal penetration into the cell contents (Wood, 1973b).

The larvae of the mosquito *Culex molestus* Forskal selectively digested the vegetative and other non-zygospore cells of *Chlamydomonas geitleri*; whereas the zygosporangia passed through the alimentary canal of mosquito larvae without being affected. The zygosporangia were not even damaged if repeatedly ingested by the larvae. Also, changes in the zygosporangium's morphology, cell structure and germination capability were not observed (Tetik et al., 1994). Only the young cultures of some algal species (*Chlamydomonas* sp., *Chlorella vulgaris*, *Pleurochloris magna*, *Dactylococcus* sp., *Klebsormidium nitens*) were consumed by nematodes *Aporcellaimellus* spp. (Wood, 1973b).

Many cyanobacteria are able to produce toxic compounds. Various strategies, either based on the selective avoidance of toxic cyanobacteria or on resistance to these toxins have been developed by algal grazers (Hietala et al., 1997; Kurmayer and Juttner, 1999). Different animals show different tolerances to the same cyanobacterial toxins, for example one soil strain of *Nostoc* cf. *calcicola* was strictly rejected by all tested oribatid mites but preferably consumed by *Enchytraeus crypticus* and *Tipula* larvae (Lukešová, unpublished data). Both oribatid mites and enchytraeids rejected toxic *Trichormus variabilis* (Krištufek et al., 1997; Lukešová, 2000). One strain of planktonic cyanobacterium *Oscillatoria aghardii*, which does not produce mammalian neuro- or hepatotoxin, was highly toxic to larval stages of the yellow fever mosquito *Aedes aegypti* (Kiviranta and Abdel-Hameed, 1994). Similarly, *Microcystis* was toxic for *Glyptotendipes paripes* larvae (Frouz et al., 2004a). On the other hand, the oribatid mite *Archegozetes longisetosus* and the astigmatic soil mite *Tyrophagus putrescentiae* graze on the microfungus *Alternaria*, despite its strong toxins (alternaria acid, patulin) (Smrž and Čatská, 1989; Smrž and Norton, 2004).

5.2. INVERTEBRATE-DRIVEN FACTORS

The ingestion and digestion of algae (and other foods) can also be affected by the size and developmental stage of animals, the size of their mouthparts and the presence of particular enzymes.

In aquatic environments, various methods may be used by invertebrates to filter algae from the water which may result in the selective consumption of micro-algae based on their cell or colony size, shape or morphology (Lampert, 1987; Frouz et al. 2004b). More complex yet, is the situation in both periphyton and terrestrial habitats (Shtina, 1984; Arens, 1994). Some groups of soil or periphyton invertebrates are specialized in feeding on a certain group of microorganisms, and are able to even search for a particular micro-algal species (Foote, 1995). Others, for example earthworms, ingest algae together with the soil.

The size of mouthparts may limit the ability of invertebrates to consume algae. The Antarctic nematode *G. villosa*, with wider mouthparts, consumed not only bacteria but also algae (*Chlorella minutissima* and *Stichococcus bacillaris*) unlike *Plectus* spp. and *Teratocephalus* spp., which, having smaller mouthparts, consumed only bacteria (Newsham et al., 2004). Additionally, the larvae of some oribatid mites were not able to feed on algae (Littlewood, 1969).

Most invertebrates have enzymes for the digestion of the intracellular contents of algae and cyanobacteria. The presence of cellulase, xylanase and pectinase which are necessary for the complete digestion of both cellulose cell walls and the gelatinous envelopes of algae (and plants) have only been proven in some species of oribatid mites, collembolan, earthworms, millipedes, isopods and snails (Nielson, 1962; Nunez and Crawford, 1976; Siepel and de Ruiter-Dijkman, 1993; Šustr, 2001; Berg et al., 2004). Mechanical damage of cells (cell rupture) is usually necessary in order to utilize micro-algae as a food source (Tarman, 1968; Hubert and Lukešová, 2001; Smrž and Norton, 2004), which leads to the preferential digestion of large cells and the intact passage of small cells through the guts.

5.3. ENVIRONMENTAL FACTORS

Most of the properties or behaviours mentioned earlier, can also be affected (both positively and negatively) by different stress factors such as extreme temperature, UV-exposure, desiccation and food supply.

The protein content and the percentage of PUFA (especially 20:5) of the Antarctic algae increased with decreasing temperature (6–9°C) (Teoh et al., 2004). Antarctic algae are known to produce and accumulate antifreezes such as polyols and sugars (Roser et al., 1992), which may help to improve the supercooling abilities of invertebrates feeding on them. Temperature also affects feeding and molting (non-feeding) activities of animals (Burn, 1981), as well as their digestive enzyme-functioning (Randall et al., 1997). The optimum temperature for feeding in the Antarctic collembolan *Cryptopygus crypticus* is reported to be around 10°C, and for molting between 10 and 15°C. However, metabolic activity is maintained at relatively high levels, even at low temperatures of 0–5°C (Burn, 1981). Additionally, no prominent differences were observed between the food intake of *C. antarcticus* feeding on algae at 10°C and 0°C (Worland and Lukešová, 2000). Bauer (1979) demonstrated that the climbing behaviour of collembola was related

to the humidity requirements of the species; the algae on tree trunks could only be exploited when they were sufficiently hydrated. Only wet films of nitrogen-fixing cyanobacteria were quickly destroyed by the larvae of Tipulidae and Sciaridae (Nekrasova et al., 1976; Lukešová and Frouz, unpublished data). Those snails feeding on cyanobacteria in the Negev desert were active only 8–27 winter days annually, after rains (Shachak and Steinberger, 1980).

A decreased digestibility was found in nutrient-limited (mainly phosphorus-limited, less so nitrogen-limited) as well as in UV-B radiation stressed algal cells (*Selenastrum capricornutum*, *Scenedesmus subspicatus*) grazed on by *Daphnia magna* and *D. pulex* (Van Donk and Hessen, 1993, 1995). The reduction in digestibility is thought to be associated with changes in the cell wall properties, increases in the cell volume and a granular appearance (Van Donk and Hessen, 1995). Indirect effects of UV have been observed in *in situ* incubated *Chlamydomonas reinhardtii* which showed, due to UV-exposure, a pronounced loss of flagella, which may also be accompanied by the excretion enzymes facilitating the uptake of nutrients (Van Donk and Hessen, 1996). Hessen et al. (1995) demonstrated a decrease for *Ch. reinhardtii* in phosphate uptake that was well correlated with flagellar loss. These changes in stressed algal cells may influence the functioning of the zooplankton community. Nutrient-limited algae are known to be a poor food source for zooplankton (e.g. Sterner et al., 1993).

6. Ecological Impact

Micro-algal mats and crusts developing in cold and hot deserts, as well as semi-deserts represent suitable shelters and food supply for invertebrates. In different nunataks and on the Schimacher Oasis in East Antarctica, nematodes reached their highest abundance in *Prasiolamats*, a high relative abundance of rotifers and tardigrades was observed in *Nostoc* (Sohlenius et al., 2004). *Nostoc commune* and crusts, dominated by *Phormidium uncinatum* (and other Oscillatoriaceae), supported considerable populations of the collembolan *G. hodgsoni* and a species of the mite *Nanorchestes* on the Blue Glacier, South Victoria Land, Antarctica (Wynn-Williams, 1985). Tardigrades, nematodes, ciliate protozoa and rotifers were observed in cyanobacterial mats dominated by *Phormidium autumnale* which developed in fellfield ecosystems in Signy Island, Antarctica (Davey and Clarke, 1992). Great microarthropod numbers are found at soil surfaces in the Australian, Great Basin, Mojave, Colorado Plateau and Chihuahuan Deserts, as has been reviewed by Belnap (2001).

It has been documented in many ecological situations that algal and cyanobacterial films quickly disappeared from the feeding activities of different invertebrates (see earlier). For example, mass growths of algae developing on the soil surface with a biomass of hundreds of kilograms per ha disappeared in 3–4 days due to consumption by algivores – nematodes, mites, collembolans and amoebae (Nekrasova, 1980). The snail *S. zonata* feeding on *Microcoleus* dominated

crusts consumed from 0.6 to 6% of the net primary production in the Negev desert. Soil crust turnover, resulting from the grazing of snails on micro-algae, was estimated at 142 kg per hectare during a 7-year study period (Shachak and Steinberger, 1980). One enchytraeid individual was able to ingest daily 1.2×10^{-3} to 4.1×10^{-3} mg of algae according to the algal species. Assuming their feeding only upon algae, enchytraeids from forest soil could utilize an algal biomass of 131–149 kg $\text{ha}^{-1}\text{year}^{-1}$ (Nekrasova and Domracheva, 1972). The consumption rates of 135 g dry weight $\text{m}^{-2} \text{year}^{-1}$ on *Prasiola*-dominated sites by the collembolan *C. antarcticus*, and a figure of 68 g dry weight $\text{m}^{-2} \text{year}^{-1}$ by *P. octooculata* were assumed in sites in Antarctica with maximal animal population densities (Burn, 1984).

A generally high assimilation efficiency (of about 42%) was reported in soil invertebrates (Schaefer, 1990). The low assimilation efficiency, of about 10%, found in desert *S. zonata*, compared with an efficiency of up to 99% in some woodland snails, is probably connected with the necessity of scraping large quantities of soil particles (Shachak and Steinberger, 1980). After feeding on algae marked with the ^{15}N isotope, earthworms and enchytraeids incorporated of about 5% of the algal cells' marked nitrogen (Shtina et al., 1981). The incorporation of carbon from species offered to the collembolan *Heteromurus nitidus* varied with the food quality, indicating the ability to adjust the proportion of food material ingested to maximal fitness (Scheu and Folger, 2004).

The importance of certain trophic chains can change during ecological succession. During primary succession, dorylaimids (nematode-colonists) are able to develop as soon as the superficial soil is covered by unicellular algae (Bongers and Bongers, 1998). During the succession from drift sand to a 150-year-old pine forest, algae-feeding dorylaimids were present in the first phase (drift sand) and disappeared during plant succession because of changing conditions (De Goede et al., 1993). In addition, terrestrial chironomids efficiently utilized micro-algae from crusts developed on abandoned sites with pioneer vegetation (Frouz and Lukešová, 1995).

Selective feeding and, usually, the incomplete digestion of all algae were observed within all groups of invertebrates (see earlier) and have also been confirmed using stable isotope methods (Nekrasova and Aleksandrova, 1982; Scheu and Folger, 2004). This can lead to changes in the structure of algal communities, for example the frequent mass developments of *K. nitens* on the soil surface is thought to be connected with this species being omitted as a food by some dominant soil invertebrates (Nekrasova, 1980). The feeding activity of invertebrates also seems to be the only explanation for daily variations in the algal abundance in soils (Nekrasova et al., 1976).

The dissemination of viable algae and other microorganisms, due to incomplete digestion by animals (collembolans, oribatid mites), in their faecal pellets into previously uncolonized micro-habitats is very important, especially in extreme environments (Broady, 1979; Worland and Lukešová, 2000). Earthworms and diplopods play an important role in algal distribution, also in the deeper soil layers (Nekrasova et al., 1976). Faecal pellets of invertebrates, enriched in

nutrients, can stimulate the development of algae (Shtina et al., 1981). Also well documented is the role of invertebrates in acceleration of the mineralization and humification of algae (Nekrasova and Aleksandrova, 1982; Nekrasova, 1987).

Algae are not only important as a source of energy, but some of them contain compounds essential for the reproduction of certain invertebrates. Feeding on *Protococcus* positively affected the development rate, size and fecundity of several species of oribatid mites (Littlewood, 1969; Seniczak, 1998). Some nematodes are able to feed and reproduce only on algae and mosses, for example *Aporcelaimus* spp. (3 species) and *Aporcellaimellus, Eudorylaimus* spp. (Wood, 1973a). A similar effect was also observed in some amoebae (Heal and Felton, 1970). Feeding on certain algae stimulated the reproduction of enchytraeids (Nekrasova and Domracheva, 1972; Krištufek et al., 1997) and feeding on a mixed diet of algae and fungi, even in combination, increased collembola reproduction (Scheu and Folger, 2004).

Sometimes more complicated interactions can be observed, for example Nekrasova (1980) found that collembolan *Proisotoma minuta* did not reproduce when feeding on *Tetraedron* sp., but reproduced well when consuming faecal pellets of diplopods feeding upon this alga. Micro-algae, hosting in their gelatinous envelopes and sheaths bacteria and other microorganisms, can affect the feeding of small amoebae or bacteriophagous nematodes indirectly. In addition, in deeper soil layers, dead algal cells activate heterotrophic microflora preferentially consumed by animals (Shtina, 1984).

Feeding on particular algae can affect the supercooling point of animal bodies, and thus, their freezing tolerance and survival in polar regions and winter periods in temperate zones (Worland and Lukešová, 2000, 2001; Zettel et al., 2002; Sinclair et al., 2003).

7. Summary

Algae and cyanobacteria represent an important food source for a wide variety of invertebrates. The extent of knowledge about their roles in food biology, related to particular groups of invertebrates is generally uneven and also depends on methods used by researchers.

Algae and cyanobacteria can serve as either the main or as a complementary food source for algivorous and omnivorous invertebrates and can be of primary importance in the energy flow of some ecosystems. Algal and cyanobacterial mats provide, moreover, a shelter for many invertebrates in extreme environments. Biologically active compounds (e.g. PUFA) produced by micro-algae may be necessary for reproduction and healthy populations of animals. The production of antifreeze compounds, such as polyols and sugars, by algae can improve supercooling abilities, and thus, the survival of polar invertebrates.

Consumption and digestion are both affected by micro-algal and animal properties, as well as by environmental factors. The reduced digestibility of

stressed algal cells may significantly alter trophic interactions and reduce the transfer of energy between primary producers and consumers, especially in aquatic ecosystems. On the other hand, by the mechanism of incomplete digestion, the dissemination of micro-algae to new, uncolonized areas and to soil profile is supported. Alivore animals can be considered as an important factor regulating the amount of algae in soil. The activities of soil invertebrates accelerate mineralization and humification of algae. Selective feeding and digestion by invertebrates leads to changes in the structure of algal communities.

Although the importance of algae in food webs is well documented, to understand the functioning of ecosystems dominated by micro-algae and invertebrates, more information about the food biology of invertebrates and the energy transformation in algae-based food chains is required.

8. References

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PART 4: PHOTOTROPHS IN COLD ENVIRONMENTS

Vincent
Singh
Elster
Komárek
Nedbalová
Mock
Junge
Benson
Harding
Day

Biodata of **Warwick F. Vincent**, author of the chapter “*Cold Tolerance in Cyanobacteria and Life in the Cryosphere*”

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COLD TOLERANCE IN CYANOBACTERIA AND LIFE IN THE CRYOSPHERE

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1. Introduction

Cyanobacteria are commonly thought of as microbial phototrophs that are characteristic of warm water environments such as hot springs (Steunou et al., 2006), stratified lakes during summer (Vazquez et al., 2005) and tropical oceans (Johnson et al., 2006). It is less widely known that many cyanobacterial taxa achieve their greatest ecological success at the opposite thermal extreme, in polar and alpine environments. One of the first discoveries of the prolific growth of cyanobacteria in the cryosphere (the ensemble of cold environments containing snow and ice) was by the Swedish-Finnish explorer Adolf Erik Nordenškiöld. In his expedition across the Greenland Ice Cap in 1870 his team discovered black sediment that he called ‘cryoconite’, cold rock dust collecting in melt holes (Leslie, 1879). On closer inspection they observed that this material was composed of not only inorganic sediments but also black-pigmented cyanobacteria, now known to be mostly the heterocystous species *Calothrix parietina* (Gerdel and Drouet, 1960). They concluded that because of its dark colouration, this cyanobacteria and its bound sediment absorbs radiation and hastens melting of the ice, a process more recently documented on glaciers (Takeuchi et al., 2001) and ice shelves in the Canadian High Arctic (Mueller and Vincent, 2006).

The early expeditions to Antarctica such as those by Scott and Shackleton also brought back evidence of cyanobacteria forming extensive communities in some south polar habitats (Vincent, 2000). Current research shows that cyanobacteria are the phototrophic dominants in many types of communities in both Polar Regions. In some meromictic lakes, picocyanobacteria are the most common photosynthetic cell type, for example in lakes of the Vestfold Hills Antarctica (Powell et al., 2005) and at the northern coastline of High Arctic Canada (Van Hove et al., 2006). They are also abundant in many arctic rivers (Rae and Vincent, 1998), although are much less common in coastal and offshore marine waters (Waleroen et al., 2007). Viable cyanobacteria are a frequent constituent of permafrost soils (Vishnivetskaya et al., 2005) and also occur commonly within and on the outside of rocks in the desert landscapes of both Polar Regions (Cockell and Stokes, 2004). The cyanobacteria-dominated assemblages that form in cryoconite holes and pools on glaciers continue to be of great interest to microbial ecologists to address questions concerning microbial distribution and diversity, biogeochemical

processes in microbial consortia and microbial strategies for survival and success in the cryosphere (Wharton et al., 1981; Christner et al., 2003; Mueller and Pollard, 2004; Porazinska et al., 2004; Stibal et al., 2006; Tranter et al., 2004). The most luxuriant growth of cyanobacteria in the Polar Regions occurs as benthic mats and films in shallow thermokarst (eroded permafrost) lakes (Vézina and Vincent, 1997; Rautio and Vincent, 2006), in ice shelf ponds of the Arctic (Vincent et al., 2004a, b; Mueller et al., 2005) and Antarctica (Howard-Williams et al., 1989; Sabbe et al., 2004; Jungblut et al., 2005), in rivers and streams (Vincent et al., 1993; Elster et al., 1997; McKnight et al., 1999; Fernández-Valiente et al., 2007) and in ice-covered lakes (Wharton et al., 1983; Hawes and Schwarz, 2001; Singh and Elster, 2007; Taton et al., 2006; Vopel and Hawes, 2006). Littoral communities of benthic cyanobacteria are also well known from cold, alpine habitats (Vinebrooke and Leavitt, 1996; Sommaruga and Garcia-Pichel, 1999).

The common occurrence of cyanobacteria in cold habitats has implications for the development of microbial ecosystems on early Earth. Although debate continues as to the thermal regime throughout the Precambrian, there is mounting evidence that extreme cold and freeze-up was experienced during several glacial periods, perhaps even during the earliest stages in the appearance and evolution of microbial life (Vincent et al., 2004a, b, and references therein). Major freeze-up of much of the planet is believed to have occurred during the Proterozoic Era, specifically during the Paleoproterozoic 2.3 billion years bp (Melezhik, 2006) and during the Neoproterozoic, 600–700 million years bp (Hoffman and Schrag, 2002). The fossil record suggests that cyanobacteria would have been present throughout these Proterozoic events, and perhaps during earlier periods of global cooling. There is evidence for the continuity of life throughout these glacial episodes, and cyanobacteria-dominated ecosystems in the modern cryosphere are increasingly viewed as analogues for such conditions (Olcott et al., 2005; Corsetti et al., 2006).

This chapter considers the range of conditions that cyanobacteria may have had to contend with in the cryosphere during glacial periods on Precambrian Earth, and the range of ecophysiological strategies that modern-day cyanobacteria in culture or in polar and alpine regions employ to deal with such conditions. There are two distinct sets of stresses imposed by a glacial environment: (i) the freeze-up process and resultant ice regime and (ii) the persistence of cold temperatures for metabolism, growth and survival in aqueous habitats. These two sets of environmental conditions are treated separately below.

2. Freeze-up and the Implications of Ice Formation

The phase transition from liquid water to ice poses a number of challenges for phototrophic growth and survival. These include the osmotic and mechanical stresses during ice formation, the need to retain viability during periods of prolonged dormancy, and an ability to capture light and achieve net growth despite the attenuating effects of the overlying ice and snow cover.

2.1. ICE FORMATION

Many polar and alpine habitats such as shallow ponds and streams freeze solid each year. During this process, solutes are excluded from the ice and the remaining water surrounding the cells can achieve high osmolarities. This process is well known in sea ice where the freeze-concentrated brines within channels between the ice crystals may achieve salinity values many times that of sea water (Thomas and Dieckmann, 2002). This process is also known in polar lakes and ponds where benthic microbial mats experience relatively freshwater conditions in late summer to salinities up to five times that of seawater, and liquid water temperatures down to -12°C , during the final stages of freeze-up in winter (Schmidt et al., 1991). Additionally, the formation of ice can destroy membranes, particularly if the crystals are formed intracellularly (Vincent, 1988; Fuller, 2004).

Cyanobacteria have a variety of strategies to minimize the osmotic and mechanical stresses of freeze-up. Like sea ice microbiota, the mat-forming species in cold environments such as on the McMurdo Ice Shelf (de los Ríos et al., 2004) form copious quantities of mucopolysaccharides (exopolymeric substances, EPS). This material likely slows the flow of liquid water during freeze-up and thaw, and may also force ice crystal formation to occur well away from the cells. Experiments on *Nostoc commune* indicate that EPS is critical to surviving desiccation as well as freeze-up (Tamaru et al., 2005).

Many cyanobacteria produce compatible solutes (Mackay et al., 1984) that reduce the osmotic effects of freeze-up, although this has not been examined in detail in polar and alpine taxa. Some of these osmoregulatory substances protect proteins from denaturation and deactivation, for example glycine betaine (*N*-trimethyl glycine) protects the photosystem II complex against dissociation (Papageorgiou and Murata, 1995).

Like other phototrophs, cyanobacteria from some polar environments are known to produce macromolecular substances that cause pitting and other modifications of growing ice crystals. The exact role of these ice-active substances is not known at present, but they are thought to play a cryoprotective role (Raymond and Fritsen, 2000).

2.2. PROLONGED DORMANCY

One of the prerequisites for life in a seasonally frozen environment is the ability to maintain viability until the next period of thaw. Freeze-drying is a method to preserve microbes, and cyanobacterial strains in some culture collections are stored in this way. In Antarctica, freeze-dried mats have been shown to resume photosynthesis and other physiological processes within minutes to hours after rethawing. It is less clear however, what the upper limit may be to survival at much longer time scales. For example, the postulated ‘snowball Earth’ scenario suggests that microbes could be frozen for up to millions of years. There is a variety of

evidence that ancient microbes immured in glacial ice, permafrost soils or salt crystals can be brought to life (e.g. Vishnivetskaya et al., 2005). In part this may strongly depend on the physical and chemical properties of the surrounding environment during dormancy, for example the extent of exposure to UV radiation and to reactive oxygen species (ROS).

2.3. LOW IRRADIANCE

Clear ice allows a high transmittance of photosynthetically available radiation (PAR) as well as UV radiation. However, bubbles, particles and snow cover have a strong effect on albedo (back reflection) and scattering, and can result in a severe reduction in underwater energy supply (Belzile et al., 2001). Some eukaryotic algal species are known to be highly adapted to such extreme shade conditions. One of the best studied examples is the chlorophyte *Chlamydomonas raudensis* UW0241 isolated from a permanently ice-covered Antarctic lake where it is one of the dominant phototrophs. This psychrophilic species has a growth optimum at 8°C and is unable to grow at temperatures above 18°C. It is a shade species adapted to optimal growth in its ambient environment of extremely low, blue-green irradiance. Among its many features tuned towards this low light regime are a low Chla:Chlb ratio, a reduction in PSI light-harvesting Chl-proteins, a high concentration of the Chlb light-harvesting protein complex and a limited ability to adjust to high irradiances (Morgan-Kiss et al., 2006, and references therein).

Cyanobacteria lack the light-harvesting chlorophyll proteins that are characteristic of *Chlamydomonas*, however their phycobilisomes provide a highly efficient protein complex of multiple phycobiliproteins for capturing light deep in the water column, within microbial mats or under ice. For example, Hawes and Schwarz (1999) have shown that microbial mats on the benthos of ice-covered Antarctic lakes are coloured pink as a result of their high levels of the phycobiliprotein phycoerythrin. Laboratory gas-exchange measurements showed that the communities have an unusually efficient light-capturing capacity, with photosynthetic quantum yields close to the theoretical maximum (Hawes and Schwarz, 2001). These results were subsequently confirmed by in situ measurements in perennially ice-covered Lake Hoare in the McMurdo Dry Valleys using oxygen micro-electrodes (Vopel and Hawes, 2006).

Cyanobacteria mats growing in exposed polar and alpine lakes are often enriched in light-protecting pigments in their surface layers (Bonilla et al., 2005), while their basal layers are deep blue-green associated with high concentrations of the phycobiliprotein phycocyanin (PC). For example, a microbial mat from Skeleton Lake in the Canadian High Arctic had a surface 1-mm thick pink layer rich in carotenoids, and a basal 2–3 mm blue-green layer that contained more than seven times the quantity of PC than in the surface layer and a 50% higher PC:Chla ratio (Quesada et al., 1999).

Phycobilisomes are now known to be dynamic light-capturing systems that are free to diffuse horizontally in the photosynthetic membranes of cyanobacteria (Joshua et al., 2005). The physical association between phycobilisomes and reaction centers modulates the distribution of excitation energy, and influences the degree of spillover between PSI and PSII. Spillover was initially thought to be a rapid bright light acclimation response, but there is increasing evidence that in cyanobacteria it is an adaptive physiological response to low light conditions (Mullineaux and Emlyn-Jones, 2005).

2.4. PROLONGED DARKNESS

In some respects, prolonged darkness in liquid water conditions may be a much greater constraint on viability than freeze-up. Respiration may continue throughout such periods and the energetic costs of basal metabolism, maintenance and repair may ultimately deplete cellular reserves. It is not known whether there may be a threshold for such metabolism, with a loss of viability below which the cells are unable to recover even when returned to available light conditions.

3. Implications of Persistent Low Temperatures

Low temperatures exert an obvious dampening effect on all metabolic processes, although the magnitude of this cold inhibition varies greatly among species. The net result is slow net growth rates, but this may not be an ecological constraint in many habitats. For example mat communities in perennial ice-covered lakes, ice shelf ponds and polar desert streams gradually accumulate biomass each year, and this biomass then overwinters to provide a large inoculum for the next year of microbial activity. In this way, cyanobacteria maintain their perennial coverage of benthic substrates, resulting in pre-emptive competitive success at the beginning of each season.

The cyanobacterial strategy of slow growth is likely to be much less successful in ephemeral habitats and in ecosystems where loss processes are more severe. For example, melting snowbanks offer a habitat for microbial growth for only few weeks each year. These ephemeral meltwaters are subject to continuous losses by percolation, and are more typically colonized by eukaryotic snow algae than slower growing cyanobacteria (Vincent, 1988). Similarly, in the marine environment of both Polar Regions, cyanobacteria are conspicuously sparse or absent, and this may reflect an inability to keep pace with substantial loss rates via turbulent diffusion, advection and grazing by a diverse spectrum of zooplankton (Vincent, 2000). In these oceanic environments, picoplanktonic phototrophs are often abundant, but they are highly adapted psychophilic eukaryotes that can achieve much faster growth rates than cold-tolerant cyanobacteria. In earlier times, such as the Paleoproterozoic before the emergence and radiation of

eukaryotes, psychrotolerant cyanobacteria may have played a more important role in cold ocean ecosystems.

A variety of other problems are imposed by persistent low temperatures. Firstly, cells may be especially prone to photobiological damage due to ultraviolet radiation and bright PAR irradiances. Photobiological effects can be direct (e.g. DNA damage through UV-B absorption) or indirect (through the production of ROS) and while these photochemical reactions are dependent on radiation exposure, they are largely independent of temperature. On the other hand, cellular repair mechanisms such as DNA and photosystem II repair processes are likely to depend on metabolic rates, and therefore reduced under low temperatures. Persistent cold therefore has the potential to shift the damage-repair balance towards net cellular damage (Rae et al., 2000). Cyanobacteria show a remarkable suite of mechanisms to reduce such effects, including the production of a great variety of photoprotective pigments. Additional challenges in the cold are to maintain membrane fluidity, transport functions and enzymatic activities, and cold-tolerant cyanobacteria provide excellent examples of this suite of acclimation abilities.

3.1. PHOTOPROTECTIVE-SCREENING PIGMENTS

One of the first lines of protection against UV and high energy PAR exposure is the production of light-screening pigments. Cyanobacteria of the cold regions show two classes of such compounds. The first are the lipid-soluble sheath pigments gloeocapsin and scytonemin. The former is present in the sheaths of some *Gloeocapsa* species, for example the taxon *G. ralfsiana* that forms rust-coloured crusts over rock in streams of the McMurdo Dry Valleys Antarctica (Vincent, 1988). This compound has been known for almost a century, with interest in the way it changes colour with pH. However it is still not characterized either ecologically, physiologically or chemically. In contrast there is a large literature on the sheath pigment scytonemin that is found in several cyanobacteria. This compound absorbs maximally in the UV-A end of the spectrum (Garcia-Pichel and Castenholz, 1991; Proteau et al., 1993) and can be in such high concentrations, for example in *Nostoc* colonies, that the cyanobacterial mats or crusts are black. High concentrations of this pigment occur in mat-forming communities in many types of antarctic, arctic and alpine communities (Vincent and Quesada, 1993).

The second class of screening pigments is mycosporine-like amino acids (MAAs). These water-soluble compounds are found within the cells and absorb maximally at the UVB end of the incident solar spectrum (Garcia-Pichel and Castenholz, 1993; Cockell and Knowland, 1999). Studies on a High Arctic cyanobacterial mat showed that these compounds were four times higher per unit Chla in the surface relative to bottom layer (Quesada et al., 1999). A novel oligosaccharide-mycosporine-amino acid found in *Nostoc* (Böhm et al., 1995) has recently been identified in microbial mats on arctic ice shelves (Mueller et al., 2005).

3.2. PHOTOPROTECTIVE-QUENCHING PIGMENTS

One of the most severe damaging effects of exposure to high solar radiation is the production of ROS such as singlet oxygen, superoxide and hydrogen peroxide. Cyanobacteria have a variety of enzymatic and pigment strategies for quenching these highly toxic photochemically produced products, including the production of superoxide dismutase and ROS-quenching carotenoids (Vincent and Quesada, 1993; Hirschberg and Chamovitz, 1994). The latter is immediately apparent in cyanobacterial mats in streams, thermokarst lakes, ice shelf ponds and other shallow, brightly lit habitats that are often pigmented orange or pink with high carotenoid pigmentation. For example, in the Skeleton Lake mat, the bright orange surface layer had a fivefold high carotenoid concentration per unit Chla relative to the phycocyanin rich bottom layer (Quesada et al., 1999). An antarctic mat-forming cyanobacterium in culture showed major increases in carotenoid pigmentation with decreasing temperature, increasing PAR and exposure to UV radiation. After a period of acclimation, the cultures growing under UV radiation had almost as high a growth rate as the controls not exposed to UV, and the observed production of photoprotective carotenoids is likely to have been one of the acclimation mechanisms allowing this near-optimal growth (Roos and Vincent, 1998).

3.3. OTHER PHOTOPROTECTIVE STRATEGIES

Several other adaptive mechanisms allow cyanobacteria to deal with bright PAR and UV radiation at the low temperatures that characterize the cryosphere. These include the choice of habitat, or exclusion from more severe, highly exposed habitats. For example, cyanobacteria in both Polar Regions are successful in habitats in rock cracks, as layers beneath the surface of rocks and underneath translucent rocks in which solar exposure is greatly attenuated (Smith et al., 2000; Cockell and Stokes, 2004). Deep ice-covered lakes and the UV-screened habitat at the base of optically thick biofilms similarly provide refugia from the damaging effects of surface insulation. Another strategy seen in many mat communities is that of motility in which trichomes are able to change their position in the vertical light gradients by way of a gliding ability. A striking example of this response has been recorded in a meltpool microbial mat on the surface of the McMurdo Ice Shelf, Antarctica (Nadeau et al., 1999).

3.4. MEMBRANE STRATEGIES

Microorganisms have a variety of adaptive strategies to maintain membrane fluidity at low temperatures. The most common mechanism observed in cyanobacteria is fatty acid desaturation to increase the production of unsaturated fatty acids that remain fluid even in the cold. Specifically, saturated fatty acids such as C16:0 and

C18:0 are converted to C16:1 and C18:1 unsaturated fatty acids, respectively, by acyl-lipid desaturases as a post-biosynthetic modification (Chintalapati et al., 2004). Considerable interest is now focused on how cyanobacteria sense the cold and initiate this process. The current model is that the signal transduction pathway involves a two component system, a histidine kinase (Hik33) sensor located in the membrane, and a response regulator (Rer1) located in the cytoplasm. Cold-shock activates Hik33 that in turn activates Rer1. This interacts with DNA and activates the transcription of the *desB* gene that produces acyl-lipid desaturatase. The resultant increase in cellular concentrations of this enzyme leads to increased desaturation of lipids that are inserted into the membrane to reduce fluidity (Suzuki et al., 2001). Other mechanisms that modulate membrane fluidity at low temperature include: alteration of the lipid head group; increased synthesis of polar lipids (e.g. zeaxanthin) that stabilize the membrane relative to non-polar lipids (e.g. β -carotene); decreases in membrane protein content, resulting in less protein–lipid interactions that limit acyl chain flexibility; decreases in fatty chain length to produce short chains that have lower melting points and that are unable to span the membrane, making it less gel-like; and an increase in the proportion of *cis*-relative to *trans*-fatty acids (Chintalapati et al., 2004).

3.5. GROWTH RATES IN THE COLD

Cyanobacteria are often conspicuous components of the modern-day cryosphere and are also likely to have been a major component of frigid ecosystems in Earth's past. Yet the growth rates of extant species in the Polar Regions show no evidence of an impressive performance at low temperatures. All species tested in our own laboratory have been found to be psychrotolerant rather than psychrophilic, with slow growth rates at the low ambient temperatures in their native habitats (Tang et al., 1997). Even under optimal conditions for growth, these species have long doubling times relative to psychrophilic, eukaryotic algae, and relative to psychrophilic, heterotrophic bacteria. A small number of psychrophilic cyanobacteria have been identified, but although these show a temperature optimum below that of most cyanobacteria and inhibition by moderately warm conditions, their growth rates are still quite low at temperatures below 10°C. Two oscillatorian strains isolated from the McMurdo Ice Shelf had growth optima at 8°C and did not grow at 24°C. However, even at the optimal temperature for growth, their doubling times were long, ranging from 8.3 to 12.5 days (Nadeau and Castenholz, 2000).

4. Conclusions

Cyanobacteria do not seem to be specifically adapted to low temperatures in that they are unable to maintain fast growth rates in the cold. On the other hand, studies in the modern-day cryosphere show that they have a wide range of adap-

tive mechanisms that allow them to survive freeze-up, growth under low irradiances such as that produced by ice cover, and periodic exposure to UV radiation and bright PAR. These mechanisms include light-harvesting pigments, light-screening pigments, ROS-quenching compounds such as carotenoids, membrane fluidity at low temperatures and cold-stable proteins. Despite the cold, polar and alpine cyanobacteria can maintain slow but steady growth. This strategy of cold tolerance has been highly successful in some habitats such as in the benthos where communities can gradually accumulate over many seasons of growth to attain prolific biomass stocks. However, psychrotolerance and slow growth in the cold is not successful in ephemeral environments such as melting snowbanks, or in ecosystems where losses are substantial, for example as a result of strong grazing pressure or removal by advection.

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6. References

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CYANOBACTERIA IN ANTARCTIC LAKE ENVIRONMENTS: *A Mini-review*

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1. Introduction

The Antarctic habitats are some of the driest and coldest ecosystems on the Earth. Earlier there was a general acceptance that polar deserts harbored little life (Priscu, 1999). But, recent studies have revealed the existence of microbes in: the snow near the South Pole (Carpenter et al., 2000), the 3.5 km deep in Vostok ice (Karl et al., 1999; Priscu et al., 1999a), exposed soils (Wall and Virginia, 1998), sandstones (Friedmann et al., 1993), meltwater ponds (Vincent, 1988), liquid water column of permanently ice-covered lakes (Priscu et al., 1999b), and the ice covers of permanent lake ice (Priscu et al., 1998; Psenner et al., 1999). Most of the microbes found in these habitats are prokaryotic (Vincent, 1988; Gordon et al., 2000; Brambilla et al., 2001). Among these microbes, one of the most important components is the photosynthetically active cyanobacteria. They provide for an adequate quantity of fixed carbon via photosynthesis to drive a well-developed ecosystem (Vincent, 1988). On the contrary, in those habitats where there is a lack of cyanobacteria, biomass production by the addition of new carbon and nitrogen is slowed. Thus, such habitats are poor in biodiversity and also poor in trophic levels. In Antarctic habitats the cyanobacteria are adapted and acclimated to their environment in terms of temperature, freeze/thaw survival photoprotection, as well as light acquisition for photosynthesis (Vincent et al., 1993a, b, c; Tang et al., 1997; Nadeau et al., 1999; Tang and Vincent, 1999; Nadeau and Castenholz, 2000). Though cyanobacteria play a significant role in ecosystem dynamics, only a few of them have been considered true psychrophiles (Tang et al., 1997; Fritsen and Priscu, 1998). They are classified as psychrotolerant or psychrotrophic due to their ability to metabolize near 0°C and also because their temperature optima for growth are typically above 15°C. Some of the cyanobacterial groups, for example, *Leptolyngbya*, *Phormidium*, *Oscillatoria*, and *Nostoc* are cosmopolitan and occur in highly divergent environmental extremes.

The distribution of microbes in Antarctic habitats can be recorded by the analysis of particulate organic carbon (POC) and DNA sequence distribution (Burkins et al., 2000; Fritsen et al., 2000; Gordon et al., 2000; Brambilla et al.,

2001; Burkins et al., 2001). The cyanobacterial mats of streams, lakes, and ponds are the primary sources of organic carbon, and support the contemporary soil metabolism (Vincent et al., 1993c; Moorhead and Priscu, 1998; Lyons et al., 2000; Burkins et al., 2001; Elster and Komárek, 2003). DNA hybridization studies between cyanobacteria and other prokaryotes have revealed that cyanobacterial mats in ephemeral streams provide the biological seed for the lake cyanobacteria (Gordon et al., 1996; Priscu et al., 1998; Gordon et al., 2000). It is clear that lakes and streams colonized by cyanobacteria are inoculum for the life-support system of the Antarctic polar deserts. They provide organic carbon to this extremely dry ecosystem and seed it with numerous microbe spores.

With these points in mind, the following polar habitat classification has been proposed (Elster, 2002). This classification was established on the bases of the water gradient, which reflects local climate; the presence or absence of water, directly or indirectly, determining the properties of the Antarctic ecosystem; and as well as on the basis of age, which determines the stage of development of the cyanobacteria and, subsequently, of ecosystem development.

Three categories, of Antarctic habitat types, which differ from each other, have been proposed – lacustrine (lake), hydroterrestrial (wetland), and terrestrial (Elster, 2002). The lacustrine (lake) environment is stable with respect to time and has a special limnological character, although the cyanobacterial benthic communities of lakes do overlap, in many cases, with the hydroterrestrial. A common distinction between hydroterrestrial (wetland) and lacustrine (lake) habitats in the Antarctic ecosystem is that wetlands freeze solid during winter, whereas most lakes do not (Hawes et al., 1992). In hydroterrestrial environments (wetlands), liquid water is available for almost the entire period of the decisive heat-giving (summer) light. In terrestrial environments, water is available in liquid form for only a short period (e.g., snow melt, summer rain or snow fall), or is only available as air humidity (vapor absorbed directly from the air).

Of course, the most of above introduced information can be applied also for the Arctic ecosystem (e.g., Vincent et al., 2000; Elster, 2002; etc.).

2. Antarctic Lake Cyanobacterial Ecology

Several studies on cyanobacterial habitats have been conducted on ice-free expanses of land in the Antarctic, some examples include: Queen Maud Land (Kashyap et al., 1998), Signy Island (Broady, 1977a, b, c), Campbell Island (Broady, 1982; Ohtani, 1986), and the McMurdo Dry Valleys (Taylor, 1916; Wilson, 1965). Lake ecosystems contain two separate algal communities: phytoplankton in the water column and the phytobenthos attached to the bottom strata. The responses to environmental variation have the potential to differ greatly between the plankton and benthos (Vinebrooke and Leavitt, 1999). Although nutrients may severely limit planktonic production, benthic phototrophs may be subject to quantitatively or even qualitatively different controls. Many Antarctic

lakes contain luxuriant benthic mosses (Sand-Jensen et al., 1999). In addition, cyanobacterial mats may achieve high standing stocks and often dominate overall ecosystem productivity (Hawes and Schwarz, 1999; Vincent, 2000a; Hodgson et al., 2004). A considerable amount of research has been conducted on benthic mats within the lakes (Wharton et al., 1983; Parker and Wharton, 1985; Simmons et al., 1993). The benthic mats and crusts are up to several centimeters thick, and may completely cover the bottom substrate (Howard-Williams et al., 1986; Davey and Clarke, 1991; Vincent et al., 1993b). There is diversity in the benthic mat composition, for example, some mat types are largely products of single cyanobacterial species, whereas others are composed of complex microbial communities, with several dominant cyanobacteria. Several studies on benthic mats and crusts (modern-day stromatolites) of deep continental Antarctic lakes have been carried out (Parker et al., 1981; Wharton et al., 1983; Parker and Wharton, 1985). Benthic mats are cohesive, skin like, mucilaginous films, flocculi of various colors, and are mainly composed of *Phormidium* and *Nostoc*, together with soft *Leptolyngbya* trichomes. Pinckney and Paerl (1996) observed that the most abundant phytopigments of Antarctic lakes were myxoxanthophyll, echinenone, zeaxanthin, and canthaxanthin. They further found that these are all markers for cyanobacteria. The species of cyanobacteria, which inhabit the melt ponds and ephemeral streams, witness and experience several environmental stresses (e.g., high radiation and freeze/thaw cycles), whereas the benthic species of lakes receive lower radiation and growth in constant liquid water. Nutrient uptake studies on periphyton of some Antarctic systems have indicated that nitrogen and phosphorus may be regulating factor for certain benthic communities (Miller et al., 1992; Vincent et al., 1993b). Except for this, Bonilla et al. (2005) also showed that microbial mat habitat also allows growth under nutrient-rich conditions despite low nutrients in the overlying water.

In addition to nutrient constraints, cyanobacterial communities of the polar regions are subject to large variations in light availability, from total winter darkness to continuous light in summer, coupled with long periods of freezing and a short-growing season. During summer ice-out conditions, the phytoplankton and benthic mats can be abruptly exposed to high levels of PAR and UV radiation (Roos and Vincent, 1998). The communities therefore require a broad set of pigment strategies embracing both light harvesting and light protection. In this connection, Bonilla et al. (2005) described the pigment characteristics of phototrophic communities and determined the array of light- harvesting and light-screening pigments that allows growth and survival under the low temperature continuous light regime of summers in the extreme polar environment.

The analyses of lake sediment layers showed that viable phototrophs and heterotrophs could occur in deep sediment layers and can become active when liquid water is available (Priscu, 1997; Priscu et al., 1998). For example, the presence of metabolically active microbial communities has been recorded in sediments 2 m below the ice cover in Lake Bonney (Wing and Priscu, 1993). POC, chlorophyll-a, primary productivity, bacterial density, and bacterial activity were

found to be associated with the depth of lake sediment. The cyanobacteria, by the process of photosynthetic inorganic carbon fixation, release extracellular photosynthate in the form of dissolved organic carbon (DOC) and ammonium. This supply of DOC is being utilized as a source of energy and carbon for growth by heterotrophic components of the microbial assemblage. The heterotrophic organism recycles CO₂, and in turn supports the photosynthesis in the cyanobacteria. Those lake sediment layers rich in ammonium concentration serve in the active regeneration of nitrogen. The adsorptive processes between soluble reactive phosphorus (SRP) and the inorganic sediment materials have been demonstrated (Fritsen and Priscu, 1998; Priscu et al., 1998). The ratio of POC to particulate organic nitrogen (PON) for the combined data from all studied ice-covered lakes in the Antarctic region averages 8.9 (g:g), which is higher than the ratio of 5.7 (g:g) that occurs during the balanced growth of photoautotrophs (Redfield, 1958). Besides this, the elevated levels of chlorophyll-a, POC, PON, ammonium, and DOC (Wing and Priscu, 1993) have also been observed in lake ice, and these findings have led to the hypothesis that the cyanobacteria occur within the ice cover. Their occurrences in the lake ice were not passive, as they grew actively. Subsequent research has shown that there was adequate liquid water present (Adams et al., 1998; Fritsen et al., 1998) to support an active prokaryotic ecosystem within the ice. This prokaryotic lake ice ecosystem consists of the cyanobacteria and a diversity of bacterial species (Pinckney and Paerl, 1996; Paerl and Priscu, 1998; Priscu et al., 1998; Gordon et al., 2000).

3. Cyanobacterial Adaptation to the Environment of Antarctic Lakes

Fritsen and Priscu (1998) have shown that despite the low photosynthetic rate of Antarctic cyanobacterial mats, a large proportion of photosynthate is incorporated into protein. This indicates that cyanobacteria have the efficient capacity for net cellular growth. Incorporation of ¹⁴CO₂ into polysaccharides, lipids, and low molecular weight metabolites averaged 39%, 4.1%, and 15.9%, respectively. However, cyanobacterial assemblages show variable biomass-specific rates of photosynthesis. Photosynthesis versus irradiance experiments have revealed that photosynthesis ranged from 0.0043 to 0.0406 µgC µg chlorophyll-a⁻¹ h⁻¹ (Pinckney and Paerl, 1996). It has been shown that ¹⁴CO₂ incorporation into protein can be used for the computation of carbon-specific growth rates. These range from 0.001 to 0.012 µgC µg chlorophyll-a⁻¹ h⁻¹ (Pinckney and Paerl, 1996). This calculation is in agreement with the pigment labeling results. Hawes and Schwarz (2001) observed the absorption and utilization of irradiance by cyanobacterial mats in Antarctic lakes with contrasting light climates. According to Fritsen et al. (1996), during the months of continuous sunlight, in situ irradiances are above what is required to saturate the photosynthetic capacity of the cyanobacteria. In addition, they further suggested that, the in situ growth rate is not likely to be light-limited when liquid water is available. The light-saturated rate of photosynthesis, normalized to chlorophyll-a for the cyanobacterial assemblage in Lake

Fryxell, increased approximately tenfold when the incubation temperature was increased from 2°C to 20°C. This corresponds to a Q10 value of 3.46. Fritsen and Priscu et al. (1996, 1998) introduced the idea that the temperature response of light-saturated photosynthesis is near 20°C, with about 10% of the maximum rate occurring at *in situ* growth temperatures. These data support the results of Tang et al. (1997) and Tang and Vincent (1999) who contend that only a few true cyanobacterial psychrophiles are associated with polar freshwater systems. The first Antarctic psychrophilic cyanobacteria were isolated and studied by Nadeau and Castenholz (2000, 2001) from a pond on the Ross Ice Shelf and Comte et al. (2007) from Antarctic peninsula. On the basis of their genetic analysis they concluded that psychrotolerant forms are more closely related to the cyanobacteria of temperate habitats, whereas the true psychrophiles probably had evolved in the polar habitat.

On the basis of above mentioned information, it can be expected that the cyanobacterial assemblages of Antarctic lakes are psychrophilic or psychrotolerant. However, Tang et al. (1997), Vincent et al. (1997), and Vincent (2000) have discussed that the presence or absence of cyanobacteria in Antarctic lake assemblages may also depend on other selection factors, apart from temperature (e.g., freeze-thaw and desiccation tolerance, tolerances to high fluxes of solar radiation, etc.). It has been reported that a photosynthesis-irradiance relationship changes in response to freezing (Lizotte and Priscu, 1992). It was shown that the photosynthesis rate is at its maximum when the irradiance is greater than 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Moreover, the rate of cyanobacterial photosynthesis is equivalent to the respiration when irradiance is up to 202 $\mu\text{mol m}^{-2} \text{s}^{-1}$ before freezing, and 308 $\mu\text{mol m}^{-2} \text{s}^{-1}$ after freezing. During the Antarctic winter dark period the cyanobacterial O_2 consumption is 33% lower than during the summer period. Measurements of O_2 consumption also showed that these values are higher before freezing, than after freezing. Thus, during the dark and after freezing the rate of respiration becomes low. It has been noted (Tang et al., 1997; Vincent et al., 1997; Vincent, 2000a, b, Šabacká and Elster, 2006) that the polar freezing and thawing cycle does not have any major adverse affect on the Antarctic cyanobacteria over long time-scales. Rather, it has been considered that freezing may be the environmental parameter that allows the cyanobacteria to be the dominant photosynthetic microorganisms in the Antarctic terrestrial ecosystem, where low light ($<200 \mu\text{mol m}^{-2} \text{s}^{-1}$) and/or complete darkness prevail. This adaptations to increase or decrease respiration within the conditions of the Antarctic environment help to protect from losses due to reduced carbon consumption and to save energy in the ecosystem.

The nutrient bioassay experiments have demonstrated (Priscu et al., 2005) that the photosynthetic activities of cyanobacteria can be enhanced by the addition of ammonium or nitrate as a source of inorganic nitrogen. Since there is an apparent inorganic nitrogen deficiency in Antarctic habitats (Priscu et al., 2005), cyanobacteria are thus a unique tool to fix atmospheric nitrogen. It has been observed that the activity of the nitrogen-fixing enzyme nitrogenase is stimulated by addition of phosphorus and iron (Priscu et al., 2005). Moreover, the molecular analyses have also been remarked (Paerl and Priscu, 1998) that cyanobacteria

and bacteria in antarctic habitats can fix atmospheric nitrogen because of the stimulatory effect of nitrogenase by light. Paerl and Priscu (1998) suggested that cyanobacteria are responsible for a majority of the phenotypic expressions of this enzyme in nature.

The additional source of inorganic nutrients, to this cyanobacterial assemblage, is the lake sediment. It has been observed that inorganic nutrients are bound to small soil particles and a considerable amount of them can be leached. It has been shown that leaching of phosphorus is significant, mainly during spring melts. The idea that sediments under ice cover leach relatively small doses of phosphorus was introduced by Priscu (Priscu et al., 2005). In addition, some measurements (Spigel and Priscu, 1998) demonstrated that there is often a deficiency of nitrogen in cyanobacterial assemblages. It has been shown (Priscu et al., 2005) that the average amounts of ammonium and phosphorus that can be leached from lake sediment is $7.1 \mu\text{gN g}^{-1}$ and $4.1 \mu\text{gP g}^{-1}$, respectively. The ratio of leachable N:P is 1.7, which is well below what is required for balanced cyanobacterial growth. Due to the differential N and P leaching, the microorganisms have a selective advantage to fix atmospheric nitrogen. Though the apparent cyanobacterial nitrogen deficiency in Antarctic habitats has been reported (Priscu et al., 2005) assemblages associated with the sediment layer indicate that their growth is supported by nitrogen contained in sediment. The isotope analysis of $^{15}\text{N}-\text{NH}_4$ shown that the uptake of ammonium is either equaled to or exceeded by microbial ammonium regeneration. It has been shown that the absolute rates of ammonium uptake and regeneration, relative to the PON pool, range from $0.7\% \text{ d}^{-1}$ to $22.0\% \text{ d}^{-1}$ and $0.1\% \text{ d}^{-1}$ to $22.1\% \text{ d}^{-1}$, respectively. The process of atmospheric nitrogen fixation apparently replenishes the ammonium pool faster than it is consumed. It has been observed that the lake bacterial ($<63 \mu\text{m}$) fraction is responsible for a majority of the ammonium regeneration. The half-saturation constant for ammonium is generally below the ammonium levels within the ice.

The concentration of dissolved inorganic carbon (DIC) has been recorded more often in lake habitats with thick benthic cyanobacterial mat at the bottom (Priscu et al., 2005). This is because of the active cycling of both the organic and inorganic carbon pools. The $^{14}\text{CO}_2$ release by radiolabeled cyanobacterial has shown pronounced mineralization of organic carbon. The cyanobacterial assemblages dominated by mixed taxa (e.g., *Nostoc* and *Phormidium*) have a greater ability to mineralize organic carbon than do the individual taxa (e.g., *Phormidium*). It has been reported that the turnover of POC in Antarctic lake assemblages ranged from $17.5\% \text{ d}^{-1}$ in the *Nostoc* to $57.8\% \text{ d}^{-1}$ in the *Phormidium* mats (Priscu et al., 2005).

4. Antarctic Lake Cyanobacteria and Evolution

A hope for cyanobacterial applications in biotechnology and as tools to help understand the early evolution of life has created interest within the research community. It is the general view that life on Earth evolved in hot environments

(Pederson, 1997; Huber et al., 2000). But the recent views have suggested alternatively that a “hot start” probably is not the only hypothesis for the origin of life (Priscu et al., 2005). The thermal origin of life is based on single gene phylogenetic relationships, that is, small subunit ribosomal RNA (16S rRNA) (Pennisi, 1998, 1999). There are strong arguments for biased construction of the “tree of life” being based on a single gene (Pennisi, 1998, 1999; Nelson et al., 1999). According to Pennisi (1998, 1999) a hierarchical universal classification is impossible. At this level of controversy, Nelson et al. (1999) has suggested a reassessment of the evolutionary pattern. Recently (Priscu et al., 2005) it has been shown that a hot origin of life is not even supported by

1. Phylogenetic trees based on genes that do not code for ribosomal RNA
2. Chemical experiments with alternative structure for the nucleic acid backbone (Eschenmoser, 1999)
3. Considerations about the thermal stability of basic molecules found in all organisms and
4. Statistical analysis of the Guanine-Cytosine content of DNA (Galtier et al., 1999)

Balter (1999) has suggested that the adaptation to life in hot environments may even be a late adaptation. Thus there is an urgent need for intensive research to determine whether life originated in hot or cold environments, or if evolution took place in parallel within both environmental types. It seems clear that cold environments have acted as a refuge for life during major glaciations. Hoffman et al. (1998), Hoffman and Schrag (2000), and Schrag and Hoffman (2001) have all suggested that during the Neoproterozoic (around 600 million years ago), early microbes endured an ice age of such intensity that even the tropic regions froze over. According to the “Snowball Earth Hypothesis,” the Earth was completely covered by ice for 10 million years, or more. The ice thickness during this period was more than 1 km, and only the deepest oceans would have contained liquid water. However, the Snowball Earth Hypothesis has been criticized (Williams et al., 1998) on the basis that such a thick ice cover over the world’s oceans would have cut off the supply of sunlight to organisms in the seawater, and thereby eliminated photosynthesis, and all life associated with photosynthetic carbon production. Williams et al. (1998) have postulated that global-scale freezing would extinguish all surface life. In addition, Koop et al. (2005) introduced the potential role of cyanobacteria in process of atmospheric oxygen build-up, which have led to massive oxidation and loss of methane and consequently global freeze-up. They proposed a simple cyanobacterial growth model incorporating the range of C, Fe, and P fluxes expected during a partial glaciation in an anoxic world with high Fe oceans content indicating that oxygenic photosynthesis could have destroyed a methane greenhouse and triggered a snowball event on time-scales as short as 1MY. Thus they concluded that cyanobacteria are directly responsible for methane green house collapse and planetary glaciation. Hoffman

and Schrag (2000), Vincent et al. (2000), and Vincent and Howard-Williams (2001) also have suggested that photosynthetic cyanobacteria and bacteria, similar to those found in the permanent ice-covered lakes, may have acted as an icy biotic refuge during these extreme cold events.

The presence of microbial populations in the polar environment suggests that there are intense chemical and biological interactions between species. According to Vincent et al. (2000) there were interactions between species for the development of symbiotic associations, which eventually developed a eukaryote during the course of evolution. Subsequently (Vincent et al., 2004) in more detail analyses have postulated that the polar freshwater microbial mats can be considered as ideal environments for evolutionary process. The microbial mats consortia consist of highly concentrated populations from diverse functional groups, which are in close contact with each other. The physical and chemical interactions can be strong in such communities and could lead to mutualism, symbiosis, even eukaryogenesis. These organisms also thrive prolonged freeze-up and dormancy, and offer insights into how complex life may have persisted and evolved, even during the glacial upheavals of the Precambrian.

On the basis of thermal microbial mat communities, Margulis and Sagan (1997) suggested a “density-speeds-evolution” theory. It seems that ice habitats also provided opportunities for microbial evolution, inducing the radiation of the eukaryotic cell type at the onset of the Neoproterozoic (Knoll, 1994; Hoffman and Schrag, 2000). The molecular responses of cyanobacterial cells to low temperature of Antarctica’s stressful environment can be divided into two steps:

1. The cold-induced desaturation of fatty acids in membrane lipids, which fluidizes membranes to compensate for decreases in membrane fluidity at low temperatures (Murata and Los, 1997; Wall and Virginia, 1998) and
2. The cold-induced synthesis of certain enzymes that are involved in transcription and translation. Such enzymes compensate for the decrease in the efficiency of transcription and translation at low temperatures (Friedmann et al., 1993; Sato, 1995).

The growth and biological activity of Antarctic taxa depend on the temperature profile. For example, cyanobacteria in the water pockets of Antarctic lake ice, where temperatures are always below 0°C, are metabolically active and retain the capacity for oxygenic photosynthesis (Paerl and Priscu, 1998). It has been observed that cyanobacteria metabolize even at -20°C in the Antarctic (Psenner and Sattler, 1998). This adaptation has been examined in *Anabaena variabilis*, *Synechocystis*, *Synechococcus* (Priscu et al., 1999a), and thermophilic species *Synechococcus vulgaris* (Priscu et al., 1998). The analysis of gene transcript during the downward shift in temperature revealed that the increase in desaturation occurs due to stimulation of expression of those genes appropriate for the desaturases (Psenner et al., 1999; Gordon et al., 2000; Brambilla et al., 2001). The impact of the cold induces the expression of several genes for ribosomal proteins (Nadeau et al., 1999; Vincent, 2000). It has also been observed that caseinolytic

proteases act as cold-shock chaperones and proteases (Nadeau and Castenholz, 2000). The fluidity of membrane lipids depend upon the extent of unsaturation of membrane lipids (Tang and Vincent, 1999). There is feedback between membrane fluidity and the expression of genes for the desaturases. This suggested the presence, in the cyanobacterial cytoplasmic membrane, of a temperature sensor that perceives a change in the physical motion of membrane lipids and transmits the signal to a mediator that then activates the expression of genes for the desaturases (Wall and Virginia, 1998; Karl et al., 1999). For cold temperature regulation there is a cold-sensing histidine kinase system in cyanobacteria. This histidine kinase might be able to regulate all the gene expression responses to low temperature shock. Recently, the studies on a mutant form of Hik33 noted that not all cold-inducible genes are controlled by this histidine kinase (Nadeau et al., 1999). Thus, it seems that some other cold sensors exist that might control gene expression by some unknown mechanism (Nadeau et al., 1999; Tang and Vincent, 1999).

Roos and Vincent (1998) also observed the temperature dependence of ultraviolet radiation (UVR) effects on pigment composition, growth rate, and photosynthetic characteristics in mat forming Antarctic cyanobacterium. Their result implies that phototrophic organisms living in cold environments may be especially prone to the damaging effects of UVR. However, offset against this evidence of toxic UVR effects was the observation that *Phormidium murrayi* gradually increases its growth rate under UVR, indicating an ability to build up tolerance against this environmental stress. This acclimation could proceed by a variety of mechanisms, including increased efficiency of damage repair. The speed of these acclimation processes will depend on biosynthetic rates and thus, like the direct effects of temperature on the damage-repair balance, will also be affected by temperature.

In addition to the above mentioned changes, which occur in cells under low temperature conditions, Antarctic cyanobacteria have a wide range of adaptation/acclimation strategies which help them to overcome extremes related to the periodic desiccation and freezing/thawing events. The main coping strategies for living in an Antarctic habitat are avoidance and protection.

1. Avoidance – Antarctic cyanobacterial communities have a diverse range of both ecological and physiological life strategies and behaviors to avoid low temperatures and fluctuations in water status (Elster and Benson, 2004). Cyanobacteria are poikilohydric organisms that are able to tolerate desiccation to differing extents. The external environment directly manages the metabolic activity of poikilohydric cyanobacteria by affecting the presence or absence of water. The poikilohydric organisms have a great ecological advantage in the severe Antarctic environment. Shelter strategies present another type of avoidance mechanism against the low temperatures and water status fluctuations. These strategies, which are mainly important in lakes because of their physically stable environment, protect against stress factors caused by dynamic temperature and water-gradients. The poikilohydricity and shelter strategies

are intercalated, and when they are combined with cellular and physiological modifications such as: cell motility; development of complex life cycles; multi-layered cell wall, sheet, and mucilage production, they afford greater advantages. Elster and Benson (2004) suggested that these avoidance regimes have thus been developed because of considerable evolutionary pressures. The mobility of cyanobacteria facilitates avoidance of the most stressful conditions, and propels the organism to a more favorable environment (Castenholz et al., 1991; Spauldin et al., 1994; Wiedner and Nixdorf, 1998). The filamentous cyanobacteria move using straight-line gliding (Castenholz, 1982, etc.). A complex life cycle is also a major avoidance strategy and this usually involves the development of resting (dormant), vegetative, and reproductive stages that change during an organism's life cycle to accommodate the seasonally incurred environmental fluctuations. The dormant stages of cyanobacteria facilitate their transportation around the world and across the Polar Regions. This has implications in terms of influencing global microbial gene flow and distribution of genomes across long distances (Elster and Benson, 2004). The dormant stages accumulate high concentrations of soluble carbohydrates which substitute for water molecules during dehydration. This stabilizes the structure and functions of macromolecules, membranes, and cellular organization (Crowe et al., 1984). The presence of protective sugars in cells enables the vitrification of the cytoplasm upon drying, and supports the formation of a high-viscosity, metastable glassy state (Bruni and Leopold, 1991). This, in turn, preserves cell viability during dry frozen storage by immobilizing cellular constituents and suppressing deleterious chemical or biological reactions that threaten survival (Sun and Leopold, 1994a, b).

2. Protection strategies – the extracellular production of protective compounds and structures such as multilayered cell walls, sheets, and mucilage is a very common phenomenon in polar cyanobacteria, and it protects them against fluctuations in water status (Elster and Benson, 2004). The sheaths, which surround the cells, are rich in carbohydrates and other amino acids. These sheaths attract other bacteria and harbor them inside, for mutual benefit. Thick sheaths have the capability to protect against ice crystal formation, so that during the prolonged cold and icy winter period the cell walls of the cyanobacterial species are not ruptured, and life hibernates during these adverse conditions.

The phylogenetic diversity of Antarctic cyanobacteria can be characterized by the analyses of 16S rRNA genes amplified from environmental DNA (Gordon et al., 1996, 2000). Gordon et al. (1996, 2000) have analyzed the cyanobacteria and bacteria colonizing sediment particles at the depth of 2.5 m in the permanent ice cover of Lake Bonney. The DNA sequencing of 198 clones and oligonucleotide probe hybridization techniques allowed the introduction of a rRNA gene clone library, which represented the cyanobacteria and other microbial components (proteobacteria, planctomycetales, acidobacterium, green non-sulfur bacteria, and actinobacteria (Gordon et al., 1996, 2000). The cyanobacterial gene clusters

closely resemble (>97% similarity) those of well-characterized cyanobacterial species, *Chamaesiphon subglobosus* (Priscu et al., 2005). The remaining cyanobacterial gene clusters resemble (less than a 93% similarity) those of *Leptolyngbya* sp. and *Phormidium* sp. The oligonucleotide probes have been made from lake ice cyanobacterial clusters, and have also been used to screen environmental 16S rDNA samples obtained from the terrestrial (soil and stream) environment (Gordon et al., 1996, 2000). It has been observed that the probes designed to hybridize to cyanobacterial 16S rRNA genes indicate that these sequences present in the lake ice are also found in terrestrial cyanobacterial mat samples. The DNA sequence analysis and physiological data of Antarctic habitats thus indicates that the cyanobacterial (and bacterial) communities dominant in lakes are also common to the terrestrial ecosystem (Gordon et al., 1996, 2000). Priscu et al. (1998) and Gordon et al. (2000) have suggested that the katabatic winds disperse microorganisms in the desert environment and provide the biological seeds for the lake ice microbial assemblages. Olson et al. (1998) have observed that the molecular characterization of the *nifH* gene of nitrogenase in lake ice also demonstrated the presence of a diverse diazotrophic assemblage. The *nifH* analysis further showed that phototrophic cyanobacteria and heterotrophic microorganisms have the potential to fix atmospheric nitrogen. Grue et al. (1996), Olson et al. (1998), and Paerl and Priscu (1998).

5. Conclusions

This chapter was written with two aims in mind:

1. To summarize our present knowledge concerning Antarctic lake cyanobacterial ecology, physiology, and molecular biology. Cyanobacteria are well adapted and acclimated to the Antarctic lake environment in terms of temperature, freeze/thaw survival, photoprotection, and light acquisition for photosynthesis. They are the major contributors of biomass to the Antarctic lake ecosystem. It is our estimation that they comprise about 80% of algal flora of the Antarctic lake ecosystem. In addition, several the most abundant cyanobacterial groups Chroococcales, Oscillatoriaceae, and Nostocales are ecologically important in the Antarctic lake ecosystem because something close to 50% (in our personal estimation) of their species are able to fix N_2 .
2. To compile the background knowledge for an Indian–Czech research project focused on Antarctic cyanobacterial lake diversity and ecology.

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GREEN CRYOESTIC ALGAE

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1. Introduction

Cryoseton inhabits one of the most extreme environments in the Earth biosphere. The phototrophic components are composed exclusively from microorganisms, adapted to life conditions of melting snow. All species occurring in cryoestatic assemblages evidently colonised the snowfields secondarily, their ancestors originating from other habitats.

Cryoestatic communities develop in snowfields and on the surface of glaciers, where the temperature surpasses 0°C periodically (daily, or over variously long time periods), and the snow changes locally from solid to liquid state. It means, that the temperature adaptability of cryoestatic species must allow to start the intense metabolic activities immediately after melting their cells accommodated in snow. Such adaptation also occurs in algae from other biotopes (in subaerophytic, endolithic and terrestrial habitats), but it is the *conditio sine qua non* in typical cryoestatic algae. Another precondition is that the cryoestatic microflora can develop only in snowfields and glaciers remaining and persisting in air temperatures above 0°C over some periods, and under convenient irradiance conditions (cf. Hoham and Duval, 2001). This situation occurs mainly in mountains and polar and subpolar regions over the spring and summer periods.

Cryoestatic communities are composed of heterotrophic and autotrophic microorganisms. Predators (mainly springtails and ice worms) also occur here, although less frequently, and they do not play any important role in this specific restricted ecosystem. They occur usually only at the edges of the snowfield, where there is close contact with soil and seepages. More extensive overview of food webs and food chains in snow was given by Hoham et al. (1993). The cryoestatic microflora also contains species from nearby sites of the mountainous or polar environmental habitats. However, the occurrence of such additional ecotypes is only facultative. Many of cryoestatic species are able to form blooms with concentrations up to 10^6 cells mL⁻¹ of melted snow. Ablation may contribute considerably to increase the cell concentration in combination with growth *per se* (Novis, 2002b). Thus, the typical snow algae represent an ecologically and physiologically very specialised group of algal species (Fig. 1).

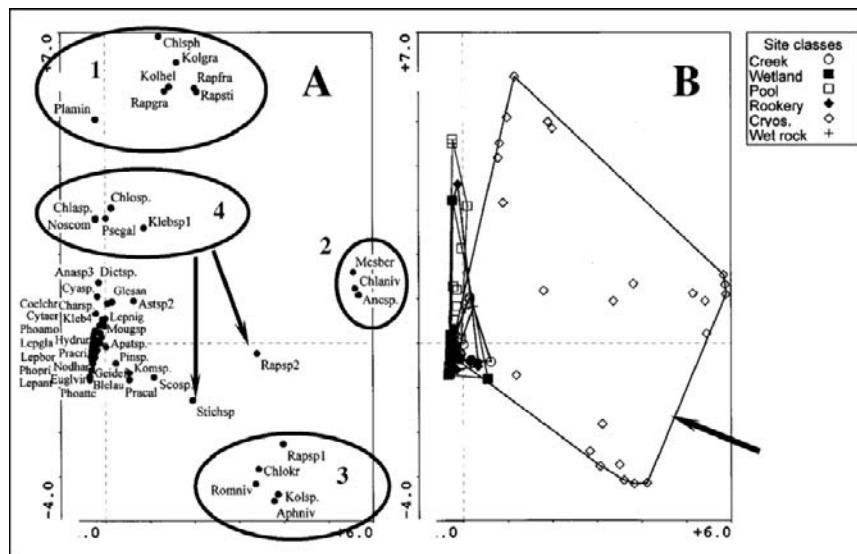


Figure 1. Detrended Correspondence Analysis (DCA) – analysis of distribution of cryoestetic microflora (thick arrow) in snowfields, in comparison with other freshwater and terrestrial habitats in the “Arctowski Station” region (King George Island, South Shetlands). The areas (A) 1–3 represent the species composition in various types of cryoestetic communities: 1 – ephemeral inland snowfields, 2 – surface of the Ecology Glacier, 3 – snowfields near rookeries, 4 – cluster of species occurring in cryoestetic algal assemblages secondarily, often on edges of snowfields. Abbreviations express the identified species from various habitats. The specific species composition in cryoeston is clearly different from all other microbiotopes of algae (B) – From Komárek and Komárek (2001); method of statistical evaluation according to Ter Braak and Šmilauer (1998).

Snow algae have been studied intensely by numerous authors. The first, “classical” period was summarised by Kol (1968), and the modern, most important review and list of literature was published by Hoham and Duval (2001). Mechanisms of adaptation and acclimation to cold environments were recently reviewed by Morgan-Kiss et al. (2006). However, many taxonomic, ecophysiological and biochemical problems remain still unsolved, and particularly methods explaining molecular phylogenies and evolution should be applied in future in higher extent. The studies of Hoham et al. (2002, 2006) yield still the first approach to this wide important problematics. The present review discusses the characters of phototrophic taxa (mainly green algae) in cryoestetic communities in relation to their ecological specificities.

2. Organisms – Biology and Life Cycles

Majority of algae occurring in cryoestetic assemblages belong to the evolutionary line of green algae from modern classes Chlorophyceae (including Chlamydophyceae), Trebouxiophyceae and Charophyceae (including Zygnemophyceae), forming most

intense green and red colouration of snow. Less frequent are diatoms, Xanthophyceae, Chrysophyceae, Dinophyceae, Cryptophyceae, perhaps Euglenophyceae and Cyanobacteria (Stein, 1963; Kol, 1968; Javornický and Hindák, 1970). The colour of green algae is often masked by carotenoids, and particularly by the xanthophyll astaxanthin (Bidigare et al., 1993) which is considered as a protection against strong irradiation, but enables also the transfers of excitation energy to chlorophyll *a* (Droop, 1955; Goodwin, 1980). Therefore, production of astaxanthin can have a special ecological importance particularly in habitats with high irradiance and low nutrients, to which snowfields belong (Fig. 2).

The study of cryoestatic species has been limited over a long period by difficulties in cultivating the organisms and studying *in vitro*. The habitat of snowfield yielded a possibility to study cryoestatic population during whole life cycle only in few cases. The different cysts and dormant stages found in natural samples were erroneously identified as different species of green algae, based on their structured cell wall surfaces, mostly without knowledge of their reproductive processes. Examples of this include members of the genera *Scotiella*, *Trochiscia* and *Cryocystis*. Redress of these problems was started by Hindák and Komárek (1968) and Javornický and Hindák (1970), who isolated the first strains of snow algae in monospecific cultures, and mainly by Hoham (1973, 1974a, b, 1975a, b), Hoham and Mullet (1977, 1978) and Hoham et al. (1979, 1983), who started to study systematically the life cycles of snow algae *in vitro*, followed by several other researchers (Ling and Seppelt, 1993, 1998; Stibal, 2003; and others). List of the main green algae occurring in cryoestatic assemblages is summarised in Table 1. Few examples of cryoestatic algae from the coloured snow see in Fig. 2.

The cryoestatic green algae are characterised according to the life form as follows:

- (1) **Species with motile stages during the life cycle.** One of the most important and most diverse groups of typical cryoestatic and exclusively cryophilic green algae is represented by several chlamydophycean species of *Chlainomonas*, *Chlamydomonas* and *Chloromonas*. Few species contain red pigments in cells. The snowfields with open exposures above timber line in mountains worldwide are generally dominated by the common *Chlamydomonas nivalis*, in which the green colour is masked by the high content of astaxanthin in cells. This species has been reported from alpine localities from all the continents as well as from polar regions, and it is regarded as a cosmopolitan cryophilic species (Kol, 1968; Duval et al., 1999b). In spite of this presumed wide distribution, its taxonomy has not been fully investigated so far. This species is mostly determined only on the basis of typical red spherical cells without any observation of flagella (Figure 2.8). It is quite probable that the reddish colouration of snow at alpine sites is caused, similarly as it is in Antarctica (Ling and Seppelt, 1993; Ling, 2001, 2002), by more species with red immotile cells. Taxonomic status of the similar populations from high mountains and polar regions has not been satisfactory investigated yet (cf. Hoham, 1974a, b).

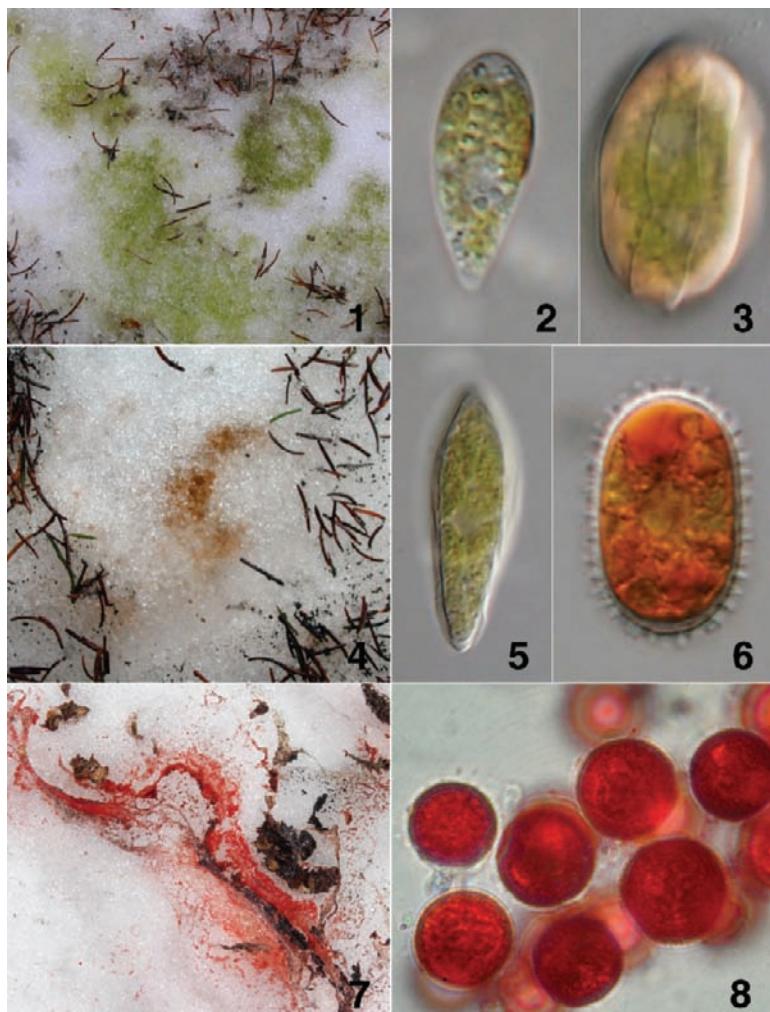


Figure 2. 1, 4, 7 – Green, brick red and red snow from the Giant Mountains (Czech Republic); 2 – Vegetative cell of *Chloromonas nivalis*, 3 – Zygospore of *Chloromonas nivalis*, 5 – Resting spore of *Chloromonas rosae* var. *psychrophila*, 6 – Zygospore of *Chloromonas brevispina*, 8 – Resting spores of *Chlamydomonas* cf. *nivalis* – Figs. 1–6 and 8 orig. photo L. Nedbalová, Fig. 7 orig. M. Kociánová.

Some strains isolated from red snow in North America were already transferred to *Chlamydomonas augustae* (Hoham et al., 2002); Ling (2002) described the species *Chlorosarcina antarctica* with red immotile cells from Antarctica. Further study of both field samples and laboratory cultures must elucidate the taxonomic position of various populations and strains.

Table 1. Main species of green algae from cryosestic habitats.

<i>Ancylonema nordenskioldii</i> Berggren 1871
<i>Chlainomonas kolae</i> [false kolii] Hardy et Curl Hoham 1974 ^a
<i>Chlainomonas rubra</i> (Stein et Brooke) Hoham 1974
<i>Chlamydomonas antarctica</i> Wille 1924
<i>Chlamydomonas augustae</i> Hoham et al., 2002
<i>Chlamydomonas nivalis</i> (Bauer) Wille 1903
<i>Chlamydomonas subcaudata</i> Wille 1903
<i>Chlamydomonas yellowstonensis</i> Kol 1941
<i>Chlorella antarctica</i> (Fritsch) Wille (1924)
<i>Chlorella vulgaris</i> Beij. 1890
<i>Chloromonas antarctica</i> Fritsch 1912
<i>Chloromonas brevispina</i> (Fritsch) Hoham et al., 1979
<i>Chloromonas chenangoensis</i> Hoham et al., 2006
<i>Chloromonas cryophila</i> Hoham et Mullet 1977
<i>Chloromonas hohamii</i> Ling et Seppelt 1998
<i>Chloromonas kerguelensis</i> Wille 1924
<i>Chloromonas nivalis</i> (Chod.) Hoham et Mullet 1978
<i>Chloromonas pichinchae</i> (Lagerh.) Wille 1903
<i>Chloromonas polyptera</i> (Fritsch) Hoham et al., 1983
<i>Chloromonas rosae</i> v. <i>psychrophila</i> Hoham et al., 2002
<i>Chloromonas rostafinskii</i> (Starmach et Kawecka) Gerloff et Ettl in Ettl 1970
<i>Chloromonas rubroleosa</i> Ling et Seppelt 1993
<i>Chloromonas tughillensis</i> Hoham et al., 2006
<i>Chlorosarcina antarctica</i> Ling, 2002
<i>Cylindrocystis brebissonii</i> Menegh. 1938
<i>Desmotetra aureospora</i> Ling, 2001
<i>Desmotetra antarctica</i> Ling, 2001
<i>Hormidiopsis verrucosa</i> Vinatzer 1975
<i>Klebsormidium</i> spp.
<i>Koliella alpina</i> (Kol) Hind. 1963
<i>Koliella antarctica</i> Andreoli et al., 1998
<i>Koliella bernina</i> (Kol) Hind. 1963
<i>Koliella chodatii</i> (Kol) Hind. 1963
<i>Koliella helvetica</i> (Kol) Hind. 1963
<i>Koliella nivalis</i> (Kol) Hind. 1963
<i>Koliella viretii</i> (Kol) Hind. 1963
<i>Koliella tatrae</i> (Kol in Görffy) Hind. 1963
<i>Koliella transsylvanica</i> (Kol) Hind. 1963
<i>Mesotaenium berggrenii</i> (Wittr.) Lagerh. 1892
<i>Prasiola crispa</i> (Lightf.) Menegh. 1838
<i>Raphidonema antarcticum</i> Kol 1972
<i>Raphidonema brevirostre</i> Scherff 1910
<i>Raphidonema fragile</i> Kom. O. et Kom. J. 2001
<i>Raphidonema nivale</i> Lagerh. 1892
<i>Raphidonema sabaudum</i> Kol 1934
<i>Raphidonema sempervirens</i> Chodat 1913
<i>Smithsonimonas abbotii</i> Kol 1942
<i>Stichococcus bacillaris</i> Nág. 1849 s.l.

^aThe original and commonly used name *Chlainomonas "kolii"* is grammatically incorrect. The species was described to the honour of Prof. Erzsébet Kol, a famous female Hungarian phycologist. According to the botanical nomenclatural rules of formation of scientific latinized names after persons, the epithet (in genitive) must be created by the addition of ending *-ae* to the personal female names, it means in our case "*kolae*" not "*kolii*"; the ending "*-ii*" is genitive of the masculine form.

The majority of species from the chlamydophycean genera mentioned were recognised and originally described (in the form of zygospores or dormant stages) as genera and species of coccoid algae (for a review of the snow algal diversity that was recognised on this basis, see Kol, 1968). They occur in this form over a long part of the vegetation period in the snowfields. Sometimes they show metabolic activity in this stage, and may also reproduce by immotile aplanospores (Stibal, 2003). The interpretation of coccoid types reproducing by biflagellate or quadriflagellate zoospores combined with aplanospores and sexual process (e.g., *Cryocystis* Kol, 1968) is therefore quite understandable. According to modern knowledge, numerous algal species from this group with motile stages and causing colouration of snowfields have complicated life cycles involving green motile cells (considered as vegetative) and immotile spores or cysts with thick walls and large amounts of various secondary carotenoids and lipid reserves.

From this point of view, the wider molecular identification of genotype relations of all cryoestetic *Chlamydomonas* and *Chloromonas* types is urgent (first prospective results see in Hoham et al., 2002, 2006). Modern generic and specific classification is based mainly on the cytology of motile zoids. However, from a taxonomic point of view, numerous species still need revision, including the most common *Chlamydomonas nivalis* (e.g., identity of populations from polar and different mountain regions, or the problem of *Chlamydomonas antarctica* Fritsch, are still open questions).

The presence of periodically changing flagellate vegetative cells and immotile resting stages is a successful adaptation to the extreme environment of mountain or polar snowfields. The formation of resistant resting stages in snow algae from the order Chlamydomonadales is one of the major adaptations to their harsh habitat. It allows them to survive periods with sub-zero temperatures, or high soil temperatures and desiccation when ephemeral snowfields completely melt (Newton, 1982; Müller et al., 2001). The life history and ecology of many species, especially from the genus *Chloromonas* were studied in detail by Hoham (1975a), Hoham and Mullet (1977), and Hoham et al. (1979, 1983, 2006). However, the life cycles of *Chloromonas* and *Chlamydomonas* still need further studies.

Vertical migration in snowfields depends on the changing irradiance intensity on the surface of the field. Motility of flagellates in liquid phase is evident; however, the strategy of motility of the population has not yet been studied satisfactorily (cf. Hoham, 1974a, b). *Chloromonas tughillensis*, published originally by Hoham et al. (1998, 2000) as *Chloromonas* sp. – D, distributes itself optimally in snowfields for irradiance and spectral composition at the time of maximum mating in its life cycle, which also take place under longer photoperiods. Migration of populations in snow were documented for *Chloromonas pichinchae* when asexual stages with flagella were most prominent during periods of high water content in snow (Hoham, 1975a; Hoham and Duval, 2001).

(2) **Simple filamentous species without motile stages.** The second large group is represented mainly by the typical cryophilic *Raphidonema* and *Koliella* species (Trebouxiophyceae). In contrast to the chlamydomonads, snow algae from these genera are characterised by the absence of special resting stages and other adaptive features. The identification of species in this complex is particularly difficult due to a high level of pleiomorphism in relation to environmental factors, and the identification of various cryoestatic morphotypes must be revised (Hoham, 1973; Komárek and Komárek, 2001; Novis, 2002a; Stibal and Elster, 2005). It has even been suggested that *Raphidonema nivale* collected in Svalbard is rather a soil than a snow species brought only occasionally onto snow surfaces by katabatic winds (Stibal and Elster, 2005).

The generic separation of both the simple filamentous genera *Raphidonema* and *Koliella* is yet discussed. Numerous unicellular stages resembling different *Koliella* species occur in *Raphidonema* populations (especially in cultures). However, this does not mean that morphologically similar, typical *Koliella*-species, which never form more-celled filaments in nature or in culture, cannot exist. Several species of each genus occurring without any intermediate forms have been observed in maritime Antarctica at one and the same locality (Komárek and Komárek, 2001). Furthermore, these genera have shown different dependences on environmental factors, with distinct ecophysiological and biochemical markers. For example, *Koliella tatrae* isolated from the type-locality in the Western Carpathians is strictly unicellular (2-celled during division), and temperatures over about 10°C were shown to be lethal (Hindák and Komárek, 1968). By contrast, American strains of *Raphidonema nivale*, disintegrating and forming *Koliella*-like stages in culture, grow well up to 15°C without a decrease in growth rate (Hoham, 1975b). The higher temperature dependence of *Raphidonema* species was confirmed by Stibal and Elster (2005). However, Hoham et al. (2002) note, that some snow algae grown at relatively high temperature in culture can change their growth optima. It seems therefore, that the limits of lethal temperature are for various strains more characteristic than the optima, which change, for example, in dependence on combined temperature and light intensities (cf. Komárek and Růžička, 1969; Růžička, 1971). Therefore, the taxonomic classification can be solved only by help of molecular methods.

Further work is required also to thoroughly examine the simple life strategy and vertical migration of filamentous green algae lacking motile reproductive cells and sexual reproduction in snow and glacier habitats. The problematics of vertical migration of immotile species in the snowfields represents a special problematics, which is shortly mentioned, e.g., by Komárek et al. (1973), but not yet satisfactorily explained. The repeated income of diaspores by winds from environmental soil habitats is supposed and discussed, but not yet proved (Marshall and Chalmers, 1997).

3. Environmental Conditions

All algal assemblages in extreme environments are usually limited by one or by few ecological factors, influencing substantially and continually the habitat. Physiological adaptations of dominant species in such habitats are even more distinct, as the influencing factors are more extreme. Cryoestatic algae are influenced by environmental factors affecting their growth and development in interaction (Komárek and Růžička, 1969; Stibal and Elster, unpublished results; Fig. 3). Temperature, radiation and nutritional background of cryoestatic habitats play the most important role and it is the magnitude of these variables that characterise the snow habitat as extreme.

3.1. TEMPERATURE

Low temperature and frequent freeze-thaw cycles represent the main characteristics of the extreme snow habitat requiring specific adaptation of organisms. Ecophysiological experiments focused on testing the growth temperature optima of snow algae were carried out particularly by Hoham (1975b). In previous studies only temperature ranges where given species survived were evaluated, and growth optima were defined only rarely.

Cryoestatic species belong in principle to two different ecological groups (Hoham, 1975b; Stibal and Elster, 2005), which differ by dependence on temperature. The typical cryophilic types grow in the range from 0° to ± 10°C, which

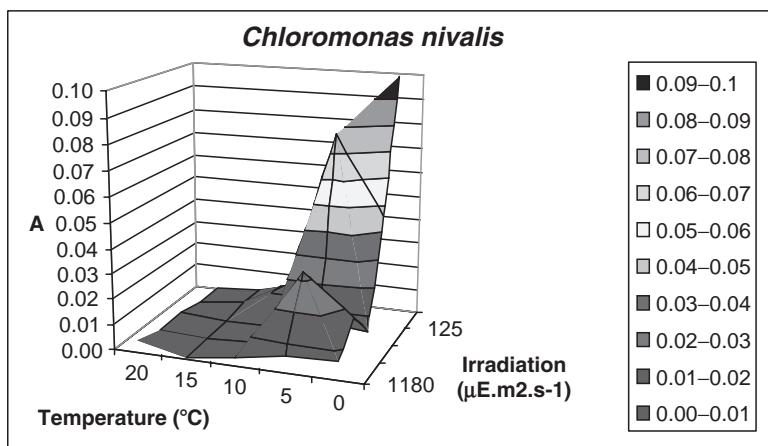


Figure 3. Combined dependence of vegetation of *Chloromonas nivalis* on temperature [°C] and irradiation [$\mu\text{mol m}^2 \text{s}^{-1}$]; (A = concentration of cells [$10^6 \text{ cells mL}^{-1}$]). – After Stibal and Elster (orig.).

usually represents the lethal temperature limits. Maximal growth *in vitro* of such types was found, for example, at 4°C (*K. tatrae* – Hindák and Komárek, 1968), 1–5°C (*Chloromonas pichinchae*, *Chlainomonas rubra*, *Chlainomonas kolae*, *Chlamydomonas nivalis* – Hoham, 1975b; *Chloromonas nivalis* – Stibal and Elster, 2005). Other species that belong to this group are *Chloromonas rubroleosa* (Ling and Seppelt, 1993), *Desmotetra aureospora* and *Desmotetra antarctica* (Ling, 2001). Among such cryophilic (psychrophilic) taxa belong species occurring almost exclusively in cryoeston and only rarely in other habitats closely connected with snow or glacier localities.

The second group of snow algae contains species with temperature optima over 10°C, which can be designated rather as cold-resistant (psychrotolerant) species, with highest metabolic activities between 10–20°C. Species occurring commonly in other habitats, such as *Cylindrocystis brebissonii*, *Stichococcus bacillaris*, *Prasiola crispa*, *Chlorella* spp., *Hormidiospora verucosa* and also several *Raphidonema*-species (e.g. *R. sempervirens*; Hoham, 1975b; Stibal and Elster, 2005) belong to this group (Fig. 4). Interestingly, some algae from other temperature-extreme habitats that do not appear in snowfields share similar temperature conditions during the vegetative season (e.g. corticolous *Desmococcus vulgaris* – O. Komárek, in litt.). However, several typical cryoestetic species, particularly from the genus *Raphidonema*, may belong also to this second group. It concerns mainly the species, the growth temperature optimum of which lies about or below 15°C, and whose upper surviving temperature is lower than 20–25°C (Elster, 1999).

Other algae found on the snow can also be psychrotolerant, that is, surviving in temperatures near 0°C. Their temperature optimum is, however, usually higher than 15°C. In contrast to typical snow species, their concentration is in most cases too low to cause snow colouration. For example, *Chlorella vulgaris*,

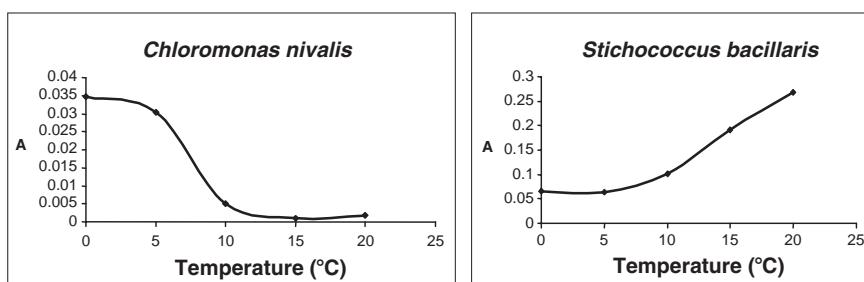


Figure 4. Growth of cryoestetic *Chloromonas nivalis* and soil species *Stichococcus bacillaris* (occurring occasionally in snow vegetation) in dependence on temperature in cultures, expressed by cell concentration in medium BG11 after 35 days of cultivation; (A = concentration of cells [10^6 cells mL^{-1}]). – After Stibal and Elster (orig.).

Xanthonema hormidioides and *Stichococcus bacillaris* are representatives of this group (Hoham, 1975b). Possibly the species of *Raphidonema* (*R. nivale*, *R. semipervirens*) belong also in this second group (Stibal, 2003; Stibal and Elster, 2005). Nevertheless, this classification is only arbitrary without any clear boundary between these groups of algae.

Remias et al. (2005) studied temperature and light-dependence of photosynthesis of the widely distributed red snow alga *Chlamydomonas nivalis* from the high Alps in Austria in laboratory experiments. This alga is generally considered to be a typical psychrophilic species (Hoham, 1975b; Kawecka and Drake, 1978). Although both photosynthetic and respiratory data showed cryophilic adaptation, no inhibition was observed at temperatures up to 20°C. High photosynthetic rates of *Chlamydomonas nivalis* from Oregon (USA) at temperatures around 20°C were also reported by Sutton (1972). The reason for these contradictory results could be different ecophysiological characteristics of flagellates and immotile cysts. In addition, there is growing evidence that the red resting cells found worldwide and ascribed to *Chlamydomonas nivalis*, can belong to different species and even to various genera (Ling and Seppelt, 1993; Ling, 1996, 2001, 2002; Hoham et al., 2002).

The adaptations of snow algae to extremes in temperature including episodic freezing were summarised by Hoham and Duval (2001). The fluidity of membranes at low temperatures can be maintained by alterations in fatty acid composition. A high proportion of unsaturated fatty acids was observed in red cells of *Chlamydomonas nivalis* collected at Hermit Island (Antarctica) by Bidigare et al. (1993). Unusual short and medium chain polyunsaturated fatty acids potentially enhancing membrane fluidity were recently isolated from the flagellated cells of the snow alga *Chloromonas brevispina* collected in the Bohemian Forest (Czech republic; Řezanka et al., in press). Other adaptations include an overall high lipid content (Margesin and Schinner, 1994), and accumulation of carbohydrates and polyols in cells (Tearle, 1987; Roser et al., 1992). In addition, a complex role in affording snow algae protection against harsh conditions is apparently fulfilled by the synthesis of secondary carotenoid astaxanthin (Bidigare et al., 1993).

3.2. LIGHT AND PHOTOSYNTHESIS

Light is not only a necessary source of energy for autotrophic organisms, but its intensity, spectral composition and photoperiodicity influence life cycles of snow algae (Hoham et al., 1998; Hoham et al., 2000; Hoham and Duval, 2001). The spectral absorption of solar radiation in snow is approximated by an exponential function, but it is strongly influenced by the water content and density of the snow, and by internal reflections within the snow. The absorption coefficient decreases with increasing snow density, so 1% of surface irradiance can reach more than 100 cm in wet summer snow (Curl et al., 1972). The light conditions in

the snow are also influenced by snow reflectance, which is highest in fresh snow (Bolsenga, 1983). Dirty snow or patches of snow algae significantly affect light transmission by decreasing snow albedo. Blue and green light have been shown to penetrate the deepest in snow (e.g. Hoham et al., 1983). An example of light penetration into a summer snowfield in the Western Carpathians during sunny and cloudy days (as influencing the vertical migration of *Koliella tatrae* in a snowfield) is shown in Figure 5 (Komárek et al., 1973).

Due to light scattering within the snow, the number of photons reaching the algal cell (photon fluence rate) is increased in comparison with incident photon irradiance measurements. The light environment of the red snow alga *Chlamydomonas nivalis* growing in a persistent alpine snowfield was described in detail by Gorton et al. (2001). The surface irradiances were commonly well above 2,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with corresponding photon flow rates up to 6,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The photon flow rates beneath the snow surface were up to about five times greater than the incident photon irradiance.

In some snow algae, the accumulation of the secondary carotenoid astaxanthin at high irradiance plays a central role in protecting cells from UV-damage and potential photoinhibition (Bidigare et al., 1993). Gorton et al. (2001) examined the spectral characteristics of individual red aplanospores of *Chlamydomonas nivalis*.

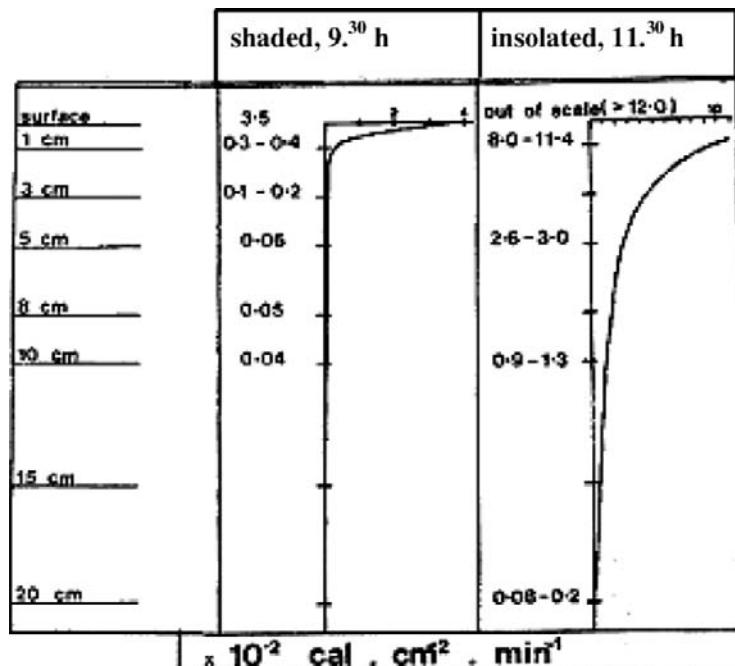


Figure 5. Absorption of the solar radiation (PAR) in a snowfield in the Western Carpathians (High Tatra Mountains) in September 17th 1965 under shaded and insolated sky – from Komárek et al. (1973).

The layer of astaxanthin efficiently blocked blue light, and only a small percentage reached the chloroplast in cells of average diameter. The accumulation of astaxanthin thus represents an effective means to survive the extremely high irradiances found in alpine snowfields.

The pioneering work on measuring primary production of snow algae *in situ* using various methods based on $^{14}\text{CO}_2$ ($^{14}\text{CO}_2$ incubation or gas chambers) was done by Fogg (1967) on the South Orkney Islands, Thomas (1972) in the Sierra Nevada (USA) and Javornický (1973) and Komárek et al. (1973) in the Tatra Mountains (Slovak Republic). Recently, Williams et al. (2003) investigated rates of CO_2 uptake in snow colonised by *Chlamydomonas nivalis* in the Rocky Mountains (USA). The light curve determined under field conditions was similar to that found in leaves of higher plants, and no photoinhibition was observed even at maximum PAR irradiance ($\sim 1,800 \mu\text{mol m}^{-2} \text{s}^{-1}$). A good correlation between gas exchange rates and algal densities was found. In heavily colonised red snow patches, the integrated CO_2 uptake reached around $2,300 \mu\text{mol m}^{-2} \text{day}^{-1}$, which represents about 10% of area-specific production of many higher plants. The combination of gas exchange measurements and remote quantification of snow algal concentrations with an airborne imaging spectrometer (Painter et al., 2001) indicates that under favourable circumstances summer snowfields can represent a significant CO_2 sink.

The mechanisms of adaptation of algae to high irradiance are well known in algae (Falkowski and La Roche, 1991; MacIntyre et al., 2002). In temperate mountains, the photon fluence rates reach up to $6,000 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Williams et al., 2003). The reaction of higher plants and lichens to the light conditions of the mountain environment was studied by Heber et al. (2000), but detailed eco-physiological studies focused on the cryoestatic algae have not yet been performed.

3.3. UV RADIATION

UV radiation affects biological systems mainly at the nucleotide and protein level. Damages to DNA include hydroxylation of cytosine, formation of cytosine-thymine bonds, linkage of DNA with proteins instead DNA, creation of photoproducts between adjacent bases and denaturation of DNA. (Vincent and Roy, 1993). Increased exposure to UV-B radiation results in limitation of protein synthesis, decline in protein content, and decrease in rates of carbon and nitrogen metabolism (Döhler, 1988, 1994). These effects can be caused by decline in supply of ATP and carbon skeletons for amino acid synthesis, damage of synthesis and activity of key enzymes in metabolism, variability in arrangement of amino acids, lipids and fatty acids, as well as inhibition of the regulatory mechanisms (Döhler, 1994). UV-B radiation also increases non-photochemical quenching and the effective quantum yield of photosynthesis is disturbed (Bischof et al., 2002). Organisms living in areas with high UV radiation, such as mountain or polar ecosystems, have developed protective mechanisms. Screening of the photosyn-

thetic apparatus sensitivity to UV-B radiation in algae representing largely different environments proved that a high fraction of UV-B resistant species was found among algae isolated from mountain sites (Xiong et al., 1996).

Snow algae are sometimes exposed to extremely high levels of UV radiation, due to rapid increase in the amount of UV radiation with altitude (Blumthaler et al., 1992). This effect is wavelength-dependent, and is therefore more pronounced for the UV-B (280–315 nm) than for the UV-A (315–400 nm) range (Blumthaler et al., 1994).

The UV-environment of *Chlamydomonas nivalis* in granular summer snow was studied in the Rocky Mountains (USA) by Gorton and Vogelman (2003). The UV radiation dropped to 50% of incident levels in the top 2 cm, with UV-B penetrating deeper than UV-A. No UV radiation could be detected below the depth of 8 cm. Gorton and Vogelman (2003) also measured UV absorbance of individual cells of *Chlamydomonas nivalis* collected at an altitude of 3,700 m above sea level in order to determine the localisation of UV-screening compounds. Most of the screening was provided by astaxanthin and its esters, concentrated in cytoplasmic lipid droplets. Isolated cell walls exhibited only a weak UV absorbance.

The UV light protective function in snow algae is thus mainly fulfilled by the extrachloroplastic carotenoid astaxanthin. The esterification of astaxanthin is considered to be a mechanism allowing efficient pigment concentration in cytoplasm (Bidigare et al., 1993). The protective role of astaxanthin in snow algal cells is apparently complex, because this secondary carotenoid is also an effective antioxidant (Tinkler et al., 1994). The water content in cells is reduced when astaxanthin concentration is high, reducing the likelihood of ice crystal formation (Hoham, 1992).

Some UV-resistant algae contain water-soluble mycosporine-like amino acids (MAAs), characterised by absorption maxima ranging from 310 to 360 nm (e.g. Karsten et al., 1998). In snow algae, the presence of MAAs has not yet been documented. Sommaruga and Garcia-Pichel (1999) did not detect any MAAs in cysts of the red snow alga *Chlamydomonas nivalis* growing on the winter cover of a high mountain lake, in contrast to planktic and epilithic cyanobacteria and algae from the same locality.

Phytophenolic compounds function as antioxidants acting as scavengers of singlet oxygen and free radicals. In contrast to higher plants, few studies have investigated the role of these compounds in algae (Foti et al., 1994). Duval et al. (1999b) studied UV light-induced changes in the total phenolic content, free proline and associated antioxidant protection factor in *Chlamydomonas nivalis* aplanospores from the Sierra Nevada, California, USA. Exposure of cells to UV-A and especially to UV-C radiation resulted in an increase in total phenolic compounds. Free proline content was not affected by UV-A, but increased markedly after UV-C exposure. Remias et al. (2005) reported an increased content of α -tocopherol (vitamin E) in young cells of *Chlamydomonas nivalis* from the Austrian Alps. These cells were characterised by lower astaxanthin accumulation

in contrast to older stages, suggesting possible shifts in the type of protection during the life cycle. The stimulation of phenolic and other antioxidant production in snow algal cells is probably an effective mechanism of their adaptation to UV-irradiation stress.

The complete and precise identification of UV-absorbing compounds in snow algal cells requires further study, encompassing a broader range of samples and species. These studies may have the additional benefit of discovering new biologically active compounds useful for biotechnological and pharmaceutical applications. Moreover, repair processes following possible UV-induced DNA and protein damage have not yet been studied in snow algae.

3.4. NUTRIENTS

Nutrient cycling in snow was reviewed by Jones (1999) and Kuhn (2001). The amount of nutrients in snow encompasses a rather broad range, and its spatial distribution is often markedly heterogeneous (Tranter et al., 1987). The reported concentration ranges of main nutrients in snow associated with algal blooms worldwide are 0–5400 µg L⁻¹ NH₄-N (Komárek et al., 1973; Müller et al., 1998; Novis, 2002b), 0–7100 µg L⁻¹ NO₃-N (Komárek et al., 1973; Hoham and Mullet, 1977; Novis, 2002b), and 0–600 µg L⁻¹ dissolved reactive phosphorus (Ohtani et al., 1998; Novis, 2002b). However, the amount of nutrients in interstitial water spaces are greater than in bulk snow, due to the high efficiency of meltwater leaching in initial fractions (Johannessen and Henriksen, 1978). There are various sources of nutrients in snow: precipitation, weathering of rocks, wind-driven deposition of particles (e.g. dust, pollen, organic debris) and animals (Jones, 1991). In the polar regions, the proximity of bird colonies may increase nutrient concentrations in snow, and the association of snow algal blooms with seabird and penguin rookeries has been repeatedly reported both from the Arctic and Antarctica (Müller et al., 1998; Komárek and Komárek, 2001). In the maritime Antarctica (King George Island), the distribution of cryoestatic communities is strongly dependent on the degree of nutrient enrichment. Oligotrophic snowfields are dominated by *Chlamydomonas nivalis*-like populations appearing as typical red aplanospores or by simple filamentous green algae from the genera *Koliella* and *Raphidoneema*. In contrast, snowfields under the heavy pressure of penguin rookeries are characterised by high content of phosphorus and a particularly intense development of green cryophilic flagellates (*Chloromonas*, *Chlamydomonas*) and *Chlorosphaera antarctica*, accompanied by the cyanobacterium *Romeria nivicola* (Fig. 1; Komárek and Komárek, 1999, 2001).

The concentration of nutrients in melting snow can also be significantly increased due to leaching of coniferous litter or other types of detritus (Komárek et al., 1973; Jones, 1987), which results in a better nutrient availability in forested areas in comparison with open exposures (Hoham, 1976). In the Tatra Mountains (Slovak Republic), the concentrations of nutrients in the

surface layer of a snowfield with a bloom of *Koliella tatrae* were comparable with eutrophic waters (Komárek et al., 1973; see earlier). Hoham (1976) reported growth stimulation in the snow alga *Chloromonas pichinchae* with increasing concentration of leaf litter and bark extracts both in laboratory and field experiments, and that *Ch. pichinchae* required a vitamin for growth, whereas *Raphidonema nivale* did not. The reaction of *R. nivale* was quite different, and an inhibition of growth by higher extract concentration was observed. This pattern corresponded to habitat preferences of these species. Besides irradiance level, concentration of nutrients seems to be one of the most important factors determining the distribution of particular species, for example, in the altitudinal gradient with respect to the position of timber line.

The growth of snow algae may result in nutrient depletions. Decreases in NO_3^- -N, NH_4^+ -N and SO_4^{2-} were observed in snow containing dense vegetative populations in contrast to surrounding snow without algae (Hoham et al., 1989; Jones, 1991). The decrease in nutrients in direct correlation with the growth of the snow alga *Chlainomonas kolae* was reported by Novis (2002b) in New Zealand. Nutrient depletions may trigger shifts in cell type dominance (Hoham et al., 1989; Novis, 2002b), indicating their role in the control of life cycles. However, an inverse pattern with lower nutrient level of unpopulated snow was characteristic for snowfields in Svalbard, which was explained by preferential colonisation of sites receiving more wind-blown material (Newton, 1982; Müller et al., 1998).

4. Periodicity, Geographic Distribution

Due to the remoteness of localities of snow algae, there are still few data on the detailed seasonal development of particular species and factors influencing their life cycles. Seasonal changes are visible during the short summer seasons (ephemeral snowfields in mountains, polar summer periods) usually mainly in intensity of colouration of snow, the qualitative changes in species composition were recognised only rarely (cf. Komárek and Komárek, 2001). A correlation between liquid water content in snow and various life cycle stages of *Chloromonas pichinchae* has been observed (Hoham, 1975b). Novis (2002b) studied the ecology of the rarely reported snow alga *Chlainomonas kolae* in New Zealand, which was previously known from the Pacific Northwest of USA (Hoham, 1974a). The growth of *C. kolae* populations occurred during major rainstorms increasing the liquid water content, and the shifts in life cycle stage were associated with decreases in nutrient concentrations as reported previously by Hoham et al. (1989). However, our knowledge of factors controlling life cycles of snow algae including cleavage of resting stages still remains fragmentary.

The geographic distribution of snow algae is still not well known. The dominant cryoestatic species are usually considered to be cosmopolitan. Furthermore, resting stages can represent an airborne inoculum, because spreading by wind is

considered to be a probable main mechanism of snow algae distribution (Marshall and Chalmers, 1997; Duval et al., 1999a). However, the genotype identity of similar species has not been studied carefully yet. Several species are only known from delimited areas. Impressive communities of snow algae with wide diversity of species develop over the summer season in coastal areas of polar regions (e.g., in maritime Antarctica), and diversity of snow algae in maritime Antarctica seems to be much richer than that from isolated high mountain American and European locations. However, numerous high mountain areas still exist, from where the knowledge of diversity of snow algae is very poor (South America, Kamchatka, many Central Asian mountain ridges, etc.). Thorough combined investigations (phenotypical, genotypical, ecophysiological) are desirable to provide a more realistic view of worldwide snow algal diversity and distribution.

In contrast to polar regions, snow algae in mountain regions of lower geographical latitudes do not grow under permanent solar irradiation during the proliferation period. It is not well known yet, how this periodicity influences the vegetation of snow algae. The snow habitats in mountains include also a broad spectrum of microscopic algae in relation to altitude and are characterised by differences in duration of snow cover, irradiance level, nutrient concentrations, etc. The snow cover in forests is exposed to lower irradiances and higher nutrient load in comparison with open exposures (Jones, 1991). The taxonomic composition, biology and ecology of snow algae in forested sites were extensively studied in North America, where at least six special species from the genus *Chloromonas* occupy this habitat (Hoham, 1975a; Hoham and Mullet, 1977; Hoham and Blinn, 1979; Hoham et al., 1983; Duval and Hoham, 2000; Hoham et al., 2006). In other continents, including Europe, reports on snow algae at forested sites are much scarcer when compared to alpine localities. Most probably, the green to orange colouration of snow caused frequently by species preferring shaded localities is generally noticed less frequently than the striking red ones. Some of the same forest species as in America were recorded in the mountains of Japan (Fukushima, 1963) and in the Giant Mountains and Bohemian Forest in the Czech Republic (Kociánová et al., 1989; Lukavský, 1993). Despite the obvious influence of altitude on snow algal distribution, no clear line can be delimited between forest and open exposures species, because of species tolerating a wide range of environmental factors (e.g. *Chloromonas nivalis*); (Hoham and Blinn, 1979; Novis, 2002b).

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PSYCHROPHILIC DIATOMS: *Mechanisms for Survival in Freeze–Thaw Cycles*

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1. Diatoms and Their Sea-Ice Habitat

Diatoms are unicellular microalgae that contribute to 20% of the global carbon fixation. This is as much as the carbon fixed by all tropical rainforests combined (Armbrust et al., 2004). Diatoms are found all over the globe, in freshwater and seawater, in hot and cold habitats. Their most distinctive feature is a silicified cell wall (termed frustule) made of hydrated amorphous silica and a small amount of organic material (sugar). The architecture of the frustule is based on silica patterns that are structured on a nano-to-micrometer scale. These nano-patterns can vary from species to species, creating unique morphotypes that are used as taxonomic keys (Fig. 1).

Psychrophilic or cold-loving diatoms are one of the most abundant groups of phytoplankton in polar oceans. This is mainly due to the presence of higher silicate concentrations in these regions and also due to their successful adaptation to the polar environment (Boyd, 2002) which is characterized by strong seasonality in solar irradiance, freezing temperatures, and extremes of salinity (Cota, 1985; Fiala and Oriol, 1990; Mock and Valentin, 2004; Ryan et al., 2004; Ralph et al., 2005). Polar diatoms are responsible for a large fraction of polar primary productivity and serve as the base for the entire polar food web, ultimately feeding krill, fish, whales, penguins, and seabirds. Due to the presence of glaciers and permafrost, photosynthetic biomass on land is negligible compared with that found in the ocean. Consequently, polar diatoms are of great interest not only because of their important role as the main food source for the whole polar food web (terrestrial and aquatic), but also because of their remarkable ability to thrive in this extreme ecosystem (Thomas and Dieckmann, 2002). To fully understand the ecology and adaptation mechanisms of these algae, one needs to understand the physics and chemistry of their main habitat, sea ice.

Sea ice being one of the largest and most extreme habitats in polar oceans is important in structuring the whole polar ecosystem (Eicken, 1992). At its maximum it covers 13% of the Earth surface. The largest expanse occurs in the Southern ocean where, during winter, 20 million square kilometers are covered by ice. In contrast to freshwater ice, sea ice is not solid but is comprised of a system of brine channels that provide a habitat characterized by low temperature (ca. -2 to -20°C),

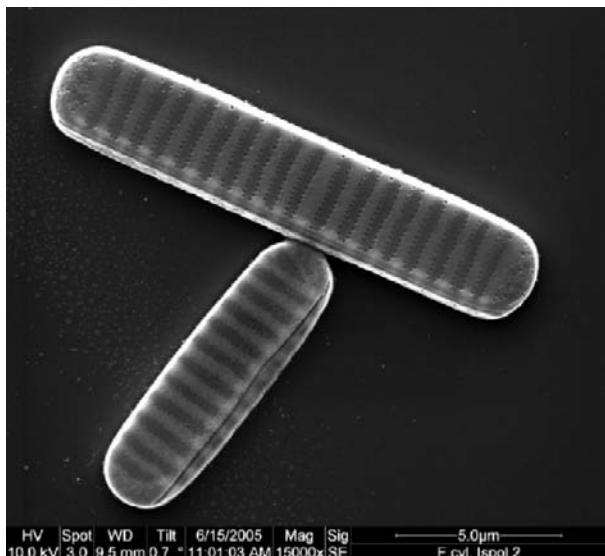


Figure 1. SEM picture of the psychrophilic pennate diatom *F. cylindrus* (Bacillariophyceae). Valve view of two cells. SEM is taken by Henrik Lange and Gerhard Dieckmann.

high salinity (35–200 ppt), high pH (up to 11), and low irradiance (below 1 μmol photons $\text{m}^{-2}\text{s}^{-1}$) (Fig. 2).

Seawater typically contains about 34 g of dissolved salts and ions (mostly sodium, chloride, sulfate, magnesium, calcium, and potassium) and does not freeze until temperatures drop below -1.86°C (28.65F). At this temperature, ice crystals begin to form and rise to the surface. These initial ice crystals (called frazil ice) vary in shape, from plates to needles, and size, from less than or equal to millimeter to centimeter in length.

Within hours, frazil ice crystals consolidate by wind and water motion to form loosely aggregated discs (called pancakes). After a few days of growth by accumulation of more and more ice crystals that form in the upper water column, pancakes can be several meters across and up to 50-cm thick. They freeze together forming a closed ice cover after 1–2 days (termed pack ice). As temperatures continue to decrease this pack ice thickens, not necessarily by the accumulation of more ice crystals, but by the growth of columnar ice at the ice–water interface. Columnar ice forms by the vertical elongation of frazil ice crystals. The proportion of frazil ice to columnar ice depends largely on the turbulence of the water in which it was formed. The more turbulent the water the more frazil ice is usually found. Antarctic sea ice contains up to 80% frazil ice as it is formed under more turbulent conditions. In the Arctic, sea ice is formed under more calm conditions containing up to 80% columnar ice. This difference is important for sea-ice biology because frazil ice provides more habitable space for organisms than columnar ice (Spindler, 1990).

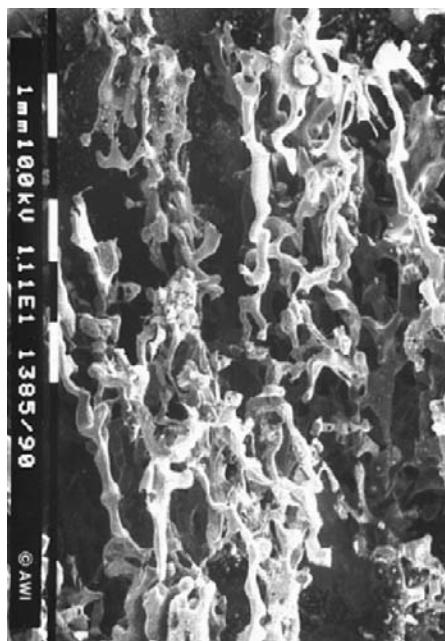


Figure 2. SEM picture of the brine channels system in columnar sea ice made visible by filling the system with epoxy resin under a vacuum. Picture by Alfred-Wegener Institute for Polar and Marine Research, Bremerhaven, Germany.

When ice is formed from seawater, salt ions and air in the water are not incorporated into the ice crystals and are concentrated as salty brine that persists as inclusions of pockets and channels within the ice or is released into the water below (Eicken, 1992). Thus, sea ice is a solid matrix penetrated by a labyrinth of channels and pores that contain highly concentrated brine and air bubbles. Brine channels vary in size from a few micrometers through several millimeters in diameter and are the main habitat for all microorganisms in sea ice (Fig. 3).

Channel volume and the concentration of salt in them are directly proportional to temperature. When temperatures decrease, brine volume decreases and salt content increases. Thus, the coldest ice contains brine channels with highly salty brines and overall fewer, smaller and less interconnected channels than warmer ice. Since ice at the sea-ice air interface is usually colder than ice in contact with the underlying water, a vertical temperature gradient exists through the ice, resulting in a gradient in brine salinity and brine volume as well (Fig. 4).

Organisms such as diatoms that live in sea ice are adapted to cope with these ever-changing physical and chemical conditions of their environment.

Diatoms are mainly introduced into the ice as it is forming. They get caught between ice crystals or simply stick to them as crystals rise through the water when it freezes in fall. During the formation of consolidated ice, diatoms can become

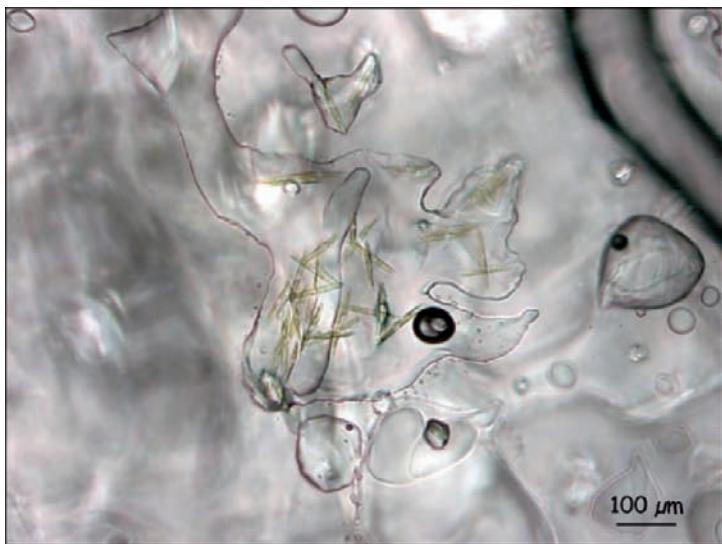


Figure 3. In situ micrograph of a brine pocket that is filled with pennate diatoms.

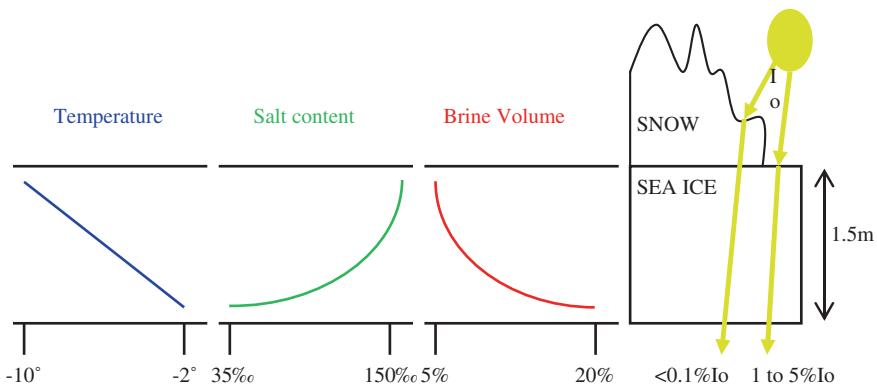


Figure 4. Vertical gradients of temperature, salt content, brine volume, and irradiance through sea ice. These general patterns may vary due to changes in temperature.

trapped within brine channels. Pennate diatoms are the most conspicuous organisms in sea ice along with other microalgae (e.g., flagellates), heterotrophic protists (e.g., ciliates), and bacteria (Thomas and Dieckmann, 2002). These micrometer-sized algae with their main light harvesting pigment being fucoxanthin can reach such concentrations in sea ice that they discolor the ice visibly brown (Fig. 5).

The time for acclimation to the new conditions in sea ice is not very long since day light hours are continually decreasing as winter approaches. Nevertheless, diatoms at the ice–water interface, where conditions are most similar to the water below the ice, are often able to adapt fast and can accumulate

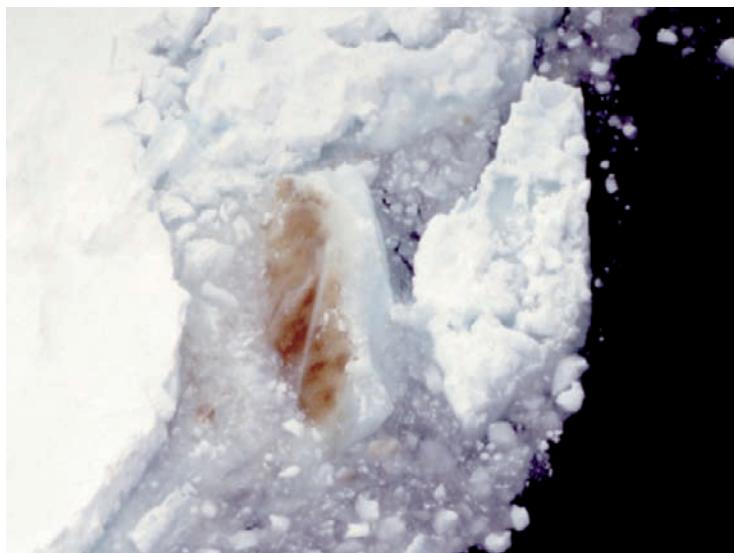


Figure 5. Ice floe (upside down, about 50-cm thick) with dense populations of diatoms at the sea-ice water interface (indicated by brown color that is caused by their main light-harvesting pigment fucoxanthin).

to high biomass even before the dark winter begins. Sea-ice diatoms are very efficient in using solar irradiance and are able to grow at irradiance levels below 1 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Light levels are minimal during polar winters not only due to short days or complete darkness but also due to snow coverage on top of the ice that is a very efficient reflector of solar irradiance.

Sea ice is mostly an ephemeral feature since after its formation and consolidation the majority of it melts again during the next summer resulting in the release of all organisms within to the underlying water. However, Arctic sea ice shows a dramatic decrease in thickness and extent due to increasing overall temperatures in Arctic regions caused by global change (Stoeve et al., 2005). Increase in solar irradiance is the most important factor that causes the ice to melt. A common feature and an indicator for the beginning of the ice melt is the formation of melt ponds on the surface of the ice. These melt ponds then can develop their own unique ice-algae assemblages and microbial communities (Brinkmeyer et al., 2004). In general, melt ponds are more common in the Arctic than Antarctic. When melting continues due to increasing water temperatures and solar irradiance on top of the ice, the ice gets thinner and more porous. Large pores and brine channels filled with seawater characterize warm ice and the ice itself has very little strength and is easily broken up. However, not all the ice that is formed in fall actually melts during next summer. If it survives the summer, refreezing occurs during the following winter that makes the ice even thicker. The longevity of the ice depends on the geographic location, on the wind, and ocean currents. Sea ice of northern Greenland and the Canadian archipelago can be several years

old with an average thickness of 6–8 m. The ice in the Southern Ocean is considerably thinner with an average thickness of only 1 m. Such differences in physical properties of the ice also result in differences in the abundance, activity, and composition of the microbial communities within.

In this chapter, we provide a comprehensive summary on what is known about how diatoms cope with the seasonal freeze–thaw cycles in their environment. To learn more about the ecology and physiology of polar algae in general, we would like to refer the reader to comprehensive reviews on this topic (Kirst and Wiencke, 1995; Thomas, 2004). In the following sections, we aim to highlight and provide a context for the few recent studies that have begun to reveal the hitherto unknown physiology and molecular adaptations that enable freeze–thaw survival in polar diatoms.

2. Physiological Adaptations: Photosynthesis, Osmoregulation, and Freeze Resistance

Psychrophilic diatoms have to cope not only with the strong seasonality of light in polar regions, but also with the resulting changes in temperature and sea-ice formation. Light limitation can occur even during the summer months when 24 h of sunlight are available. This can be caused either by the higher mixing depth of polar seawater or by thick sea ice that persists throughout the summer. Furthermore, sea ice covered with snow is a very effective barrier to irradiance so that diatoms within have to cope with low irradiance to thrive under these conditions. Thus, one would expect to find that polar marine diatoms are more adapted to lower light intensities compared with their temperate counterparts. Early studies of algae obtained from the sea-ice water interphase – the darkest place in sea ice – described this flora as shade-adapted. Indeed, it is found that most polar diatoms can actively photosynthesize at irradiance intensities as low as 0.01% of incoming solar irradiance corresponding to irradiance levels at the bottom of snow-covered sea ice (e.g., below 0.5 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$; Mock and Gradinger, 1999). However, a recent study of short-term effects of temperature on the photokinetics of sea-ice algae from the surface layers of Antarctic sea ice during the spring–summer transition (Ralph et al., 2005) showed that some sea-ice diatoms are also able to tolerate irradiance levels as high as 350 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ for several hours (Ralph et al., 2005) when they get trapped from inflowing seawater into the upper parts of the ice during melting. At temperatures around the freezing point microalgae used nonphotochemical quenching (NPQ) to prevent damage to their photosystems (Ralph et al., 2005) and showed no obvious photosynthetic damage or limitation. However, when temperatures dropped below -10°C the algae only tolerated irradiance levels below 50 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$. Thus both light and temperature appear to be interacting factors that determine photosynthetic activity in ice diatoms.

Effective harvesting of photons under very low photon flux densities is regulated in diatoms by a dense package of pigments, mainly fucoxanthin, chlorophyll *a* and *c* (Falkowski, 1980; Boczar and Palmisano, 1990). Most of the cellular carbon under such conditions is actually stored in these pigments and their associated proteins (termed **fucoxanthin chlorophyll *a*-**, *c*-binding proteins: **fcps**) resulting in light-saturated growth below $20 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ (Cota, 1985). Fucoxanthin is the main light-harvesting pigment particularly under conditions of extremely low irradiance. This pigment is known to harvest wavelengths of the green-blue spectrum that can penetrate sea ice and deep water in the open ocean. Fucoxanthin is physically connected with chlorophyll *a* and *c* by fcps into higher-order complexes such as dimers and trimers (Buechel, 2003). To know the abundance of different genes such as fcps in the genome as well as the different molecular organization of such pigment protein complexes would greatly improve our understanding of acclimation or adaptation to different irradiance levels in psychrophilic diatoms. Adaptation per se is related to evolutionary changes in gene composition (e.g., gene duplications or deletions) and/or regulatory components of gene expression and translation. Genome-wide investigations of gene families (e.g., fcps) or regulatory genes (e.g., transcription factors) would be an appropriate method to address in-depth questions of molecular light adaptation in ice diatoms.

In diatoms (as in all plants) effective light harvesting is accompanied by efficient electron transport to the primary reductants such as carbon dioxide, nitrate, and sulfate. The fluidity of the thylakoid membranes is crucial for this electron transport because the correct folding and mounting of multi-subunit membrane complexes (e.g., photosystem II, cytochrome-b-6f-complex) as well as the mobility of the primary electron acceptors such as plastoquinone (A and B) relies on the fluidity and lipid composition of the thylakoid membrane. The main lipid classes of the thylakoid membrane of diatoms are galactolipids (MGDGs and DGDGs). Only recently the fatty-acid composition of these lipid classes has been investigated for polar diatoms (Mock and Kroon, 2002a, b). It was shown that both lipid classes contained high concentrations of certain polyunsaturated fatty acids (termed PUFA; 16:4, 20:5 and 22:5) that also increased under lower irradiance levels (Mock and Kroon, 2002a). These high levels of unsaturated fatty acids in both lipid classes may aid in assembly of the D1 reaction center protein in photosystem II and the correct folding of light-harvesting complexes as known from higher plants. It has also been shown that the psychrophilic green alga *Chlamydomonas raudensis* (which is more closely related to higher plants than to diatoms) exhibited a significantly higher unsaturated fatty acyl bond index in comparison with the mesophile *Chlamydomonas reinhardtii*. All chloroplast galactolipids of *C. raudensis*, which made up more than 75% of the total lipid content, contained high levels of PUFA (Morgan-Kiss et al., 2002). These results indicate a general importance of these lipids for maintenance of membrane fluidity and therefore photosynthesis functioning at low temperature in psychrophilic algae.

Carbon acquisition in psychrophilic diatoms has received relatively little attention despite the fact that dissolved carbon dioxide and oxygen concentrations in sea ice can differ significantly from polar seawater (Michel and Beardall, 1996). In the absence of biological processes, concentrations of dissolved carbon dioxide and oxygen present in air-saturated waters at the freezing point and salinity of seawater (-1.8°C , 34ppt) would be 1.5 times greater than those in the same water at 15°C . Concentrations of dissolved carbon dioxide and oxygen, however, are also influenced by photosynthetically active algae. In semi-enclosed or closed systems such as brine pockets dissolved carbon dioxide can become rapidly exhausted due to carbon acquisition by ice algae (Gleitz et al., 1995). The ability to use bicarbonate and to accumulate dissolved inorganic carbon (DIC) and/or store carbon in organic acids as proposed for the temperate diatom *Thalassiosira weissflogii* (Reinfelder et al., 2004) is likely to be as – if not more – important in ice diatoms to cope with likely carbon limitation situations.

The following two paragraphs describe what is currently known about freeze–thaw survival; the physiology of diatoms when entrained into sea ice in fall, persisting in the ice during winter and being released to the water after it melts in summer. It is experimentally very challenging to study the physiological processes of diatoms that are incorporated into newly formed sea ice at *in situ* conditions. Nevertheless, recent experiments using gene arrays of the polar diatom *Fragilariaopsis cylindrus* after a temperature shift from $+5$ to -1.8°C indicated that the Calvin cycle enzymes glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and ribulose-1,5-bisphosphate-carboxylase/oxygenase (RUBISCO) were strongly down-regulated after the shift (Mock and Valentin, 2004). However, inferences cannot be easily made as polar diatoms experience a different temperature shift (from -1.8 to ca. -5°C) when they get enclosed into newly formed sea ice, also accompanied by an increase in salt concentration. Nevertheless, based on these gene array results, it seems likely that carbon dioxide fixation and growth is reduced during the transition phase from seawater to sea ice during fall. When irradiance levels decrease below critical values for net photosynthesis to occur, diatoms stop their growth and enter diapause for the winter months (Antia, 1976; Doucette and Fryxell, 1983). Whether they take up dissolved organic matter to keep metabolic rates active at minimum levels as known from temperate diatoms is still under debate (Horner and Alexander, 1972; Palmisano and Garrison, 1993). Even so, it has been shown that heterotrophic growth appears to play only a minor role over wintertime with the heterotrophic capacity of psychrophilic diatoms estimated not to be higher than 0.3% of photosynthesis (Palmisano and Garrison, 1993).

Light conditions improve greatly and relatively quickly during spring and summer so that diatoms can build up to huge biomass in sea ice and seawater in a short amount of time. Sea ice can even turn brown at the ice–water interface or in internal layers where enough space and nutrients are available for growth. If enough light penetrates through these layers of algae, increased oxygen concentrations may stimulate photorespiration and production of radical oxygen species (Schriek, 2000; Morgan-Kiss et al., 2006). Even though irradiance levels increase during

spring and summer, the temperatures remain relatively low in sea ice as long as the ice is present (and not melting). This imbalance between constant low temperature and increasing irradiance may cause an imbalance between energy absorbed versus energy utilized (Morgan-Kiss et al., 2006). Psychrophilic diatoms are growing below their optimum temperature for growth (ranging between 7 and 10°C, Fiala and Oriol, 1990) in sea ice. Thus, under typical sea-ice conditions, enzymes are less active, operating below their optimal capacity, resulting in reduced growth. Furthermore, under these conditions only relatively small amounts of irradiance are needed to grow at maximum capacity (but still far below optimum). When photon flux densities are higher than that needed, dissipation of energy is necessary to avoid damage of the cell. This can already occur under relatively low irradiance levels if growth is strongly limited by very low temperatures (Tilzer et al., 1986; Robinson et al., 1997; Ralph et al., 2005). One mechanism to reduce the total input of excitation energy is the reduction of light-harvesting antenna size and/or the effective absorption cross-section area of photosystem II (Dubinsky et al., 1986; Falkowski and Chen, 2003). Another mechanism is dissipation of excitation energy by increasing concentration of carotenoids as shown for ice algae (Robinson et al., 1997; Mock and Valentin, 2004). Thus, a reduction in temperature can mimic an increase in irradiance and therefore acclimation to high irradiance even though the irradiance level does not change (Maxwell et al., 1994). The molecular basis of this phenomenon has only recently been elucidated (Mock and Valentin, 2004; see Section 3). The formation of reactive oxygen species (ROS) caused by excessive light is reduced by the production of antioxidant enzymes (e.g., catalase) and by photorespiration (Schriek, 2000). The Antarctic diatom *Entemoneis kufferatii* shows high catalase activity at temperatures below 0°C and high photon flux densities (Schriek, 2000) indicating a cold-adapted and high-light-insensitive enzyme. It is also known that these diatoms exhibit relatively high rates of nitrate uptake. A study with the temperate diatom *Thalassiosira pseudonana* under low temperature stress indicated that the reduction of nitrate was used as an electron sink under high excitation pressure (Parker and Armbrust, 2005). However, when nitrate levels were depleted, the photorespiration pathway became the major sink for excess electrons. Regulatory processes under conditions of high excitation pressure and the potential to acclimate or adapt to very low photon flux densities may be responsible for the dominance of diatoms in permanently low-temperature environments such as sea ice.

Other requirements for low temperature ice algae growth (beside the dynamic regulation of photon flux densities discussed earlier) are an adequate response to osmotic stress and the avoidance of freeze damage by growing ice crystals. The ability of diatoms to acclimate to high salt concentrations (i.e., osmotic stress) in brine channels is accomplished by the regulation of the ion concentration and composition through the accumulation of free amino acids such as proline and other cryoprotectants such as DMSP (DiTullio et al., 1998; Plettner, 2002). The intracellular concentrations of these compounds depend on environmental conditions and on the physiological potential of each diatom species. Some

diatoms are able to produce more of these cryoprotectants (e.g., *Chaetoceros gracile*, *Amphiprora kufferathii*) and are therefore able to grow under lower temperatures and higher salinity (Plettner, 2002). These different acclimation potentials indicate niche separation in sea ice. In contrast, pelagic diatoms are less prolific producers of DMSP and proline. Studies of both the Antarctic pack ice and fast ice have shown a huge production of high concentrations of DMSP by ice algae assemblages reaching concentrations of over 1,500 nM. This is much higher than seawater values (typically ranging from 0 to 50 nM; DiTullio et al., 1998).

Besides intracellular acclimation to osmotic stress, there is evidence that polar diatoms are able to secrete ice-binding proteins (IBPs), which deform growing ice crystals (Fig. 6) and possibly enable them to survive freeze–thaw cycles by avoiding damage to the cell membrane (Raymond and Knight, 2003; Janesch et al., 2006).

These proteins were recently identified in several polar diatoms but have not been found in temperate and tropical diatom species (Janesch et al., 2006). IBPs acting as antifreezes, ice recrystallization inhibitors, and ice nucleators have been found in many organisms that are exposed to the cold, including fish, insects, plants, fungi, and bacteria (Cheng, 1998). IBPs of diatoms resemble plant antifreezes in that they do not lower the freezing point. However, they are more closely related to fungal antifreeze proteins than to other plant antifreeze proteins (see Section 3 for more detail). IBPs contain signal peptides and are possibly post-translationally modified before they are excreted into the extracellular space where they bind to ice crystals (Janesch et al., 2006). One possible function of IBPs is to prevent freeze injury to membranes and the frustules of diatoms protecting



Figure 6. Irregular-shaped ice crystals caused by secreted ice-binding proteins from diatoms. This phenomenon is termed ice-pitting and prevents the cells from damage by growing ice crystals. Image courtesy by James Raymond.

the cell by preventing the recrystallization of external ice. Recrystallization is a process in which large grains of ice grow at the expense of small grains, which are thermodynamically unstable. The growth of the larger crystals is thought to be physically disrupting to cell membranes (Knight et al., 1995). Protein recrystallization inhibitors have been found in several cold-hardy plants such as ryegrass and Antarctic hair grass (Doucet et al., 2000). However, other functions of diatom IBPs cannot be ruled out. IBPs are just one group of substances that are excreted into the extracellular space. The vast majority of secreted substances is not characterized yet and operationally defined as extracellular polymeric substances (EPS) presumably composed of different kinds of polysaccharides, amino acids, and proteins. A recently developed *in situ* microscopical approach provided the first evidence that EPS can be highly concentrated in brine channels (Fig. 7) (Krembs et al., 2002). It is likely that EPS function also to retain salts in sea ice resulting in increases of the liquid brine fraction and hence the habitable pore space at any given temperature. Diatoms are the main EPS producers in sea ice

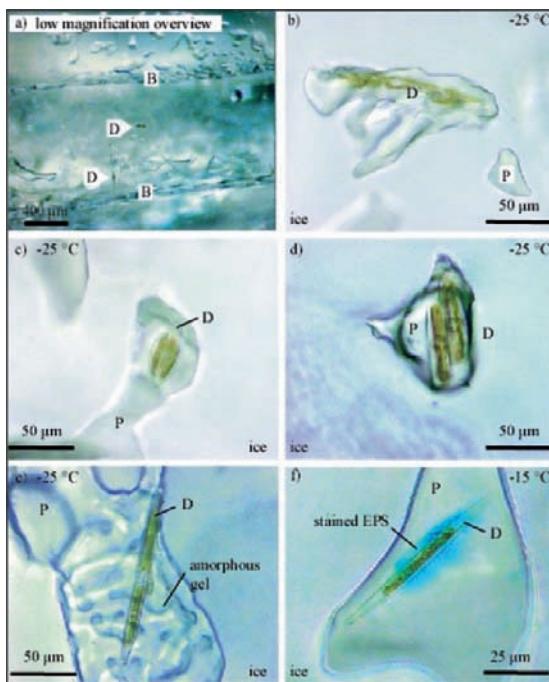


Figure 7. Microphotographs of pennate diatoms residing within pore spaces at a depth of 112 cm in an ice core. (a), low-magnification image showing ice texture, brine layers (B) and diatoms (C); (b), damaged diatom cell in a pore with encroaching ice crystals and an empty pore space (P); (c), diatom in a pore connected to a brine layer; (d), two diatoms in an isolated pore; (e), diatom in a pore with indications of amorphous transparent gel-like exopolymeric material; and (f), diatom surrounded by an EPS matrix stained with Alcian blue. Images courtesy of Christopher Krembs.

that can maintain high local concentrations of EPS at the cell periphery (Krembs et al., 2002). Such an ability to retain salts could be another important extracellular factor beside IBPs and intracellular osmoregulation that could explain the predominance and success of diatoms in sea ice.

3. Molecular Adaptations: Gene Composition and Expression

To investigate molecular adaptations in polar diatoms, sequence information of functional genes is necessary. The first whole genome sequence of a diatom, *T. pseudonana*, became available in 2003 (Armbrust et al., 2004) and the second genome sequence from *Phaeodactylum tricornutum* will be available soon. Only recently the Joint Genome Institute (JGI, USA) approved the whole genome sequencing of the polar diatom *F. cylindrus* (<http://www.jgi.doe.gov/sequencing/why/CSP2007/fragilariosis.html>). Comparative genomics with both temperate diatoms (*T. pseudonana*, *P. tricornutum*) and *F. cylindrus* will uncover genes that only occur in *F. cylindrus* (such as IBPs) and will therefore elucidate potential roles in conferring freeze tolerance. The most prominent genera of diatoms in polar ecosystems are *Fragilaria*, *Nitzschia*, *Navicula*, *Corethron*, *Thalassiosira*, and *Melosira*. Among these genera, the species *F. cylindrus* is considered to be an indicator for cold water because it is known to only occur in the Arctic Ocean and Southern Ocean (there, from sea ice to polar frontal zones) (von Quillfeldt, 2004). *F. cylindrus* can form large ice-edge blooms (Kang and Fryxell, 1992). Because of its widespread distribution in polar oceans this diatom is an ideal candidate for the investigation of its adaptation to the polar environment. Consequently, *F. cylindrus* was selected to prepare the first EST (expressed sequence tag) libraries for a polar marine alga (Mock et al., 2005). In this study, diatom cells were grown either at freezing temperature (cold stress) or with added salt (salt stress), both environmentally relevant conditions. For cold stress, RNA was obtained 5 days after chilling the cells to the freezing point of seawater; for salt stress, RNA was taken at different time points after adding salt (60 ppt). To date, 2,485 *F. cylindrus* EST sequences have been generated – 996 from the cold-stress library and 1,691 sequences from the salt-stress library. All ESTs are deposited at the dbEST-databank at NCBI. Furthermore, about 200 gene-specific oligonucleotides (70mers) are for functional gene-array experiments available. All EST-sequences were compared against the genome of *T. pseudonana* and *P. tricornutum*, and 11 additional algae and plant databanks were consulted to annotate those sequences that were not found in the genomes of both diatoms. Nevertheless, only less than 50% of all sequences displayed similarity to known sequences in these databanks and to both diatom genomes even when using a comparatively high e-value of $\leq 10^{-4}$ (Mock et al., 2005).

In the cold-stress library, the most abundant functional categories were related to translation, post-translational modification of proteins, and transport

of amino acids and peptides by ABC-transporter. Some of these ABC-transporters displayed homology to bacterial permeases; whereas others appeared to be involved in translational control or post-translational processes. However, most of them have no assigned function at this point. The presence of six different DNA/RNA helicases in the cold-stress library indicated that DNA and RNA coiling and uncoiling are important under freezing temperatures. Minimizing the likely formation of secondary structures and duplexes of mRNAs under low temperature stress is necessary to initiate translation. However, protein domains of DNA/RNA helicases are also the eighth most abundant protein domain in the genome of *T. pseudonana* and therefore more evidence is necessary to conclude that these enzymes are essential to cope with freezing temperatures (Armbrust et al., 2004). The most abundant sequences in this library in terms of their redundancy were either sequences that were related to energy generation (e.g., fucoxanthin-chlorophyll *a*, *c*-binding proteins) or completely unknown sequences (Mock et al., 2005).

In the salt-stress library, the most abundant functional categories of sequences were related to post-translational modification of proteins (e.g., hsps) and ion-transport (Krell, 2006). Most of them were heat-shock proteins (hsps) and different ionic transporter genes reflecting the requirement to reestablish homeostasis under salt stress. Several sequences of different kinds of V-type H⁺-ATPases and antiporters for various ions such as sodium, potassium, and calcium were found in this library. V-type H⁺-ATPases are of great importance to establish an electrochemical proton gradient across the tonoplast to drive sodium sequestration into the vacuole (Shi et al., 2003). One important organic osmolyte under salt stress in diatoms is the amino acid proline. Many genes involved in proline synthesis were found in the salt-stress-EST library indicating that this pathway was active under the experimental conditions. The gene pyrroline-5-carboxylate reductase (P5CR, catalyzing the final step in proline synthesis) could be identified among the most abundant sequences in the salt-stress library, which indirectly indicates that this gene was at least moderately expressed under salt stress and therefore important for salt acclimation (Krell, 2006).

One of the most interesting discoveries in the salt-stress library was a gene involved in antifreeze processes. The protein sequence (translated from the nucleotide sequence) of this gene showed high sequence similarity to an isoform protein of the snow mold *Typhula ishikariensis*. The *T. ishikariensis* protein has been shown to reduce the freezing point of seawater by approximately 0.1°C (Hoshino et al., 2003). However, this is not a strong freezing point depression when compared with the antifreeze ability from numerous antifreeze proteins identified from fish, insects, plants, and bacteria. Thus, they might represent a new class of IBPs. Using a proteomics approach and another polar diatom (*Navicula glaciei*) the corresponding protein was also discovered (Janesch et al., 2006). This protein is probably unique in polar diatoms since none of the temperate diatoms tested so far had this class of genes and the two whole genomes available for diatoms (temperate)

did not contain the gene (Janesch et al., 2006). Furthermore, in contrast to polar diatoms with IBPs, temperate diatoms when subjected to freeze–thaw cycles were not able to survive (Janesch et al., 2006). Using nearly pure IBPs, 2-D polyacrylamide gel electrophoresis yielded a spot of approximately 25 kDa with a pI of about 5.0. Tandem-mass spectrometry-sequencing of the band yielded four peptide sequences. These sequences were found to be similar to several antifreeze isoforms of the snow mold *T. ishikariensis*. The amplification of the *Navicula* gene revealed strong similarity to the gene from *F. cylindrus* and the corresponding *T. ishikariensis* genes. The N-terminal sequences of the identified IBPs of *N. glaciei*, *F. cylindrus* IBPs, and each of the *T. ishikariensis* antifreeze isoforms are most likely signal peptides and have low probabilities of being mitochondrial- or chloroplast-targeting peptides.

These IBPs also show some similarity (between 43 and 58% amino acid sequence identity) to hypothetical proteins from gram-negative bacteria such as *Cytophaga hutchinsonii* and *Shewanella denitrificans*, species of genera that have frequently been isolated from Arctic sea-ice (Junge et al., 2002). The former species belongs to the Cytophaga–Flavibacterium–bacteroides (CFB) phylum group – a bacterial group that appears to be especially important in well-established sea-ice algal assemblages (Bowman et al., 1997) and the coldest (wintertime) sea ice (Junge et al., 2004). Several psychrophilic species of *Shewanella* have been isolated from sea ice, often in association with algal assemblages (Bowman et al., 1997). Whether polar sea-ice bacteria also express IBPs remains to be explored. The sequence similarity observed to bacterial and fungal IBPs also invites questions about the origin of these genes. Horizontal gene transfer from fungi is one possibility, because basidiomycotic fungi (which include *Typhula*) are also known to inhabit sea ice and are believed to have arisen hundreds of millions of years before diatoms appeared. Horizontal gene transfer from members of the CFB-group of bacteria is another possibility because of their known association with algal assemblages in sea ice and their relation to species with IBP-like genes. In other organisms, antifreezes appear to have arisen from a variety of proteins with other functions, although some retain the original functions (Cheng, 1998). Overall, the evolution of IBPs remains to be explored further.

The presence of genes in a genome only indicates the potential for physiological adaptation, but knowledge of the expression and regulation of genes and their respective proteins leads to an actual understanding of how these diatoms cope with the extreme polar conditions. Expression analysis can be done by focusing on single genes (e.g., northern blots or quantitative PCR = qPCR) or by using gene arrays that are either composed of known genes (gene-only arrays) and/or the whole genome sequence (tiling arrays). In a recent study (Mock and Valentin, 2004), about 200 oligonucleotides (70mers) were obtained for gene-only arrays based on both EST libraries from *F. cylindrus*. Nylon-membrane-based macro-arrays were designed to investigate the expression of genes involved in photosynthesis and cold acclimation of *F. cylindrus*. Short, mid-, and long-term acclimation to the freezing point of seawater was investigated to differentiate acclimation between fast-changing environmental conditions (representing

sea-ice formation in fall) and long-term acclimation (e.g., to different geographic locations and therefore different temperature regimes).

One of the most dramatic environmental changes in polar marine ecosystems is the freezing of seawater and the melting of the ice. The inclusion of organisms into newly formed sea ice represents a strong selection pressure. Only those organisms can survive and thrive in sea ice that are capable to acclimate to the relatively fast-changing conditions of temperature, irradiance, and salinity. To date, only one experiment has been conducted using a polar diatom (*F. cylindrus*) to investigate gene expression changes from non-freezing to freezing temperatures in order to obtain molecular information of the acclimation potential (Mock and Valentin, 2004). Macro-arrays composed of genes from different pathways were specifically designed for these experiments. The short-term response to freezing temperatures, which simulates the incorporation into newly formed sea ice during fall, was characterized by down-regulation of genes encoding proteins for photosystem II (psbA and psbC) and carbon fixation (RUBISCO large subunit, rbcL), regardless of light intensity used (3 and 35 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$). However, under the higher irradiance (35 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) up-regulation of genes encoding chaperons (hsp 70) and genes for plastid protein synthesis and turnover (elongation factor EFTs, ribosomal rpS4, and plastidial ftsH protease) was observed. Freezing accompanied with a reduction in irradiance (from 35 to 3 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) showed a typical response to low-light acclimation by up-regulation of genes encoding specific fucoxanthin-chlorophyll *a*,*c*-binding proteins (fcps) without signs of a cold stress response. Fcps are a diverse gene family composed of genes involved in light harvesting as well as dissipation of light (see Section 2). Up-regulation of stress response genes and genes for protein turnover only under higher light intensities and decreasing temperatures indicates that a decrease in temperature at such light intensities mimics a further increase in light that could be more stressful than the actual decrease in temperature was by itself. This phenomenon is probably part of a cold-shock response that is also known from temperate plants when they get exposed to lower temperatures (Allen and Ort, 2001). In contrast to temperate plants and diatoms though, psychrophilic plants and diatoms are able to acclimate to higher irradiances under low temperatures (Streb et al., 1998; Mock and Hoch, 2005; Ralph et al., 2005; Morgan-Kiss et al., 2006). Long-term acclimation experiments to higher irradiances at freezing temperatures when compared with the same light intensity but higher temperatures (+5°C) revealed that cells kept at lower temperatures showed a typical response known from high-light acclimation: higher NPQ, up-regulation of the gene psbA, and up-regulation of high-light fcps that are involved in energy dissipation (Mock and Hoch, 2005). A reduction in expression of other photosynthesis-related genes (such as rbcL) was not observed anymore after several months under freezing conditions indicating that long-term acclimation had been achieved. Temperature effects that are less dependent on adjustments of the energy flow under freezing temperatures could also be identified by gene expression analysis (Fig. 8).

For this study, genes were selected that either were abundant in the EST libraries (e.g., ABC transporters) or were important for general acclimation to

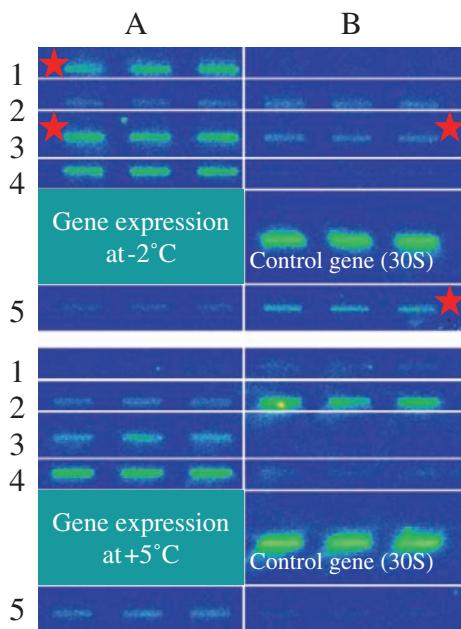


Figure 8. Two macro-arrays that show gene expression for selected genes from *F. cylindrus* at +5 and -1.8°C. Gene-specific oligonucleotides (70mers) were spotted in triplicate. Red stars indicate significant up-regulation (>twofold differences) of genes at -1.8°C. Genes that were selected: A1: ABC-transporter no. 1; B1: ABC-transporter no. 2; A2: Polyketide-synthetase (PKS); B2: High-light-inducible protein (HLIP); A3: Delta-5-desaturase; B3: Ice-binding protein; A4: unknown protein no.1; B4: unknown protein no. 2; A5: Sigma factor protein; B5: fucoxanthin-chlorophyll *a*,*c*-binding protein (FCP).

freezing temperatures (e.g., IBP, fatty-acid desaturase). Three unknown but abundant genes (in EST libraries) were also selected to see whether at least one of them is up-regulated under freezing temperatures. Expression of these genes was investigated at +5°C and 9 days after reducing the temperatures to -1.8°C (Table 1). Up-regulation of a gene encoding a delta5-desaturase under freezing temperatures indicated the necessity for production of PUFAs to maintain membrane fluidity at lower temperatures. Delta5-desaturases produce omega3-fatty acids such as EPA (20:5 *n* - 3), one of the most abundant fatty acids in diatoms and the main fatty acid in the galactolipids MGDG and DGDG. Thus, it can be assumed that more EPA is necessary under freezing temperatures to keep the thylakoid-membrane fluid for electron transport or other membrane-bound processes. In contrast, a delta-12 desaturase gene also known for producing PUFAs was not up-regulated in temperate cyanobacteria (Nishida and Murata, 1996). This indicates a different mechanism of gene regulation for this enzyme in psychrophilic diatoms.

Strong up-regulation of a gene that encodes an IBP (approx. 50-fold) was also measured under freezing temperature supporting the hypothesis that these

Table 1. Relative gene expression based on macro-arrays (Fig. 8) for selected genes from *F. cylindrus* that might be involved in freeze resistance.

Encoded Protein	Column A or B	Row number	Relative expression	
			+5°C	-2°C
ABC transporter (1)	A	1	7 ± 6	690 ± 177
ABC transporter (2)	B		65 ± 14	11 ± 2
PKS	A	2	128 ± 19	107 ± 23
HLIP	B		738 ± 90	189 ± 22
Delta-5-desaturase	A	3	284 ± 69	818 ± 61
Ice-binding protein	B		3 ± 5	170 ± 15
Unknown protein (1)	A	4	1,005 ± 70	972 ± 93
Unknown protein (2)	B		60 ± 19	28 ± 6
Sigma Factor	A	5	173 ± 25	78 ± 3
FCP	B		16 ± 3	293 ± 15

Numbers in bold indicate up-regulation (>twofold difference to expression at +5°C) at freezing temperature. $n = 3$, \pm indicates standard deviation (STD), PKS = polyketide-synthetase, HLIP = highlight inducible protein, FCP = fucoxanthin–chlorophyll *a*, *c*-binding protein

proteins are of great importance not only under salt stress but also under freezing temperatures to protect the cells from injury by growing ice crystals (Table 1). One uncharacterized ABC-transporter gene was strongly up-regulated at -1.8°C. However, the family of ABC-transporters is composed of genes with very diverse functions (see earlier) so that there is no direct evidence, based on gene expression only, that this gene has actually something to do with transporting molecules. One of the unknown genes being twofold up-regulated under freezing temperature indicated some importance for acclimation to low temperatures. In contrast, another unknown gene was down-regulated at -1.8°C, and the third gene didn't change its expression at both temperatures but was relatively strongly expressed under both temperatures. Overall, these initial results indicate that psychrophilic diatoms have specific genes to cope with freeze–thaw cycles that are also known from other psychrophilic organisms but are not present in temperate diatoms. However, it remains to be seen whether they also have a unique set of genes that is specific to them.

4. Summary

Psychrophilic diatoms are regarded as one of the most extremophilic eukaryotes on our planet. They are responsible for a huge fraction of primary productivity at the poles (>50%) because of higher silicate concentrations and the ability to cope with the strong seasonality of light at temperature at or below the freezing point of seawater. Polar diatoms serve as the base of the entire food web, ultimately feeding krill, whales, penguins, and fish. Since particularly polar krill and fish are commercially used, polar diatoms also contribute significantly to human food sources. Global change already has a tremendous impact on the

Arctic ecosystem by a reduction of sea ice cover with consequences for all sea-ice-associated life. Such dramatic impacts of the global increase in temperatures on sea ice appear to be less pronounced in the Southern Ocean (Wolff et al., 2006). Clearly, diatoms being one of the key players in this ecosystem have developed unique adaptation mechanisms (such as IBPs) to survive and adapt to freeze–thaw cycles of seawater. Molecular studies on gene composition and expression were found to be very useful in finding unique adaptation mechanisms and in highlighting the interplay of photosynthetic activity and light, temperature, and/or salinity. Genome-wide comparison between a polar diatom (*F. cylindrus*) and diatoms from temperate regions (*T. pseudonana*, *P. tricornutum*) are planned to uncover more genes that are responsible for their unique adaptation.

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ALGAE AT EXTREME LOW TEMPERATURES: *The Cryobank*

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1. Introduction

In vitro cryopreservation is the storage of viable cells at ultra-low temperatures (196°C), usually in liquid nitrogen or its vapor phase. Under these conditions it is assumed that metabolism is arrested and cells are stable for indefinite periods, so long as liquid nitrogen supply is maintained. The fact that cells tolerate cryogenic temperatures is remarkable as survival after cryopreservation is common to a wide range of biodiversity. The in vitro cryobank is one of the most, if not the most extreme low-temperature environment that an organism, or component part thereof, will ever encounter on earth. It is fascinating to speculate how, with the aid of cryoprotection (Fuller, 2004) so many diverse life-forms survive such extreme cold. Cryopreservation has important applications for astrobiology and in vivo studies of extremophiles; as water and temperature are physical determinants of life, indeed water is a prerequisite for life. This chapter considers cryoconservation in a wider context, appraising the comparative utilities of both natural and artificial cryobanks as repositories and research tools that may be used to help understand how life survives extreme cold. Algae are the subject of choice as they are one of the oldest and most diverse groups of organisms; their ancestral, fossil remains have been found in strata dating from 1.4 billion to 2.1 billion years (Cloud et al., 1969; Han and Runnegar, 1992). Algae are ubiquitous primary producers and formidable extremophiles, yet, compared with other biological resources, their preservation in cryobanks (Day et al., 2005) and their utilization as a valuable economic resource remains limited.

1.1. PRINCIPLES OF IN VIVO AND IN VITRO CRYOPRESERVATION

Water is nature's solvent, and its behavior at subzero temperatures is influential to recovering life from the "frozen state" (Fuller et al., 2004). Understanding survival in natural and artificial cryogenic environments requires multidisciplinary

knowledge, especially regarding the effect of temperature on water behavior and the formation of its different states, which comprise liquid, vitreous (glass) and solid (ice) forms.

1.1.1. Survival in the Naturally Frozen State

Water is a molecule of unusual chemistry (Ball, 2003); it is bipolar, the H atoms carry a positive charge and the O atom a negative charge, making it able to form H-bonds (hydrogen bonds), both with itself and other charged molecules. H-bonds are key to understanding the cryobiology of water. In its liquid state, water's H-bonds are in dynamic equilibrium and continually form, break and reform bonds with one another. As the temperature is lowered, ice is formed as molecules of water link together to form four H-bonds with adjacent molecules. To form ice, H-bonds must orientate themselves into a more organized stable structure and in doing so they move slightly apart, becoming less compact. Therefore, water molecules accommodated in the crystalline lattice of ice form open spaces interconnected by H-bonds; water thus expands as it freezes and this is why it floats on water. When heated to a temperature $>0^{\circ}\text{C}$ the H-bonds are broken and the H_2O molecules are free, once more, to move closer together. The density of water changes with temperature and is greatest at 4°C , a point above which its molecules begin to expand again as molecular motion increases. Only above 4°C does the density of water behave more normally, declining with increasing temperature. This unusual property allows aquatic life to survive freezing temperatures. If ice were to have a greater density then water bodies would freeze solid from the bottom up and life in aquatic habitats would be virtually impossible. When a water body cools, ice forms at the surface and insulates the water columns below, reducing further freezing. Aquatic organisms survive below the surface, for algae survival can also be dependent upon entering dormant states and forming "resting" stages (e.g., akinetes, aplanospores) during which metabolism is arrested to accommodate seasonal limitations in nutrients, temperature and light (Laybourn-Parry, 2002).

In the case of terrestrial organisms, survival at extreme low temperatures requires a different strategy that often combines adaptive stress biochemistry with complex life cycles. Algal survival is influenced by freezing and desiccation tolerance as water is "locked up" in ice in frozen terrestrial environments. External ice nucleators can prove problematic with respect to the equilibration of osmotic gradients during differential extra and intracellular freezing. Because of the evolutionary pressures incurred on extremophiles, Polar terrestrial algae have developed complex adaptive behaviors (Elster and Benson, 2004). These are manifest as avoidance, habitat protection and cryoprotection, and include adaptive behavioral patterns related to shelter, motility and symbiotic protection. Biochemical cryoprotection by psychrophilic organisms has applications in *in vitro* cryobanking and include cold-shock protein production, cryoprotective sugar synthesis and the moderation of ice nucleation and growth by antifreeze proteins (Elster and Benson, 2004; Ponder et al., 2004).

1.1.2. Assisted Survival in Cryobanks

Ensuring survival in cryobanks requires an understanding of cryoinjury (Fleck et al., 2006) and cryoprotection. Historical perspectives of plant and animal cryopreservation have been summarized by Benson (2004) and Leibo (2004), respectively. In brief, Maximov pioneered studies of cryoprotection in plants, postulating the importance of sugars in natural freeze tolerance (Diller, 1997). Polge et al. (1949) made the serendipitous discovery of the cryoprotective effects of glycerol using avian spermatozoa. This marked the start of modern cryobanking (Fuller et al., 2004; Leibo, 2004) and its use for assisted reproduction via in vitro fertilization (IVF) and cell, tissue and organ storage. Phytodiversity cryopreservation has also been advanced considerably due to the implementation of improved cryoprotection strategies (Day et al., 2005; Benson, 2006). The basic principles of cryoprotection are common to most organisms, and water plays an important part. Cells exposed to controlled rates of cooling do not usually freeze at 0°C, but supercool, in the absence of ice nucleators, to lower temperatures, to a minimum of -40°C, the point of homogeneous ice formation. When controlled cooling rates are applied to cryopreserve cells, ice first forms extracellularly. This creates a water vapor pressure gradient between the inside and the outside of the cell causing water to cross the membrane to rebalance osmotic equilibrium. This leads to intracellular dehydration and the concentration of cell solutes, resulting in two consequences for survival: (i) the intracellular compartment loses water that would otherwise be available for the formation of ice inside the cell, an event which is almost always lethal and (ii) solutes become concentrated as cell viscosity increases. Balancing the excursion of intracellular water, the formation of ice and cell solute concentration is critical to successful cryopreservation. These parameters underpin the two-factor theory of cryoinjury forecasted by Maximov (Diller, 1997), substantiated by Lovelock (1953), Meryman (Meryman and Williams, 1985) and Mazur (2004). The factors are: ice damage and dehydration injury and their elucidation gave rise to the colligative theory of cryoprotection (Lovelock, 1953; Meryman and Williams, 1985; Mazur, 2004). This has two key attributes: (i) cryoprotectants must penetrate the cell and (ii) they must be nontoxic at concentrations required for their efficacy. To understand colligative cryoprotection it is necessary to consider the effect of solute concentration on freezing point. As more extracellular ice forms, water is withdrawn from the cell and the intracellular freezing point is depressed further. If the effect is too extreme, the integrity of the cell is compromised as it approaches a potentially injurious minimum cell volume. Penetrating cryoprotectants are efficacious because when added at relatively high concentrations they become equally distributed at the same concentration across the cell membrane, thereby contributing to the overall osmolality of the cell. This is a count of the total number of osmotically active components of a solution on the basis of osmoles of solute per kilogram of solvent. Hence, the level of water needed to be frozen to achieve osmotic equilibrium is less, as is the extent to which dehydration occurs in the cell (Meryman and Williams, 1985). Another

protective advantage of colligative additives is that they depress freezing point, and as viscosity is enhanced, the rate of ice nucleation is reduced. Once ice is formed, crystal growth is inhibited and any crystals produced at the point of freezing may be so few and so small they are rendered innocuous (Meryman and Williams, 1985). Importantly, increased viscosity also raises the glass transition temperature (T_g); a glass is a metastable, amorphous solid liquid which lacks crystalline structure and, as such, is less damaging to cells. Vitrification of water is a possible end point of colligative cryoprotective; whilst extracellular freezing does occur, the cells do not freeze intracellularly and they are cryopreserved in a partial “glassy state.”

Vitrification for the cryogenic storage of plant and algal germplasm has been considered for different cryoprotectants (Harding et al., 2004; Benson et al., 2005; Benson, 2006). Totally ice-free cryopreservation is one of the main approaches for preserving plant germplasm (Benson, 2004) and is now also used for algal storage (Harding et al., 2004). Enhancement of cell viscosity is usually brought about by two manipulations, the addition of cryoprotective additives at very high concentrations and removal of water by evaporative desiccation and/or osmotic dehydration. It is cautioned that vitrification can be injurious; as additives may be toxic when applied at high concentrations, they may also cause osmotic injury. Importantly, devitrification and ice formation can occur on rewarming, and osmotic dehydration by evaporative means presents the problem of desiccation sensitivity. The application of different types of cryoprotective strategies for algal cryobanking is therefore dependent upon the resistance of each type of organism or cell type to cryoinjury and the potentially deleterious effects of cryoprotection. Controlled rate cooling and colligative cryoprotection requires an organism to survive chilling, extracellular freezing and osmotic stress. Vitrification, on the other hand, necessitates high tolerance to osmotic stress and desiccation injury (Harding et al., 2004). A major problem for algal cryoconservationists is that their organisms have high phenotypic and genotypic diversity. This makes developing widely applicable cryostorage protocols a major challenge; how and why this challenge is being addressed will now be discussed.

2. Algal Cryobanks: Valuable Research Resources

Algae are virtually ubiquitous contributing to approximately half of the Earth's photosynthetic activity and underpinning food chains for ca. 70% of the world's biomass (Wiessner et al., 1995; Andersen, 1996). They are valuable research resources and a relatively underexploited source of novel products. One of the most important aspects of algal research pertains to their use as indicators of environmental health and ecosystem sustainability (McCormick and Cairns, 1994) and as such this chapter will primarily focus on this aspect of their cryobanking.

2.1. USING ALGAL CRYOBANKS FOR BASIC AND APPLIED RESEARCH

All comparative studies and publishable science require the use of “standards” to facilitate the repetition and further development of work by other researchers. In whole-cell research, biological standards are ideally authenticated using documented strains of organisms, preferably held in internationally recognized culture collections. These collections, or more broadly, Biological Resource Centers (BRCs) are a mixture of academic, public service, private, government and commercial activities, delivering cultures as “seed” stocks for multiple purposes. Including: teaching, academic research, development of industrial/biotechnological processes, the provision of reference strains for biological assays, the endorsement of published scientific literature, the use of type strains for taxonomical studies and to support biodiversity conservation.

Algal culture collections normally hold strains under defined maintenance conditions (Lorenz et al., 2005) providing them, and their associated data-sets, access to a wide-ranging user community including academic and commercial organizations. This approach has the advantage in that it ensures strain samples can be provided rapidly as a master culture which can be “split” and/or used as a large (>10%) inoculum to produce a healthy vigorous culture within 1–3 weeks, depending on the growth rate of the alga. However, serial subculturing of microalgae has significant disadvantages: it is labor intensive, requires considerable consumable resources for maintenance and has an inherent risk of contamination and/or mislabeling. Furthermore, it does not arrest evolutionary change due to mutation or genomic rearrangement and cannot guarantee phenotypic stability (Jaworski et al., 1988). Serial culture is susceptible to risks of inadvertent strain loss due to failure of the facility; hence there is a requirement for the stabilization and conservation of algal cultures. As drying and freeze-drying have limited value for long-term algal preservation (McLellan et al., 1991; Day and Brand, 2005), cryopreservation is now adopted as the most appropriate storage method.

2.1.1. *Practical Operations of a Cryobank*

Outlined below are key factors for the operation of cryobanks; more detailed guidance can be found in specialist literature (Hawksworth, 1999; Kirsop, 1999; Stacey and Day, 2006). The single most important issue is cryostat maintenance at an ultra-low temperature (<−135°C) that stabilizes the stored material in the long term. For security purposes and in the case of potentially catastrophic equipment failure, it is also advisable and cautionary to split cultures between separate storage vessels and ideally to have an additional off-site storage location. Accurate records of stored materials are crucial for the maintenance of “stock-control” and managing the cryostore. Commercial database systems are designed for managing electronic records, but it is important to select a system that is flexible over the full range of user requirements. It is safest to maintain up-to-date

hard copy printouts so that amendments to storage records that reflect sample additions or withdrawals can be made at the storage site to avoid transcriptional errors. The minimum data that should be recorded are: strain number, genus and species, date cryopreserved, protocol employed, cryostorage inventory location, number of stored cryogenic vials and their removal dates and an indication of poststorage viability.

2.1.2. Worldwide Distribution of Algal Cryobanks and International Networking

In recent years, there has been increased interest in the application of cryopreservation to microalgae and cyanobacteria and most of the largest collections including ATCC, USA (Lee and Soldo, 1992), CCALA, Czech Republic (Lukavský, 2003), CCAP, UK (Morris, 1978), CCMP, USA (Andersen, 2005), Coimbra, Portugal (Osorio et al., 2004), NIES, Japan (Watanabe et al., 1992), PCC, France (Rippka et al., 2002), SAG, Germany (Friedl and Lorenz, 2002), UTCC, Canada (Kuzmina, 2004) and UTEX, USA (Bodas et al., 1995) now employ this method to maintain some of their holdings. This move toward cryopreservation has, in part, been stimulated by concerns about genotypic stability of conserved strains, as well as the major collections reaching a size (3,000 strains) beyond which an expansion of their holdings requires significant additional resources and staffing levels. A further stimulus has been methodological developments (Bodas et al., 1995; Day et al., 2000; Harding et al., 2004; Day and Brand, 2005; Rhodes et al., 2006), which have facilitated the cryopreservation of a much broader range of freshwater, terrestrial and marine algal taxa. The EU-funded “COBRA” project (EU FP5 project QLR1-CT-2001-0164) resulted in the cryopreservation of >3,000 algal strains, approximately 50% of the total holdings of the 5 participating European culture collections (Day et al., 2005).

2.2. USING ALGAL CRYOBANKS FOR ENVIRONMENTAL RESEARCH

Algae are one of the few groups of photosynthetic organisms for which long-term *in vitro* and *in situ* “cryogenic storage” is possible. This is due to their remarkable ability to survive over long periods the extreme conditions of the Polar regions. Therefore, comparative explorations into their survival in natural and artificial cryobanks afford unique opportunities for low-temperature and environmental research.

2.2.1. In Situ Cryobanks

Elster and Benson (2004) consider two natural terrestrial low-temperature environments: (i) stable (glacial ice, permafrost, subglacial systems, glacial melts and temporary snowfields) and (ii) unstable (soils, lithophytic/aerophytic communities, on, or within, rock substrata, hydro-terrestrial and lacustrine niches). Longevity of algae in natural permafrost provides some of the most spectacular

reports of extreme survival. Ponder et al. (2004) describe a wide diversity of microorganisms surviving in deep permafrost exposed to temperatures $\leq 0^{\circ}\text{C}$, for at least 2 years. These regions include 85% of Alaska, 55% of Russia and Canada, 20% of China and the major part of Antarctica; amounting to almost 20% of the Earth's surface being a "natural cryobank." Their extreme to low subzero temperatures range from -20 to -65°C in the Arctic and Antarctic respectively, these regions have low water contents (1–7%) and are exposed to long-term gamma radiation; constraints not unlike those of artificial cryogenic storage.

Ponder et al. (2004) postulate that due to the stability of the low temperatures in the Earth's most extremely cold environments, permafrost may be considered one of the most important depositories for ancient biomolecular markers and most significantly viable cells. *In situ* cryopreserved algal archives can span thousands of years and in terms of environmental research, they may be utilized to facilitate explorations into microbial taxonomy. Particularly with respect to the evolution of adaptations to extreme cold and desiccation for which sampling substrates (Sojna et al., 2004) containing flora representative of geologically significant timelines constitutes a powerful research tool. Perhaps one of the most compelling applications of natural algal cryobanks is in helping to tackle the increasingly pervasive problem of climate change. Particularly in understanding the dynamics of the Earth's ancient carbon caches and the prehistoric photosynthetic organisms presently trapped in ancient ice. It is currently speculated that organic carbon products, created in the early Holocene and "locked up" in frozen Siberian peat-lands pose a problem if released by global warming. Smith et al. (2004) caution that predicting how peat carbon stocks trapped in a frozen, but warming Arctic, will impact the global climate poses a complex problem.

Significant releases of methane gas are associated with the end of severe ice ages and the same is proposed if the present rate of anthropogenically induced climate change ensues. One potential scenario is that if the Earth's temperature continues to rise, the ancient carbon residues currently frozen in Siberian peat and permafrost will release methane, a greenhouse gas known to contribute to climate change. Smith et al. (2004) have predicted what the consequences may be if this does occur (assuming no appreciable change in global CO_2 uptake) and indicate that it will significantly increase carbon release to the atmosphere. Estimating over the next 500 years there will be a release of ca. $140 T_{\text{g}} \text{ C Year}^{-1}$ and enhancing contemporary levels of CO_2 in the atmosphere by 0.07 ppm, approximately 4% faster than the present rate. Relating the significance of CO_2 release projections from permafrost peat with respect to the concomitant possibility for reanimating potentially viable, photosynthetic microorganisms archived in frozen depositories presents an interesting challenge. Central to which will be the need to gain a greater insight into how organisms survive in arrested animation when subjected to extreme cold for periods of geologically significant time. Integrating our knowledge of survival and stability in algae exposed to artificial cryopreservation may be useful in gaining an insight into the recovery of organisms in natural ancient and *ex situ* cryobanks and vice versa.

2.2.2. *Ex Situ Cryobanks*

Ex situ cryobanks are important environmental research resources but they are not a substitute for conserving biodiversity *in situ*. Rather, they provide a complementary means of “capturing in time” the genetic and physiological state of the organism when introduced into the cryobank. By comparison, the *in situ* population from which the cryopreserved progenitor sample was derived will continue to be changed by environmental factors, ageing and evolutionary progression. *In vitro*-cryopreserved strains are thus living “snapshots” captured at the instant they are secured in liquid nitrogen. In situations where environmental factors may be attributed to anthropogenic activities (pollution, habitat disturbance and climate change) it is possible to compare the effects on organisms that remain *in situ* with the parallel “stress histories” of those deposited in cryobanks at progressive sampling times. Strategic cryobanking of organisms sampled across timelines of *in situ* disturbance history (e.g., pollution episodes) is a powerful tool, particularly for monitoring changes in organisms sampled from pristine, sensitive environments such as Antarctica (Elster and Benson, 2004). It is best practice in environmental profiling to use organisms that may be compared with, or obtained from, *in vitro*-validated, axenic strains held in internationally designated culture collections under stable storage (Day et al., 1999). For algae used in phytoremediation and environmental monitoring (Johnstone et al., 2006a, b) deposition of standard test strains in *in vitro* cryobanks ensures best practice parity across different laboratories.

An increasingly important application of *in vitro*-cryopreserved culture collections is in the preservation of strains from endangered and at risk provenances. *Ex situ* conservation safeguards against loss of phycological diversity through environmental erosion and protects precious strains for future reinstatement (Watanabe et al., 2005) and environmental impact monitoring (Johnstone et al., 2006a, b). Many extremophilic algae reside in remote or conservation-sensitive habitats such as the Polar regions; this poses access and economic difficulties for their collection (Johnstone et al., 2002). Holding stable, *in vitro*-cryopreserved cell lines obviates the need for their continuous sampling from protected reserves such as Antarctica. Similarly, comparing recovery experiences (Vishnivetskaya et al., 2000) of viable organisms retrieved from ancient cryobanks with those from *in vitro* cryopreservation (Day and Harding, 2006) would benefit both fields of interest.

3. Stability of Algae in Natural and Artificial Cryobanks

Stability relates to how long a viable organism, or part thereof, can be maintained in and retrieved from cryobanks, unaltered at molecular genetic, physiological, biochemical and functional levels. Understanding the mechanisms that confer cell longevity and stability at low temperatures, and that allow a resumption of normal function is important for *ex situ* cryoconservationists, extremophile researchers and paleoecologists.

3.1. TIMELINES OF LOW-TEMPERATURE LONGEVITY IN CRYOBANKS

Antarctica is the coldest place on Earth, the lowest temperature ever recorded there was -89°C at the Russian Vostok Station in 1983. Usually, low Polar temperatures are within the range -40°C to -55°C for Antarctica, and -20 to -35°C for the Arctic (Elster and Benson, 2004; Ponder et al., 2004). In vitro cryogenic storage temperatures are much lower at -196°C for liquid nitrogen and -135 to -190°C for its vapor phase. The rationale for using cryogenic storage to extend longevity and arrest biological change is based on the premise that metabolic rate declines with temperature and that all biological activity ceases at the temperature of artificial cryobanks. When comparing longevity and stability in *ex situ* and *in situ* cryobanks, time must however, be considered across geological, evolutionary and biomolecular scales.

Algae have played the most influential role in the development of life on Earth which was formed about 4.6 billion years ago, with the first plants and animals emerging around 534 million years. Biomarker evidence (Lane, 2002) found in iron deposits in Western Australia provide the earliest dating of cyanobacterial remains at about 2.7 billion years. Megascopic fossils of eukaryotic algae have also been discovered in 2.1 billion-year-old iron deposits in Michigan (Han and Runnegar, 1992). In addition, viable green algae have been isolated from permafrost samples in Siberia and Antarctica's dry valleys. Ponder et al. (2004) summarize that the highest biodiversity of green algae was evidenced in 10,000-year-old Holocene sediments. Green algae were found at higher frequencies and a greater diversity level than cyanobacteria. Examples reported in ancient Arctic permafrost include *Chlorella* and *Pseudococcomyxa* isolates, *Mychonastes* sp., *Chlorococcum* sp., *Chodatia* sp., *Stichococcus* sp. and *Scotiellopsis* sp., and for Antarctica, *Mychonastes* sp. and *Pedinomonas* sp.

The longevity of viable algae surviving *in situ* cryobanks can extend to thousands of years and potentially even millennia, as is the case for organisms trapped in ancient permafrost. Their time span for survival is dictated by the type of habitat or niche in which they reside; thus, Vorobyova et al. (1997) report that permanently low, stable temperatures of permafrost are key to stabilizing microbial life. Sediment studies revealed that for substrates of different lithological structures and age it was the level of subzero temperature and its duration that determined viability. This was categorized by Vorobyova et al. (1997) into: (i) hypometabolic cells in a reversible state capable of active proliferation and (ii) viable, but nonculturable, "deep resting" cells. The ratio between the groups is affected by the stability of the low temperatures to which the organisms were exposed. Preservation of undamaged cell structures sampled from the frozen sediments correlated with a resistance of up to 100 freeze-thaw cycles (Vorobyova et al., 1997). Viable green algal components of microbial flora isolated from Arctic deep sediments were also dated to 5,000–7,000 years of entrapment in a frozen state and were composed of green algae and cyanobacteria. Recovered isolates

grew at slow rates at 20–25°C and were sensitive to photooxidation (Vorobyova et al., 1997). Chlorophyll and carotenoid pigments were found in sediments across different ages; however, no plant residues were detected in Antarctic samples, although there were some trace pigments, unfortunately, attempts to retrieve and culture viable algae were unsuccessful (Vorobyova et al., 1997).

Entering contemporary times, Day et al. (1997) performed the first long-term longevity study for *ex situ* cryopreserved algae, demonstrating that the process did not significantly reduce viability after 22 years of storage under liquid nitrogen. Furthermore, mammalian sperm has been demonstrated to remain motile and fertile after 37 years cryostorage (Leibo et al., 1994). In both of these examples, the samples were frozen employing a two-step controlled rate cooling protocol with the materials being maintained in liquid phase nitrogen at -196°C. This study contrasts with that of a similar investigation performed by Walters et al. (2004) who found a measurable change in germination of dried seeds stored in liquid nitrogen for >10 years, with considerable variability in deterioration between species and storage accessions. This study questions the basis for cryobank storage and highlights the importance of conducting stability assessments of organisms and germplasm maintained in the artificial cryogenic state.

3.2. HOW STABLE IS THE CRYOGENIC STATE?

It has been generally assumed that viable cells could be maintained in liquid nitrogen cryobanks in a “dormant” cryopreserved state ad infinitum and that once at these ultra-low temperatures all metabolic activity ceases. However, two key questions must be posed: (i) How stable are cells held in cryobanks? (ii) How changed are they when they come out? To address these queries, it is necessary to explore the relationship between physical, physiological and molecular aspects of cryogenic stability (Fahy et al., 2004). Algal culture collections use cryopreservation to circumvent the genetic change that may ensue if organisms are maintained in active serial subculture. In essence, cryobanking halts the potential for genetic change occurring through *in vitro* culture selection. Similarly, knowledge of the stability of algae entrapped in ancient ice fields, “fixed” at a point along an evolutionary timeline is highly relevant. Most especially for interpreting and understanding the basis of their adaptive and taxonomic progression as compared with their modern-day contemporaries. However, substantial evidence is now emerging from studies of other organisms and germplasm types held in *ex situ* cryogenic storage that ultra-low storage temperatures are not as stable as was once thought.

3.2.1. Biophysical Stability of Cryobanks

The longevity storage study conducted by Walters et al. (2004) on seeds held in liquid nitrogen vapor and liquid phases (-135°C to -196°C) showed a deterioration with time in cryogenic storage. Although in some cases seeds were stored at

“traditional” storage temperatures of -18 to -20°C prior to subsequent storage in liquid nitrogen, Walters et al. (2004) generally concluded (through the assistance of predictive models) that the lowering of storage temperature progressively increased seed longevity. These researchers also caution that molecular mobility, albeit limited, can occur at the cryogenic temperatures of liquid nitrogen storage (Walters, 2004; Walters et al., 2004). Importantly, they developed a half-life model for predicting longevity of cryopreserved lettuce seeds containing $0.065\text{ g H}_2\text{O g dry weight}^{-1}$, such that 50% of the seeds will survive 524 years at -135°C and 3,377 years at -196°C . This may be considered an adequate projection for continuing to pursue artificial cryobanking, but one which will be affected by the physiological status of the germplasm, as well as the storage process. Therefore, it is most important to build on these studies and consolidate best-practice cryobanking procedures as well as constructing research strategies which will help elucidate the contributory processes that determine the stability of viable biological materials held in cryogenic storage.

At the most fundamental level, stability involves moderating the biophysical state with respect to molecular mobility, the behavior of water and the stabilization of transitions between the glassy, solid and liquid phases of water. Walters et al. (2004) and Walters (2004) rightly question the view that all biological activity ceases at $<-130^{\circ}\text{C}$ (the T_g of pure water) and using thermal analysis studies supported by Arrhenius plots and kinetic equations conclude that molecular mobility is sufficiently active at this temperature to allow ageing reactions to proceed. Thus, Walters et al. (2004) attributed a decline in stored seed viability to an inadvertent warming through the T_g which promoted and accelerated deteriorative processes and possibly the formation of intracellular ice. This most likely occurred when seed accessions were removed from the cryobank, an explanation that can be used to inform practical recommendations for good-practice cryobanking. Specifically: (i) care in maintaining temperatures of banked cryomaterials on the withdrawal of samples from the bank; (ii) consideration of precryogenic storage handling and physiological health status and (iii) careful choice of storage regime (i.e., liquid- or vapor-phase nitrogen) with respect to the stability of the glasses formed which will be primarily affected by choice of cryoprotectant strategy applied. Knowledge of cryoprotectant mode of action is thus critically important for predicting the long-term stability of the glassy state as incurred by different cryoprotective regimes. For example, Volk and Walters (2006) applied thermal analysis to elucidate the mode of action of the cryoprotectant Plant Vitrification Solution 2 (PVS2), leading them to postulate that a key reason for its efficacy is in its ability to restrict molecular mobility and disorganize ice crystal structure.

3.2.2. The Biochemical and Molecular Genetic Stability of Cryobanks

Studies of stability must integrate knowledge of the physical impacts of cryobanking with an understanding of the biological processes that determine longevity. Cell-ageing mechanisms are extensively researched in medical disciplines

and include: (i) correlation of increased formation of reactive oxygen species (ROS) in cells that have higher metabolic rates and that are short-lived (Adelman et al., 1988); (ii) telomere shortening (Keys et al., 2004) and (iii) oxidative damage to DNA (Adelman et al., 1988). Reducing metabolic activity by ultra-low temperatures and low water status thus reduces ROS formation, arrests cell division and enhances longevity by slowing down or stopping the ageing process (Benson and Bremner, 2004). Molecular mobility in cryopreserved and desiccated systems, as dictated by water state stability and glass transition temperature, are likely to be critical factors in determining whether, or not, oxidative stress and DNA damage ensue in vitrified and partially vitrified cells (Buitink et al., 2000; Murthy et al., 2003; Benson and Bremner, 2004).

The maintenance of genetic integrity throughout the cryogenic process is particularly important for plant and algal germplasm conserved in *ex situ* cryobanks used by designated algal culture collections. For cultured organisms, stability assessment does not only include the impacts of exposure to the cryogenic process per se, but also cryoprotection and pre- and poststorage manipulations (Harding, 2004; Harding et al., 2005). Assessment of genetic stability in organisms or individuals regenerated from cryobanked germplasm has, to date, been largely techniques driven (Harding, 1999) comprising studies at phenotypic, histological, cytological, biochemical and molecular levels. There is a paucity of information as to the genetic stability of algae held in *in situ* cryobanks, although Müller et al. (2005) observed no genotypic differences, on the basis of AFLP patterns between *Chlorella vulgaris* CCAP 211-11b that had previously been cryopreserved for >20 years and isolates that had been maintained by serial transfer over the same period. In vitro cryopreservation has, over the past 25 years, been applied to over 100 higher plant species, and to date, reports of genetic stability assessments are positive, favoring the assumption that storage in LN maintains genetic stability. Exemplars of stability in cryobanked plants include: Random Amplified Polymorphic DNA (RAPD) analysis (Jokipii et al., 2004) and RAPD and Amplified Fragment Length Polymorphism (AFLP) analysis (Liu et al., 2004). However, as advances in diagnostic molecular technologies progress, it will be essential to consider the appropriateness and stringency of the diagnostic tools applied (Harding, 2004).

4. Algal Cryobanks: Their Potential Use in Astrobiological Research

Terrestrial extremophile research is important for space exploration and investigations of the Earth's lower and upper atmospheres and beyond, particularly with respect to profiling the presence and movement of microorganisms therein (Hoover, 2006). Natural algal cryobanks can provide test organisms for astrobiological studies (Day et al., 1999; Vishnivetskaya et al., 2003) and *ex situ* repositories may be used to secure precious organisms retrieved from the stratosphere and putative microbial life-forms recovered from space (Mileikowsky et al., 2000; Wainwright et al., 2004; Wallis and Wickramasinghe, 2004; Hoover,

2006;). Extremophilic cold-tolerant algae and cyanobacteria are primary producers, and their study may be extrapolated to the conditions required for supporting life in extraterrestrial environments (Rothschild and Mancinelli, 2001; Vishnivetskaya et al., 2003; Rosing, 2005). Manned planetary missions and space stations need reliable life support systems that guarantee food, water and oxygen supply. The only sustainable means of achieving this, particularly for the increasingly long-term requirements of manned spaceflight and station missions, is to use photosynthetic organisms (Kanervo et al., 2005). Algae are the biota of choice due to the technical difficulties of maintaining higher plants under extreme and prohibitively resource-restricted conditions. Microalgae are more amenable to storage in, and recovery from cryobanks located on spacecrafts and stations and cryopreserved backup cultures offset risks of life-support systems failure. Space environment simulations using *Synechocystis* sp. PCC 6803 as a model demonstrated no negative effects on photosynthetic O₂ production (Kanervo et al., 2005) giving confidence in their fitness of purpose.

The concept of Panspermia proposes that microbial life is ubiquitous in the Universe (Wickramasinghe, 2004) and presupposes a potential for interstellar transfers of planetary microorganisms (Wallis and Wickramasinghe, 2004) and the survival of microbial life in the stratosphere, on comets and meteorites. Wickramasinghe (2004) considers the Universe a “cryogenic habitat for microbial life,” integrating survival knowledge of organisms cryobanked at liquid nitrogen temperatures (77 K), or in permafrost may enable research into the existence of life at comparable extraterrestrial temperatures (e.g., 20–50 K for interstellar dust and comets). Terrestrial cryobanking expertise may help develop strategies for the retrieval, reanimation and confirmation of viabilities for organisms collected from the stratosphere. Particularly by applying strategies developed for recovering cryopreservation-recalcitrant algae (Benson, 2004; Fleck et al., 2006) and bacteria from permafrost (Vishnivetskaya et al., 2000). Similarly, cryobanking requires the stringent use of robust viability (Kobabe et al., 2004; Day and Harding, 2006) and molecular stability tests (Harding, 1999) as well as the use of specialist cryogenic instrumentation (Benson et al., 2005). These technologies may also be adapted to study vital signatures in astrobiological samples (Wainwright et al., 2004; Hoover, 2006) and to help ascertain the potential for viable interstellar transfers of planetary biota (Mileikowsky et al., 2000; Wallis and Wickramasinghe, 2004).

To conclude, comparative studies of algae exposed to natural and artificial cryobanks and astrobiological conditions present unique opportunities to investigate research issues of shared relevance. Principally the impacts of extreme cold, desiccation and anaerobia on organisms held in vacuum, in space; under liquid nitrogen in artificial cryobanks or, entrapped in ancient permafrost. Understanding how algae withstand these analogous extremes offers unique opportunities to share technologies and information concerning their adaptive responses to cold, desiccation and radiation stress. Particularly through gaining insights into mechanisms of cryoprotection, tolerance and cryoinjury as well as the

extent to which extreme cryogenic temperatures are able to impede molecular mobility and secure genetic stability. Clearly an exchange of knowledge and technologies across these areas of common interest presents exciting potential for research synergy between the fields of cryobanking and astrobiology in the future.

5. References

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PART 5:

PHOTOTROPHS IN HOT ALKALINE AND ACIDIC ENVIRONMENTS AND NON-THERMAL ACIDIC HABITATS

**Pinto
Miller
Ionescu, Oren
Hindiyeh, Malkawi
Brown, Allen, Mummey, Sarkisova, Mckay
Novis, Harding
Aguilera, Amaral-Zettler, Souza-egipsy,
Zettler, Amils
Pinto, Ciniglia, Cascone, Pollio
Weber, Barbier, Shrestha, Horst,
Minoda, Oesterhelt
Teske**

Biodata of **Gabriele Pinto** author of the chapter “*Cyanidiophyceae: Looking Back–Looking Forward*”

Gabriele Pinto is Professor of Botany at the University “Federico II” of Naples. The research field of Prof. Pinto deals with the biology and the economic utilization of extremophilic microalgae (acidotolerant and acidophilic microalgae). On these organisms multi disciplinary studies were carried out by Prof. Pinto leading to the reclassification of several algal genera and species or to the institutions of new taxa. He has also worked on the biotechnological utilizations of microalgae and has isolated numerous algal strains which have been used as ecological indicators for the identification of toxic pollutants, as source of useful lipids in industry and in the biotransformation of selected molecules giving products which could be hardly synthesized through conventional methods.

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CYANIDIOPHYCEAE: *Looking Back–Looking Forward*

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1. Taxonomic History

An Italian scientist, Giuseppe Meneghini (1839) (Fig. 1) was the first to study and describe the thermo-acidophilic algae inhabiting the sulphur hot-spring of Acquasanta (Ascoli Piceno, Italy). He observed “small and very small globules” (0.2–2.0 µm) and he proposed the new species *Coccochloris orsiniana* (Cyanophyta) without any diagnosis.

In the following 100 years, these algae, always considered as one species, captured the interest of many phycologists who tried to clarify their systematic position. Because of their simple morphology and the absence of sophisticated means of investigation, these algae were variously identified as *Palmella orsiniana* (Chlorophyta) (Kützing, 1849), *Chroococcus varius* (Tilden, 1898), *Protococcus botryoides* f. *caldarium* (Tilden, 1898), *Pleurococcus sulphurarius* (Chlorophyta) (Galdieri, 1899), *Pleurocapsa caldaria* (Collins et al., 1901), *Palmelloccoccus thermalis* (West, 1904), *Pluto caldarius* (Copeland, 1936), *Cyanidium caldarium* (Geitler and Ruttner, 1936), *Dermocarpa caldaria* (Drouet, 1943) and *Rhodococcus caldarius* (Hirose, 1958). By using morphometric analyses of field samples, De Luca and Taddei (1970) identified for the first time, two thermo-acidophilic algae (Fig. 2). These were provisionally named *Cyanidium caldarium* forma A and *Cyanidium caldarium* forma B. In the early 1980s the Neapolitan school used morphological, physiological and ultrastructural data to invalidate the previous descriptions, and made a definitive revision of the taxonomy of these algae. Two species were formally recognized: *Cyanidium caldarium* (Tilden) Geitler (Fig. 3) and *Galdieria sulphuraria* (Galdieri) Merola (Merola et al., 1981) (Fig. 4).

2. Expanding Knowledge

The interest in these extremophilic organisms led many scientists to undertake a series of geographical expeditions all over the world documenting that these algae, previously described only from a few European and American sites (Acquasanta and Campi Flegrei in Italy and Yellowstone National Park in the USA) are present on all the continents, everywhere where there are environmental



Figure 1. Giuseppe Meneghini (1811–1889).



Figure 2. Cyanidiales at Pisciarelli (Campi Flegrei, Naples, Italy).

conditions that allow them to survive. The only exceptions are Africa and Antarctica where there are no records of these algae.

All data collected to date suggest that thermo-acidic environments throughout the world are inhabited by mixed communities of species of Cyanidiales. In addition, new thermo-acidophilic algae from other phyla have been discovered and described. De Luca et al. (1978) described *Cyanidioschyzon merolae* De Luca, Taddei and Varano, an alga characterized by very small dimension ($1.5 \times 3.0 \mu\text{m}$)

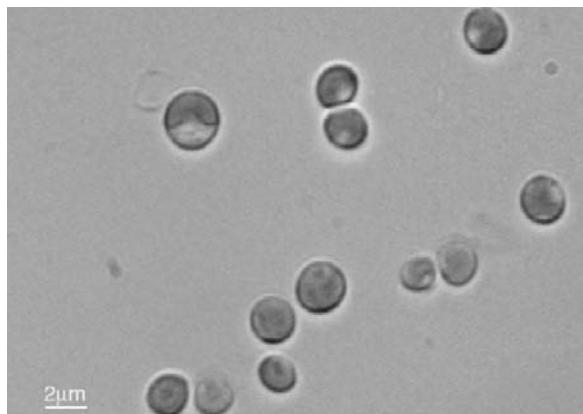


Figure 3. *Cyanidium caldarium*.

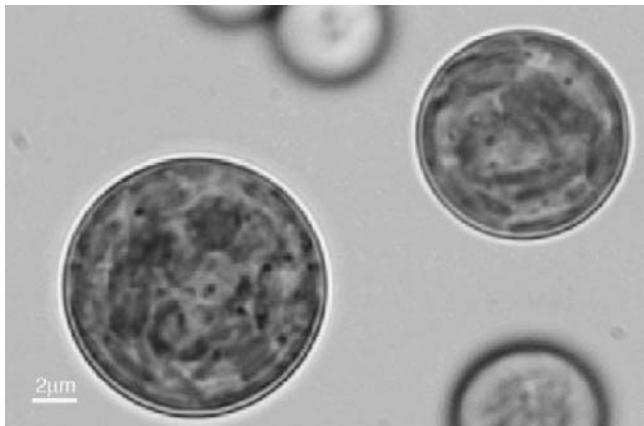


Figure 4. *Galdieria sulphuraria*.

reproduction by binary fission, one mitochondrion, one plastid, and absence of a cell wall (Fig. 5). Fifteen years later, Sentsova (1994) isolated three new species of *Galdieria*, *Galdieria partita* Sentsova (Figs. 6–7) and *Galdieria daedala* Sentsova (Figs. 8–9) from Kamchatka peninsula (Russia) and *Galdieria maxima* Sentsova (Fig. 10) from Kunashir Island (Russia).

3. Modern Perspective on Taxonomy and Evolution

Until the end of the twentieth century, little was known about the biodiversity of Cyanidiales, their population structure and their phylogenetic relationships. Indeed, in a major review of red algal taxonomy and evolution Garbary and Gabrielson

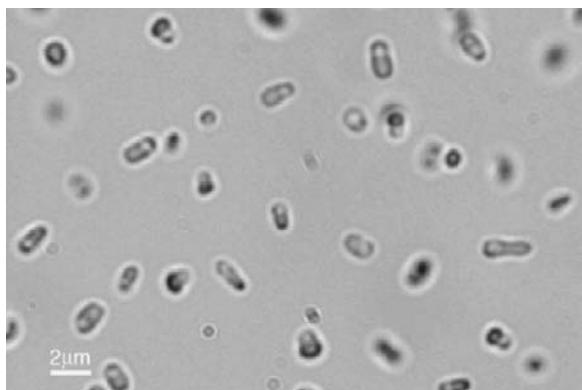


Figure 5. *Cyanidioschyzon merolae*.

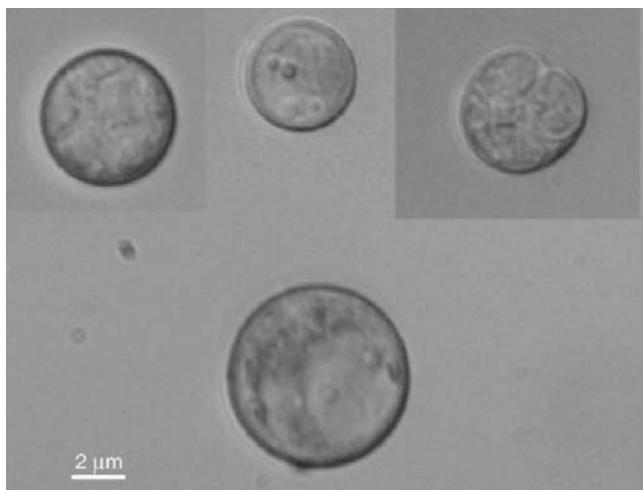


Figure 6. *Galdieria partita*.

(1990) still retained these organisms in the Porphyridiales. By means of the new methods of molecular biology, the study of thermo-acidophilic algae received new impetus. This research revealed an unexpected hidden biodiversity among Cyanidiales that also revised current thinking about the phylogeny of these algae. The phylogenetic analyses supported the presence of four distinct lineages of Cyanidiales: the *Galdieria* spp. lineage (excluding *Galdieria maxima*), the *Cyanidium caldarium* lineage, a novel monophyletic lineage of mesophilic *Cyanidium* spp., and the *Cyanidioschyzon merolae* plus *G. maxima* lineage (Ciniglia et al., 2004). Moreover, different molecular studies confirmed that these extremophilic algae are

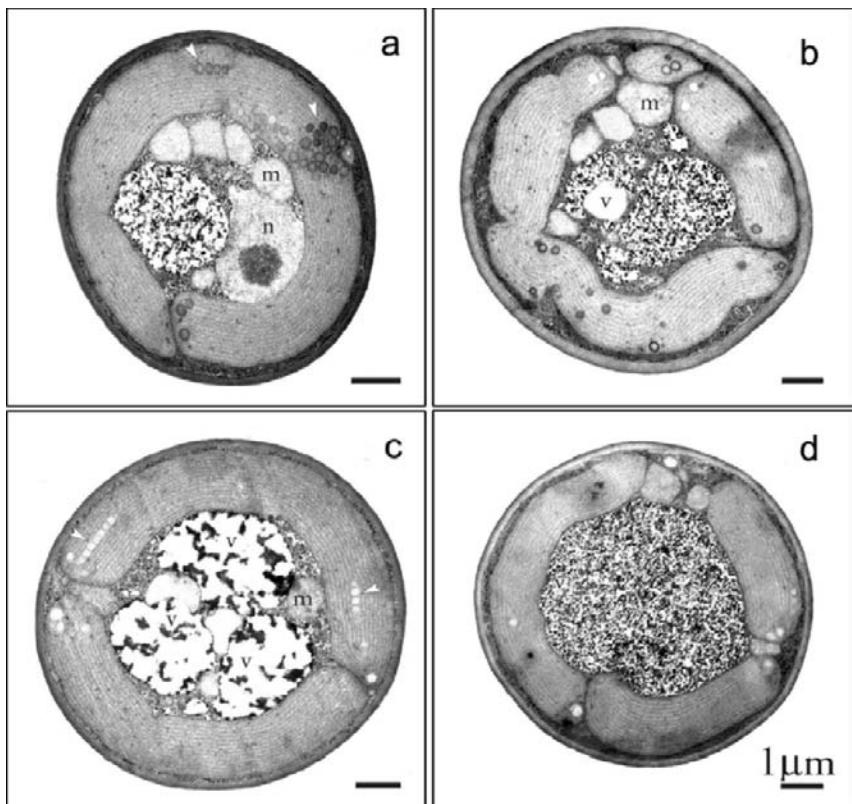


Figure 7a-d. TEM micrographs of *Galdieria partita* cells. a: young cell; arrowheads show plastoglobules grouped or forming rows interspersed within intertylacoidal space. b-d: mature cells showing a different number of vacuoles. c. arrowheads indicate rows of plastoglobules. Abbreviations: m = mitochondrion, n = nucleus, v = vacuole. The scale bar in all figures is 1μm. (from Pinto *et al.*, 2003).

evolutionary distantly related to other red algae and that there was an ancient split (ca. 1342 ± 22 million years ago) among the Cyanidiales and all other red algal or red algal derived plastids (Yoon *et al.*, 2002). On the basis of these data, Yoon *et al.* (2006a) proposed a new classification of the Rhodophyta, establishing the new subphylum Cyanidiophytina that included the single class Cyanidiophyceae.

Recently, Yoon *et al.* (2006b) studied the Cyanidiales population structure at endolithic and interlithic habitats in Pisciarelli (Naples, Italy) and Larderello (Tuscany, Italy). Biomolecular data and ecophysiological tests supported the institution of a new *Galdieria* species which has been named *Galdieria phlegrea* (Pinto *et al.*, X this book) (Fig. 11).

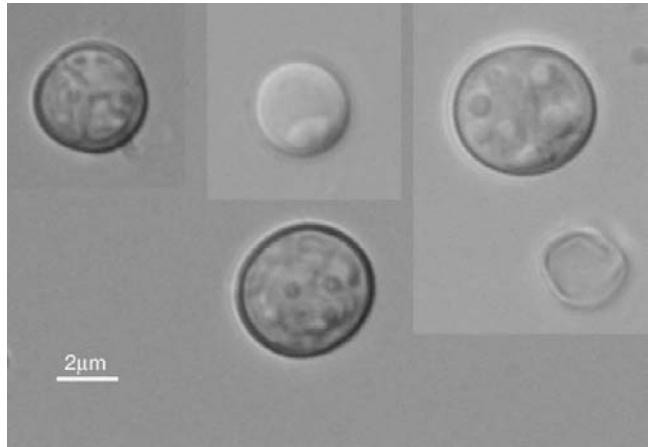


Figure 8. *Galdieria daedala*.

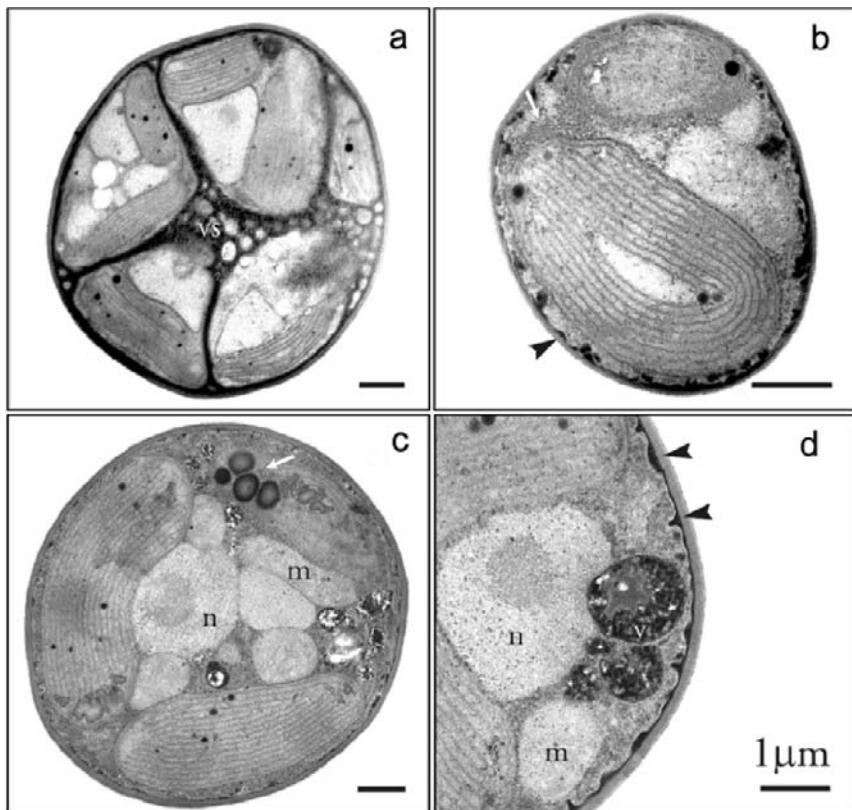


Figure 9a-d. TEM micrographs of *Galdieria daedala* cells. a: sporangium. b: young cells; arrowhead shows the protrusion of the innermost layer of the cell wall (transfer-cell like structure); arrow indicate dictyosomes. c: mature cell; arrow indicates osmiophilic globules in the plastid stroma. d: a detail of a mature cell showing the protrusion of the inner layer of the cell wall (arrowheads). Abbreviations: m = mitochondrion; n = nucleus; v = vacuole; vs = vesicles: The scale bar in all figures is 1 μm (from Pinto *et al.*, 2003).

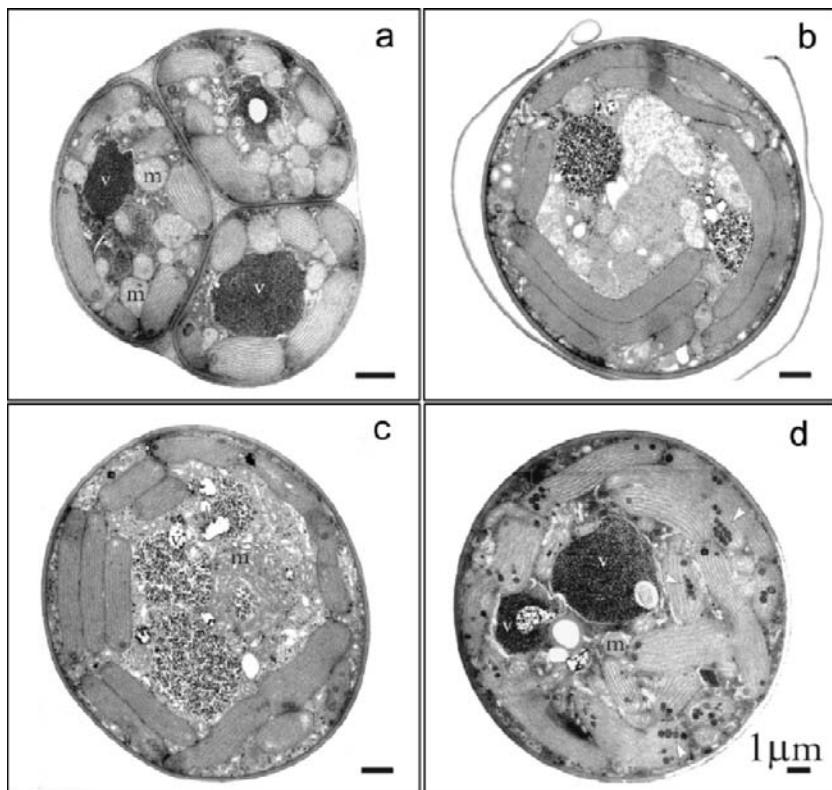


Figure 10a–d. TEM micrographs of *Galdieria maxima* cells. a: sporangium. b: young cell; c–d: mature cells. d: arrowheads shows rows of plastoglobules. Abbreviations: m = mitochondrion, n = nucleus, v = vacuole. The scale bar in all figures is 1 μm . (from Pinto *et al.*, 2003).

4. Cyanidiophyceae as Model Systems in Biology

Interest in Cyanidiophyceae has increased following the total genome sequencing of *Cyanidioschyzon merolae* (Matsuzaki *et al.*, 2004). This thermo-acidophilic alga contains the smallest genome among photosynthetic eukaryotes. *C. merolae* has just one plastid, one mitochondrion and a minimal set of small membrane bound compartments. As a consequence, this species is a valid biological model for providing information on the basic and essential genes that support life processes in photosynthetic eukaryotes. In addition, this species is now a model system for understanding mechanisms of chloroplast and mitochondrial division (Kuroiwa *et al.*, 2006; Yoshida *et al.*, 2006).

Barbier and collaborators are also sequencing the total genome of *G. sulphuraria*. This species, as the other of its genus, has a unique position within the Cyanidiophyceae because, in contrast with the obligately photoautotrophic

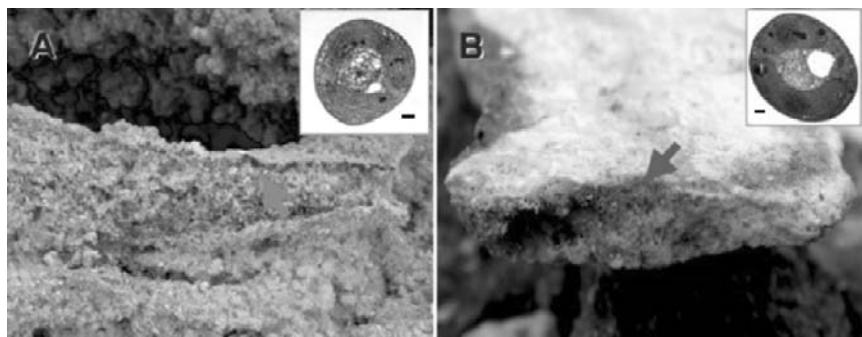


Figure 11. Endolithic Cyanidiales at Larderello (A) (Tuscany, Italy) and at Pisciarelli (B) (Naples, Italy). The arrows indicate the biomat that thrive inside the rock at these sites. The TEM images show *Galdieria sulphuraria* (A) and *Galdieria phlegrea* (B) from the endolithic sites. Scale bars = 1 μm (from Yoon *et al.*, 2006b).

Cyanidium and *Cyanidioschyzon*, it is also able to grow mixotrophically and heterotrophically. These peculiarities, in addition to its resistance to high salt, toxic metals and abiotic stressors, make *G. sulphuraria* an interesting model for studying adaptation to extreme environments.

Recently, *Galdieria* has been also utilized in microalgal biotechnology. Its extremophily, the above mentioned metabolic capabilities, and the ability to retain its photosynthetic apparatus, including the blue pigment phycocyanin, make this alga potentially useful and promising (Sloth *et al.*, 2006). For further information on these thermo-acidophilic algae, see Seckbach (1994).

In conclusion, the study of these thermo-acidophilic organisms can provide important answers on the biology of extremophiles. In addition, new research is also highlighting the potential of these organisms for applications in biotechnology.

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DIVERSITY OF THE COSMOPOLITAN THERMOPHILE *MASTIGOCLADUS LAMINOSUS* AT GLOBAL, REGIONAL AND LOCAL SCALES

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1. A Natural System for Investigating Microbial Biogeography

A recent theme in the study of microbial diversity has been the issue of whether and how the genetic and phenotypic variation of microorganisms is distributed along a geographic transect. Population genetic theory and the results of experimental evolution studies in the laboratory (e.g., Wright, 1931; Atwood et al., 1951; Bennett and Lenski, 1993) suggest that spatially structured microbial populations in nature should rapidly diverge from each other, provided that migratory gene flow among them is low, thereby creating geographic patterns of variation. Recent reports confirm that divergence of geographically isolated populations indeed outpaces the homogenization of genetic variation by migration, indicating the presence of dispersal barriers for microorganisms (e.g., Miller and Castenholz, 2000; Papke et al., 2003; Whitaker et al., 2003; Miller et al., 2006). These observations run counter to the longstanding idea that the abundance of a microorganism at a location is not limited by dispersal but is determined solely by environmental factors (Baas-Becking, 1934), a view that has been recently championed for eukaryotic microorganisms on the basis of morphological criteria (Finlay, 2002). Here, I will summarize our recent investigations of the biogeography of the moderately thermophilic, filamentous cyanobacterium, *Mastigocladus (Fischerella) laminosus*.

This bacterium provides an excellent system for investigating the organization of microbial diversity at a variety of geographic scales. *M. laminosus* is present virtually worldwide, though not necessarily in great abundance, in alkaline hot springs at temperatures below approximately 58°C (Castenholz, 1996). For reasons that are not entirely clear, it tends to dominate fast-flowing thermal streams of appropriate temperature (Castenholz, 1978). Like other members of the Stigonematales, *M. laminosus* forms true branches as a result of changes in division plane during growth, typically resulting in a tuft of thick primary trichomes and narrow secondary trichomes. This complex morphology makes it easily recognizable during microscopic examination of field samples.

M. laminosus is also capable of differentiating specialized structures in response to changes in the environment. Under conditions of nitrogen limitation, it develops heterocysts for spatially separating the biochemically incompatible processes of nitrogen fixation and oxygenic photosynthesis. Other specialized

structures are hormogonia, motile dispersal structures released from the non-motile parental trichome by lysis of a necridial cell (Hernández-Muñiz and Stevens, 1987), and akinetes, which are freezing and desiccation-resistant resting cells that are produced in response to nutrient and/or light limitation.

The ability to produce akinetes may explain why *M. laminosus* is an excellent colonizer of new, often geographically distant hot spring habitat. A few examples serve as a testament to this bacterium's dispersal abilities. On the island of Surtsey, *M. laminosus* was observed around steam vents within several years of its formation by volcanic eruptions beginning in 1963 off the southern coast of Iceland (Castenholz, 1972). The nearest hot springs are 75–90 km away on the Icelandic mainland. It has also successfully colonized hot springs on Mount St. Helens formed following the 1980 eruption (Castenholz, 1996), as well as the former thermal effluent channels at Savannah River Nuclear Plant (Brock, 1978), where the closest site known to contain *M. laminosus* is Hot Springs, Arkansas. The above cases are notable for the absence of *Synechococcus cf. lividus*, a common resident of alkaline hot springs in western North America, which does not produce resting cells.

2. Global Diversity: A Cosmopolitan Bacterium with Local Flavor

To investigate the genetic diversity of *M. laminosus* from thermal areas around the world (including Yellowstone National Park, Iceland and New Zealand), we have characterized 51 of the laboratory isolates of this cyanobacterium that have been deposited in the University of Oregon's Culture Collection of Microorganisms from Extreme Environments. Each of the isolates belonged to one of seven closely related groups based on the complete identity of a partial sequence (835 bp) of the 16S ribosomal RNA (rRNA) gene (Fig. 1). The level of dissimilarity at this locus for the two most divergent lineages was under 3%, similar to that observed for A and B clades of *Synechococcus* (Miller and Castenholz, 2000). That is, global *M. laminosus* diversity is comparable to the degree of genetic differentiation between *Synechococcus* lineages that have diverged in thermal ecology and may be separated by only millimeters along a hot spring channel. Whereas a few of these 16S rRNA groups were endemic to a single location, many were found at multiple, geographically disparate sites (Fig. 2), highlighting this bacterium's dispersal capabilities.

A closer look at the genetic diversity of these isolates tells a more complicated story, however. We have also obtained sequence data from these strains for three functional genes involved in nitrogen metabolism: *nifH*, encoding the iron protein of nitrogenase; *narB*, the gene encoding assimilatory nitrate reductase; and *devH*, which codes for a DNA-binding protein required for the development of a functional heterocyst (Ramírez et al., 2005). These additional data revealed 23 distinct multi-locus haplotypes (Fig. 2). Although a few of these haplotypes were observed in geographically distant populations (e.g., haplotype 16 in Oman,

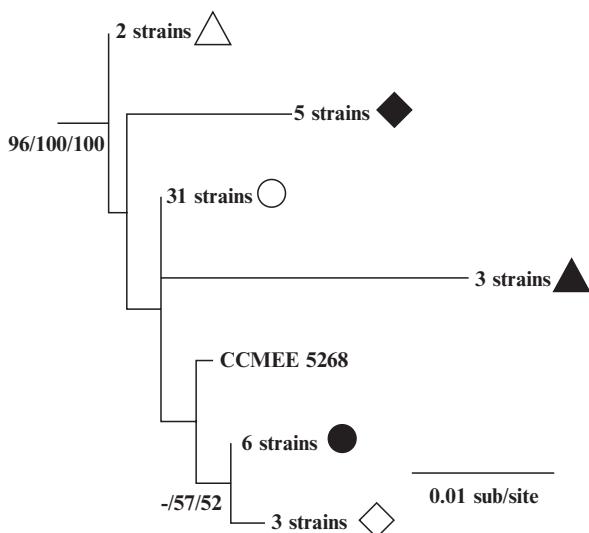


Figure 1. Maximum likelihood phylogeny of 51 *Mastigocladus* isolates reconstructed from 835 nucleotides of the 16S rRNA gene, rooted with outgroups *Chlorogloeopsis* PCC6718 and *Chroococcidiopsis* PCC7203 (not shown). The model of DNA substitution used (HKY + G + I) was selected by a hierarchical likelihood ratio analysis implemented in ModelTest 3.06. Symbols for each lineage appear in Fig. 2. Bootstrap values are indicated for likelihood, neighbor joining, and parsimony analyses for 1,000/10,000/10,000 pseudoreplicates.

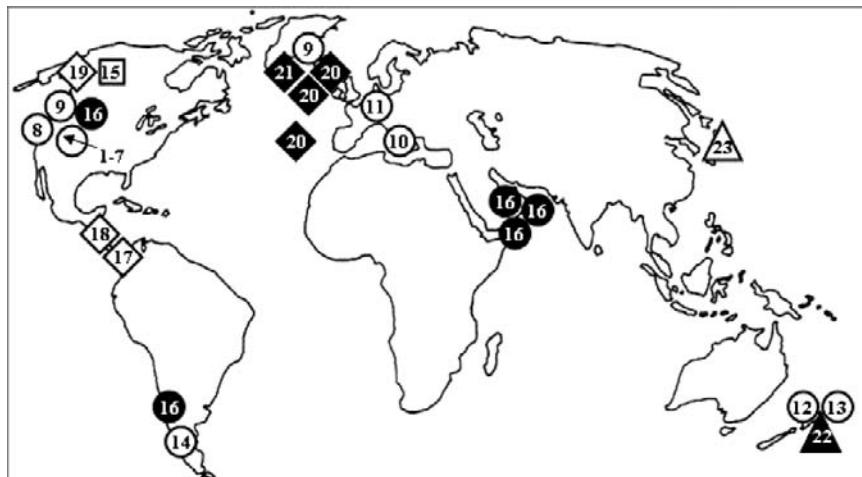


Figure 2. Genetic diversity of *M. laminosus* on a global scale. The symbols represent the seven 16S rRNA groups depicted in Fig. 1. Numerals refer to individual haplotypes observed in the survey based on approximately 3 kbp of sequence data obtained for three functional genes involved in nitrogen metabolism (see text for details).

Montana, and Chile), most haplotypes were only observed at a single location. For example, within Yellowstone National Park, haplotypes 1–4 are unique to White Creek in the Lower Geyser Basin, haplotype 5 to Boiling River, haplotype 6 to Chocolate Pots and haplotype 7 to Obsidian Pool. This suggests that genetic differentiation between populations generally occurs on very local geographic scales, despite the greater dispersal capability of *M. laminosus* compared with other thermophilic cyanobacteria such as *Synechococcus*.

Why this local differentiation in an apparently strong disperser? Factors in addition to dispersal capability appear to be required to explain the existence of both the wide geographic distribution of some haplotypes and the genetic differentiation among local populations of *M. laminosus*. While dispersal probability is the primary factor determining colonization of new suitable habitat, the probability of a migrant successfully establishing itself at a populated site is expected to depend not only on its ability to disperse to the location, but also on other factors, including its relative fitness compared with the extant population. For example, an established population could act as a preemptive barrier to migration simply by occupying substrate.

Because geographic barriers to migration appear to be important in this system, we would expect there to be a positive association between the amount of genetic divergence between a pair of strains and the geographic distance that separates their sites of origin. We investigated whether there is indeed evidence for isolation by distance in *M. laminosus*. To do so, we used correlation analysis to examine the relationship between nucleotide diversity (the probability of two sequences having a different nucleotide at a site) and the distance separating each pair of sequences for members of the most abundant 16S rRNA group in the dataset (open circles in Fig. 1). There was a strong and statistically significant positive relationship between these variables (unpublished data), providing further evidence for the importance of dispersal barriers in shaping the observed geographic patterns of genetic diversity in this group.

The major phylogenetic groups identified in the CCME survey exhibit interesting differences in thermal performance, particularly with respect to the maximum temperature permissive of growth. Whereas the thermal limit of strains from certain 16S rRNA lineages (open triangle and open circle in Fig. 1) was observed to be 55°C in batch culture experiments, others were capable of growth at 57°C (strain CCME 5268, closed circle and open diamond in Fig. 1). There was no obvious relationship between the temperature of the collection from which the strain had been isolated and its thermal maximum. The ecological importance of these physiological differences therefore requires further investigation. Because the phylogeny of these 16S rRNA lineages is currently unresolved (Fig. 1), it is also not possible at the present time to infer with confidence the direction that phenotypic evolution has taken during *M. laminosus* diversification (e.g., whether strains with a higher thermal limit were derived from less thermotolerant ancestors).

3. Regional Diversity: Life Under Different Nutrient Regimes

As noted above, populations of *M. laminosus* within geographic regions such as the greater Yellowstone area are genetically differentiated from each other. This raises the question of whether populations are locally adapted to the prevailing chemical and physical conditions of their environment, which is a fundamental question in the study of microbial diversity. We have investigated this issue for two populations of *M. laminosus* within Yellowstone National Park that exhibit dramatic phenotypic differences in situ as a result of environmental differences in nitrogen availability. White Creek is a thermal discharge fed stream in the Lower Geyser Basin that is dominated in biomass by *M. laminosus* where mean annual temperature is between 39 and 54°C (unpublished data). Combined nitrogen is undetectable in this system, and heterocyst-forming *M. laminosus* fixes nitrogen at appreciable rates as assayed by acetylene reduction (~150 nmol ethylene produced per µg Chl *a* per hour; Miller et al., 2006). In contrast, Boiling River, a short (~150 m) channel of Mammoth Hot Springs outflow located approximately 50 km from White Creek, is nitrate-replete (130 µg/L). *M. laminosus* from this site does not develop heterocysts, and ethylene production in acetylene reduction assays did not differ from background levels (Miller et al., 2006).

We have recently investigated the degree of genetic differentiation between these populations in considerable detail. Despite their close proximity, *M. laminosus* from White Creek and Boiling River are genetically distinct. No genotype was shared between populations, based on the distribution pattern of 25 polymorphic nucleotide sites from approximately 8 kbp of sequence data collected for six nitrogen metabolism genes (Miller et al., 2006). The average amount of divergence observed between a randomly chosen pair of sequences from the total sample was comparable to that observed for the human global population at noncoding autosomal loci (Yu et al., 2004), indicating that identity of these Yellowstone strains of *M. laminosus* at the 16S rRNA gene and the internal transcribed spacer of the *rrn* operon belie substantial genetic diversity. This diversity is simply at a different scale of evolutionary differentiation than is typically investigated in microbial ecology. Another way to quantify the amount of genetic differentiation between populations is with F_{ST} . This parameter takes on values between 0 (when different populations harbor the same alleles at a locus in the same proportions) and 1 (when the populations are fixed for different alleles). Observed values of F_{ST} at polymorphic loci were considerable, and ranged between 0.22 and 0.94 for the six nitrogen metabolism genes. For comparison, F_{ST} for two Yellowstone populations of the hyperthermophilic archaeon *Sulfolobus* was estimated to be 0.37 (Whitaker et al., 2003).

But are these genetically differentiated populations adapted for the utilization of the different nitrogen sources available in their respective environments? Given sufficient evolutionary time, we would expect that the relative fitness of each population will have increased on its available nitrogen source in response to

selection, and, conversely, for the ability to assimilate an unutilized nitrogen source (either because it is not available in the environment or because it is not preferred) to have declined due to relaxed selective constraints on loci involved in its metabolism. As a first step toward testing whether local adaptation has occurred in the Boiling River and White Creek populations, we assayed growth with either nitrate or dinitrogen as sole nitrogen source for ten randomly selected strains from each population (Miller et al., 2006). No differences in performance were observed between the populations when grown on either kind of medium: Growth rate constants μ (h^{-1}) for each population were approximately 0.02 when grown in the presence of nitrate, and about 0.017 under nitrogen-fixing conditions (Miller et al., 2006).

Why haven't these populations apparently adapted to the prevailing nutrient conditions of their respective environments? After all, they appear to spend generation after generation expressing idiosyncratic suites of nitrogen metabolism genes, while repressing others. Population genetic models suggest that local adaptation is mutation-limited, but also that the populations are expected to continue to diverge due to low migratory gene flow (Miller et al., 2006). This suggests that locally adaptive mutations may ultimately become fixed in their respective environments given sufficient time.

4. Local Diversity: Population Structure Along a Thermal Gradient

Whereas the changes in microbial community composition along certain environmental gradients are comparatively well understood (e.g., see Ward et al. (1998) for a review on community shifts along hot spring gradients), how *population-level* microbial diversity is partitioned along environmental gradients is still unknown, in large part due to the predominant use of slowly evolving markers such as the 16S rRNA gene. Yet there are several compelling reasons for exploring this largely hidden component of microbial diversity. Of central importance is the recognition that genetic differences between individuals within populations provide the raw material for *all* evolutionary diversification, whether that variation is generated by new mutations or by the acquisition of novel DNA from a donor. Consequently, it is at the population scale that one can investigate the *process* of diversification that produces the resultant patterns that we describe when we investigate diversity at the macroevolutionary (phylogenetic) scale. Second, genetic differences within populations may prove to be important for community function, particularly if different population members specialize on different regions along an environmental gradient. A population-genetic perspective on microbial diversity would also enable the empirical evaluation of predictions derived from experimental evolution studies of microbial populations in the laboratory.

One of the intrinsic challenges of investigating this issue with microorganisms is the difficulty of unambiguously identifying a distinct population that is distributed along a clear environmental gradient. *M. laminosus* from White Creek

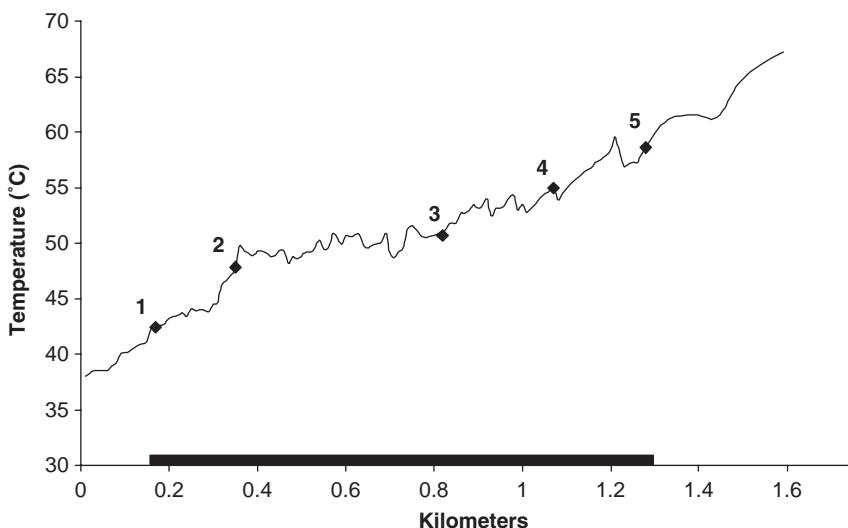


Figure 3. White Creek thermal gradient on September 11, 2004, with the region occupied by *M. laminosus* indicated by the shaded bar. Collection sites for population structure analysis are also shown (diamonds).

in the Lower Geyser Basin of Yellowstone National Park provides just such a system. Benthic streamers of this bacterium dominate the biomass where the mean annual temperature is between approximately 39 and 54°C, a region of the channel that stretches for greater than a kilometer (Fig. 3). For perspective, this 15°C difference in mean temperature is comparable to that observed for a 15° latitudinal gradient in eastern US Atlantic coastal waters.

To test whether analogous differences in allele frequencies and thermal performance exist among *M. laminosus* that occupy different regions along the White Creek thermal gradient, we first made multiple live collections from five sites spanning the range of *M. laminosus* habitat (Fig. 3). Individual trichomes were directly isolated in laboratory culture from these collections to provide a representative sampling of *in situ* diversity that is not biased by selective enrichment. Clonal isolates were next characterized both genetically and phenotypically. Though these bacteria share identical nucleotide sequences at slowly evolving loci including the SSU rRNA gene and the downstream internal transcribed spacer, *M. laminosus* from White Creek had been found in a previous study to be variable at several nitrogen metabolism loci (Miller et al., 2006). We genotyped approximately 150 isolates at four of these loci (*narB*, *devH*, *nifH*, and *nirA* (nitrite reductase). A total of 14 multi-locus haplotypes were observed in our sample (Table 1). These were not distributed evenly across sites, indicating the existence of population structure along the White Creek gradient. Most haplotypes were found

Table 1. *M. Laminosus* Haplotype Frequencies Along the White Creek Thermal Gradient.

Haplotype ^a	Frequency at site (%)				
	1	2	3	4	5
2232	8.3	8.3	50.0	29.3	27.9
2122	41.7	16.7	—	—	—
2132	—	—	8.8	2.4	30.2
2121	—	16.7	—	—	—
2231	—	—	—	—	11.6
3131	8.3	8.3	23.5	19.5	2.3
3121	—	8.3	5.9	9.8	7.0
3122	16.7	33.3	—	—	—
3232	—	—	—	17.1	16.3
3331	—	—	—	—	2.3
3321	—	8.3	—	—	—
3421	—	—	11.8	22.0	—
4323	16.7	—	—	—	2.3
4233	8.3	—	—	—	—

^aFour-digit haplotype designation indicates allelic identities at *narB*, *devH*, *nifH*, and *nirA*.

at two or fewer sites, and only two were observed at all sites. Some haplotypes were most abundant at gradient extremes, e.g., 2122 and 2132 are the most frequently observed haplotypes at site 1 and site 5, respectively. An analysis of molecular variance (AMOVA) indicated that roughly 13% of the total variation in the sample was due to differences in genetic composition among sites (unpublished data), which is comparable to the amount of molecular variation observed among geographic regions for the human global population (Excoffier et al., 1992).

The observed nonrandom associations between haplotype frequencies and environmental temperature may be the result of adaptation along a selection gradient. Preliminary investigations suggest that some haplotypes have indeed diverged with respect to thermal performance. For example, haplotype 2122 (only found at lower temperature sites 1 and 2) and haplotype 2132 (abundant only at site 5 and absent from sites 1 and 2) exhibit dramatic differences in thermal dependence of growth despite sharing identical alleles at three of the four loci examined. Whereas 2122 strains outperform 2132 at 37°C (the approximate temperature of site 1), haplotype 2132 strains double ~2 times faster than 2122 strains at 55°C (the approximate temperature of site 5; unpublished data). These results suggest that the population structure observed along the White Creek thermal gradient may in part reflect differences in thermal ecology among genetically divergent population members, and that at least some haplotypes have specialized on the local conditions of their respective environments.

Temperature dependence of dry weight-normalized phycobiliprotein content is qualitatively similar to that of growth, although the difference between haplotypes in the amount of these light-harvesting proteins is most striking at

37°C (unpublished data). At this temperature, haplotype-specific differences in phycobiliprotein content explain a large fraction (86%) of the variation in fitness between 2122 and 2132. By contrast, differences in phycobiliprotein content at 55°C explained little (7%) of the differences in fitness. These differences in the relationship between light-harvesting pigment composition and fitness at 37 and 55°C, respectively, suggest that the genetic basis of adaptation to temperature extremes along the White Creek gradient differs. At the former temperature, 2132 strains may be experiencing either chronic nitrogen deprivation and/or light stress. Production of phycobiliprotein-containing phycobilisome complexes requires a heavy investment in protein, and, consequently, degradation of these supramolecular light-harvesting antennae is an early response to perceived nitrogen limitation and light stress in cyanobacteria (e.g., Collier and Grossman, 1992). Elucidating the genetic basis of observed differences in thermal performance of these haplotypes at gradient extremes will be an exciting challenge!

5. Summary

Recent studies of the distribution of genetic diversity in distant hot springs have demonstrated the existence of geographic structure for the cyanobacteria *Synechococcus* and *Oscillatoria cf. amphigranulata* (Papke et al., 2003) and for the acidophile *Sulfolobus solfataricus* (Whitaker et al., 2003). Here, I have described examples of genetic differentiation at distant as well as much finer geographic scales for the cosmopolitan cyanobacterium *M. laminosus*.

With accumulating evidence for microbial biogeographical structure from extreme environments as well as other habitats (reviewed by Hughes et al., 2006), the next step is to better understand how these patterns are generated by evolutionary and demographic processes. For example, gene networks (Posada and Crandall, 2001) can provide insight into the relative ages of alleles at a locus, thereby potentially enabling inference of the centers of dispersal from which younger alleles have spread by migration. Gene networks reconstructed from the patterns of global sequence variation observed for *M. laminosus* at nitrogen metabolism loci suggest that alleles sampled from western North America are older than, and in some cases gave rise to, alleles found in other parts of the world. This suggests that the evolutionary origins of much of the extant *Mastigocladus* diversity on the planet might be traced back to geothermal activity associated with the hot spot that currently lies under Yellowstone National Park. Enhancing our understanding of the evolutionary origins of microbial diversity will also require the linking of genetic and phenotypic variation on a geographic scale. Our evidence for differences in thermotolerance of *M. laminosus* strains at different phylogenetic scales, from between 16S rRNA groups to within a population distributed along an environmental gradient, highlights the likelihood that genetically divergent microorganisms will often exhibit phenotypic differences of potential ecological importance.

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THE THERMOPHILIC CYANOBACTERIA OF THE ZERKA MA'IN THERMAL SPRINGS IN JORDAN

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1. Introduction

Thermal springs are found all over the world. Many such springs are found in Africa, Iceland, Italy, Japan, and New Zealand, with Yellowstone National Park, USA having the largest concentration of hot springs (Brock, 1978). The source of the heat in these waters varies with location and can be due to direct contact of the water with magma in volcanic active location such as at Yellowstone. In other cases the water can penetrate to such depths that it comes in contact with hot rocks.

The Dead Sea Rift, being part of the Syrian–African Rift Valley, is rich in thermal springs. Some are freshwater springs, others are saline or hypersaline. Examples are Hamat Gader (up to 52°C) and the hot springs of Tiberias (up to 60°C). The area of the Dead Sea, on the border between Jordan and Israel, contains many springs which differ in their physical and chemical properties. The eastern bank of the Dead Sea is especially rich in thermal springs. These include the springs of Zara on the Dead Sea shore (the ancient Kallirhoe; Donner, 1963), with temperatures up to 59°C, and the hot springs of Zerka Ma'in, 5 km inland, up to 63°C (Abu Ajamieh, 1980) (Fig. 1).

Specialized macro- and microorganisms inhabit the springs, each adapted to its habitat, and the cyanobacteria form a prominent part of these biota. Hot springs worldwide are inhabited by dense communities of cyanobacteria adapted to life at high temperatures. The most thermophilic microorganisms known thrive at temperatures above 110°C (Blöchl et al., 1997; Kashefi and Lovley, 2003). However, a temperature around 74°C appears to be the upper limit for photosynthesis (Castenholz, 1969; Brock, 1978). The cyanobacteria most tolerant to high temperatures are unicellular forms (*Thermosynechococcus*), which thrive in North America (e.g., the hot springs of Yellowstone), Japan and the eastern Mediterranean (Castenholz, 1969) but filamentous cyanobacteria also abound in hot springs worldwide (Copeland, 1936; Castenholz, 1969, 1984, 1996; Brock, 1978).

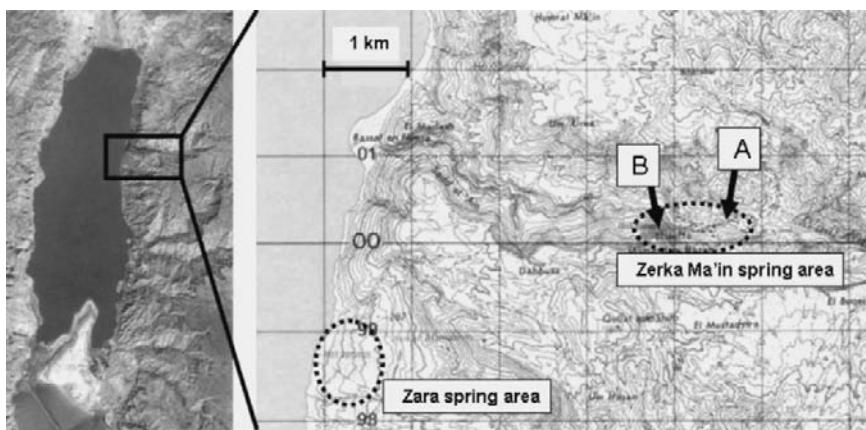


Figure 1. Map showing the location of the Zerka Ma'in and the Zara hot springs east of the Dead Sea; the sampling sites in the Zerka Ma'in spring area are indicated (right panel).

Very little is known about the phototrophic communities living in the hot springs in the Dead Sea area. A morphological description of the cyanobacteria of the Tiberias hot springs has been given (Dor, 1967), and taxonomic identifications of the species present in the Zara springs (limited to areas in the temperature range 35–40°C) were published long ago (Frémy and Rayss, 1938). The highest temperatures are encountered in the Zerka Ma'in springs, and information on the microbial communities inhabiting these springs is altogether lacking. The German explorer Ulrich Jasper Seetzen (1767–1811) wrote after his visit to the hot springs of Zerka Ma'in in 1807: “In dem Wasser wuchs eine grüne schleimige Conserve” [In the water grew a green slimy microscopic alga] (Seetzen, 1854). Blanckenhorn (1912) provided a somewhat more detailed macroscopic description of the green material in the springs, but to our knowledge no additional information has been published.

In this chapter we present a preliminary survey of the cyanobacterial community of the Zerka Ma'in hot springs, using both classic morphological characterizations and molecular ecological techniques.

2. The Sampling Site

The Zerka Ma'in springs (Abu Ajamieh, 1980, 1989; Swareih, 2000) consist of a large number of both natural and man-made orifices, outflow channels and waterfalls. Figure 1 shows the location of these springs, indicating the sampling sites. The main source of the water (sampling site A1) is located at a small shallow pond with walls partly covered by microbial mats (Fig. 2A). The water from site A1 flows via a pipe into a bigger pond, sampling site A2 (Fig. 2B). This



Figure 2. Photographs of the Zerka Ma'in sampling sites: (A), site A1 – the source of water; (B), site A2 – a pool into which water flows from site A1. The margins of the pool are covered with floating microbial mats; (C), site B1 – the three water pipes (arrows) that are source of water to site B; (D), site B2 – the end of the channel starting at B1. The water flow is separated into two channels. The main channel concentrates the majority of the water flow, while the water in the side channel (arrow) flows slowly, allowing for the development of a variety of colored microbial mats.

second pond is larger, deeper, surrounded by vegetation, and on one side covered with floating green microbial mats. The second area sampled is located about 500 m west of site A. The water at sampling site B1 flows out through three pipes (Fig. 2C) into a 50-m long channel (Fig. 2D), after which, creating a spectacular waterfall, it runs through wadi Zerka Ma'in towards the Dead Sea. The water running from site B1 forms several small basins and channels close to the edge of the cliff (sampling site B2). The temperature of the Zerka Ma'in water ranges from 63°C at site A to 58°C at site B. Table 1 presents data on the chemical properties of the waters.

3. Morphological Characterization of the Thermophilic Cyanobacteria of the Zerka Ma'in Springs

The microbial mats at sites A1 and A2 are colored dark-green. At sites B1 and B2 and along the channel connecting them, the dominating color is orange, with prominent patches of green material being present as well. Similar

Table 1. Chemical and physical characteristics of water samples collected at the different sampling sites of the Zerka Ma'in springs, as analyzed at the department of earth and environmental sciences, Yarmouk University.

	Sampling site			
	A1	A2	B1	B2
Temperature (°C)	63	62	58	58
pH	6.69	6.62	6.38	6.81
H ₂ S (mM)	0.3	0.3	0.03	NA
Conductivity ($\mu\text{S cm}^{-1}$)	2140	4180	2460	2470
Total dissolved salts (ppm)	1306	1267	1428	1445
Alkalinity (ppm)	120	110	130	130
Hardness (ppm)	560	520	540	580
[Cl ⁻] (ppm)	860.1	810.4	900.9	790.6
[NO ₃ ⁻] (ppm)	0	1.02	0.72	0
[SO ₄ ²⁻] (ppm)	197	196	210	211
[HCO ₃ ⁻] (ppm)	239 ¹	ND	ND	ND
[Mg ²⁺] (ppm)	90	91	93	92
[Ca ²⁺] (ppm)	185	186	193	195
[K ⁺] (ppm)	33	35	37	38
[Na ⁺] (ppm)	86.3	85.6	78.5	78.2
[Sr ²⁺] (ppm)	3.3 ¹	ND	ND	ND

ND = not determined.

¹As determined by I. Gavrieli, The Geological Survey of Israel.

colors have been reported from hot springs in other thermal environments (Brock, 1978).

Microscopic examination of the samples collected from the Zerka Ma'in springs shows a highly diverse community of cyanobacteria, unicellular as well as filamentous (Fig. 3). Site A1 was characterized mainly by unicellular organisms (Fig. 3A), while thin filamentous cyanobacteria dominated at site A2 (Fig. 3B). The in situ diversity of cyanobacteria at site B appears larger than that of site A. Spirally wound filaments reminding the genus *Spirulina* were abundantly found at site B (Fig. 3C and D). Similar tightly coiled filaments were previously reported from Mammoth Hot Springs, Yellowstone (*Spirulina* cf. *labyrinthiformis*), where they occurred at an upper temperature limit of 51–52°C (Castenholz, 1977). They have been found in other hot springs worldwide (Ward and Castenholz, 2000). In previous descriptions of hyperthermal springs, including the Zara springs in Jordan (Deranieh, 1990), orange mats were often reported to contain green anoxygenic photosynthetic, *Chloroflexus*-like organisms. We detected the fatty acids 15:0 and 17:0 in the orange material, found also in *Chloroflexus* mats at Yellowstone (Zeng et al., 1992). However, a microscopic examination of orange mats in the Zerka Ma'in springs showed prominent presence of a unicellular *Gloeocapsa*-like cyanobacterium (Fig. 3E), and their carotenoids probably contribute most of the color.

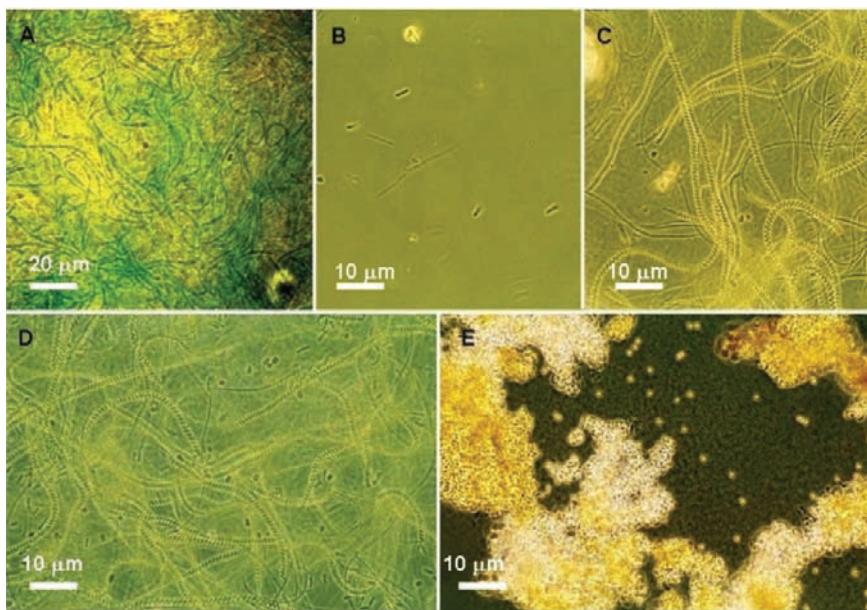


Figure 3. Phase-contrast microphotographs of cyanobacteria collected from site A1 (A), site A2 (B), site B1 (C), and site B2 (D). (E), Shows material from an orange mat developing inside the channel connecting sites B1 and B2.

We have set up enrichment cultures to isolate representative thermophilic cyanobacteria from the Zerka Ma'in springs. Part of these cultures were set up in BG-11 medium (Stanier et al., 1971); others contained only water collected from the site without further nutrient enrichment. After incubation at 55°C for periods up to 1 month, we obtained a variety of both unicellular and filamentous types (Fig. 4A–B and Fig. 4C–F, respectively). Some of the isolates resembled those seen in the original samples; others were of types not seen before. An example of the latter is seen in Fig. 4E and F: *Fischerella/Mastigocladus*-like filaments, which rapidly became dominant in part of the enrichment cultures. The orange-brown *Gloeocapsa*-like cells observed in the springs (Fig. 3E) were also obtained in culture (Fig. 4A and B). Cultured cells showed a thick capsule, which was less prominent in field-collected material.

The filamentous cyanobacteria depicted in Fig. 4E and F, being of a type not seen in the original samples, are of interest because in culture they developed heterocysts (Fig. 5). The occurrence of heterocysts in thermophilic filamentous cyanobacteria has been previously documented in *Fischerella* (*Mastigocladus*) spp. (Nierzwicki-Bauer et al., 1984; Stevens et al., 1985).

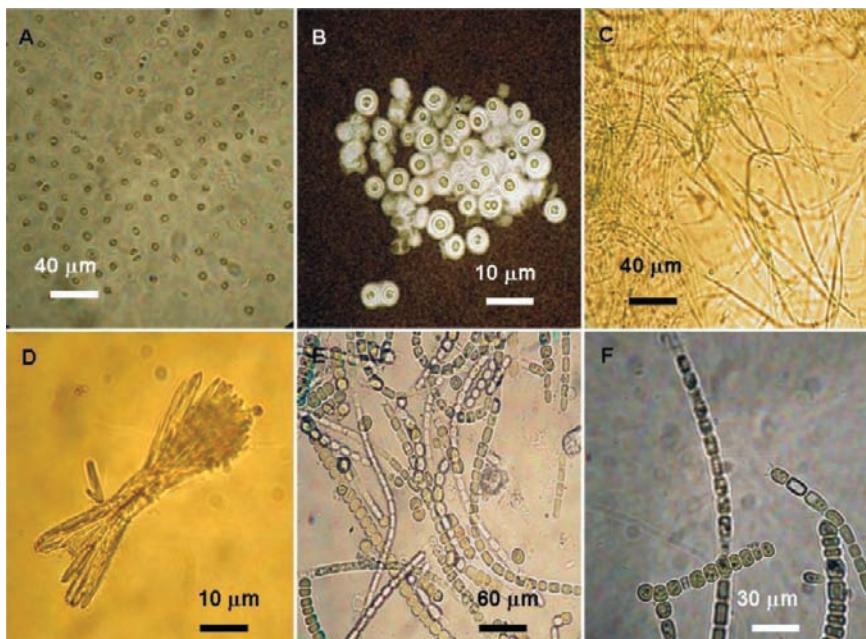


Figure 4. Phase-contrast photomicrographs of cyanobacteria obtained in culture: *Gloeocapsa*-like cells from site B1 suspended in water (tBTRCCn 23; A) and in India ink to better visualize the capsules surrounding the cells (tBTRCCn 28; B), thin *Oscillatoria*-type filaments from site B1 (tBTRCCn 302; C), a bundle of short trichomes from site B1 (tBTRCCn; D), and *Mastigocladius/Fischerella* filaments from sites A1 and B2 (tBTRCCn 101; E and tBTRCCn 403, F). The enrichment cultures are marked as temporary Bridging The Rift Culture Collection number (tBTRCCn).

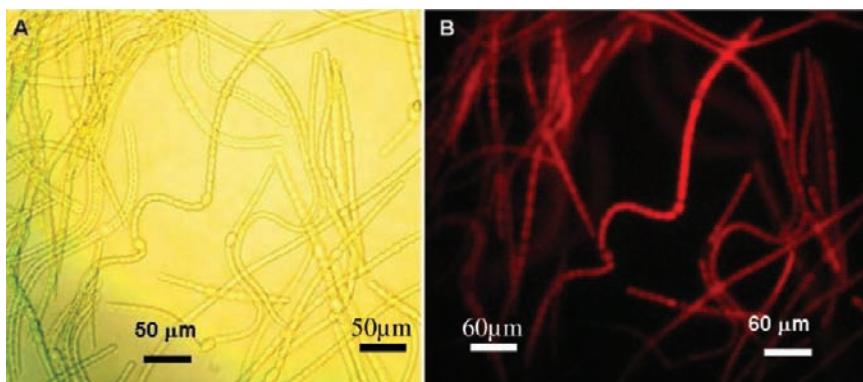


Figure 5. Heterocyst differentiation in a filamentous cyanobacterium (tBTRCCn 101) obtained in enrichment culture, as viewed by phase-contrast microscopy (A) and in the fluorescence microscope (B). Some of the heterocysts are indicated by arrows. Heterocysts do not show the red autofluorescence of vegetative cells caused by chlorophyll *a* and phycobilin pigments.

4. Phylogenetic Characterization of the Cyanobacterial Isolates from Zerka Ma'in

Morphological data on the isolated strains were supplemented with phylogenetic analyses based on 16S rRNA gene sequencing. A set of four primers was used to amplify the 16S rRNA genes, two of which are cyanobacteria-specific, and two are general eubacterial primers (Table 2). This allowed us to sequence over 1,400 bp of the gene.

The sequences were aligned and plotted as a tree using the neighbor joining algorithm (Fig. 6). The heterocystous cyanobacteria strains designated temporary Bridging The Rift Culture Collection number (tBTRCCn) 101 and 403 (Fig. 4E and F) showed 98% 16S rRNA gene similarity with *Fischerella major*, and cluster together with some other filamentous cyanobacteria from thermal environments in Australia and in the Philippines. The unicellular cyanobacterial strains tBTRCCn 23 and 28 (Fig. 4A and B) cluster together with *Chroogloeocystis siderophila*, with which it shows 98% 16S rRNA gene similarity. Interestingly, the recently isolated *C. siderophila* was originally found in iron-rich thermal environments in Yellowstone, and requires as much as 30 µM iron for optimal growth (Brown et al., 2005). Chemical analyses of the Zerka Ma'in spring waters showed far lower iron concentrations, ranging from 0.4 µM (Khoury et al., 1984) to 2.2–3.6 µM (Rimawi and Salameh, 1988). The thin filamentous isolate tBTRCCn 302 (Fig. 4C) clusters among other filamentous organisms, some of which (such as *Oscillatoria J24*) originate from thermal environments.

Culture-independent 16S rRNA gene sequence-based approaches have often been used for the characterization of cyanobacterial communities in hot springs since the pioneering work of Ward and coworkers (1990, 1994, and 2000) in Octopus Spring, Yellowstone National Park. *Synechococcus* sequences were recovered from the Yellowstone cyanobacterial mats developing at 50–55°C, and these were different from the sequences of the strains cultured from the site (Weller et al., 1992). We are currently applying such culture-independent techniques to the cyanobacterial mats of Zerka Ma'in to obtain a better picture of the types that dominate in the mats.

Table 2. Primers used to amplify cyanobacterial 16S rRNA genes.

Primer	Target organism	Sequence
27F	General ¹	AGA GTT TGA TTT ACG CGA CA
809R	Cyanobacterial ¹	GCT TCG GCA CGG CTC GGG TCG ATA
740F	Cyanobacterial ²	GGC YRW AWC TGA CAC TSA GGG A
1494	General	GGY TAC CTT GTT ACG ACT T

¹Derived from Pomati et al. (2004).

²F. Goh, University of New South Wales (personal communication).

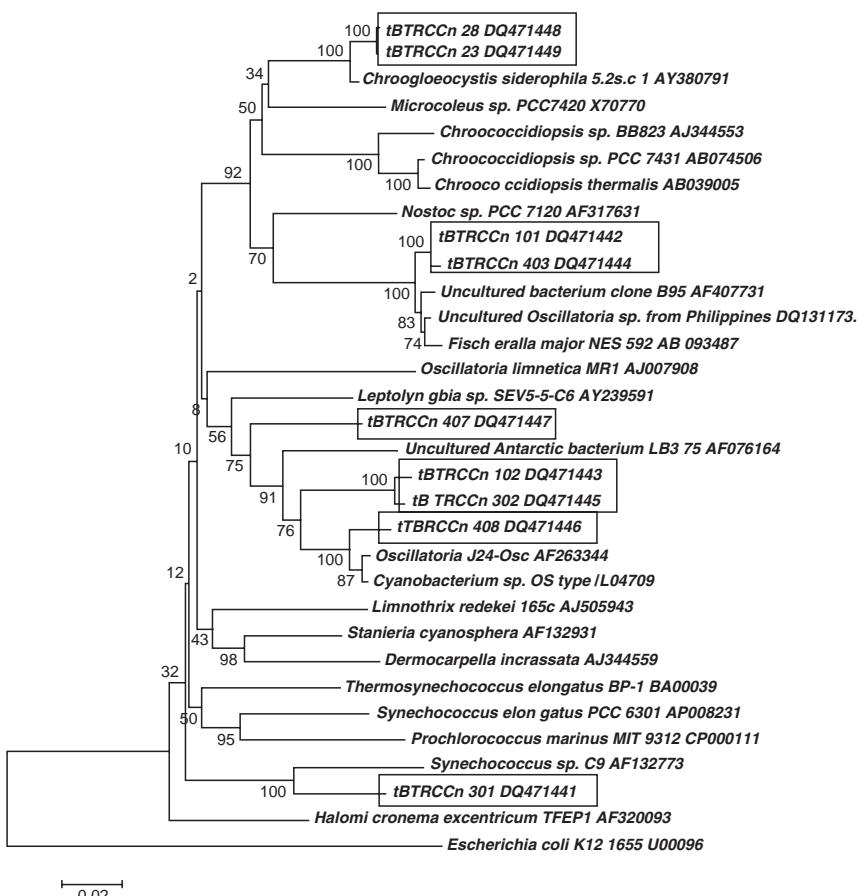


Figure 6. Phylogenetic tree showing the 16S rRNA gene sequences of cyanobacteria obtained in enrichment cultures from the Zerka Ma'in springs. The sequences were aligned using the ClustalW algorithm and were plotted as a tree using the Neighbor Joining algorithm. *Escherichia coli* K12 was used as the out-group. The enrichment cultures are marked as temporary Bridging The Rift Culture Collection number (tBTRCCn), and GenBank accession numbers are given after the strain designations.

5. The Potential for Nitrogen Fixation by the Zerka Ma'in Microbial Community

The Zerka Ma'in springs are low in inorganic nitrogen. No ammonium and nitrate-nitrogen was detected in the springs in September 1977 and between January and May 1978. Ammonium was found at concentrations between 0.4 and 1 ppm in October–December 1977, and 0.9–1.8 ppm nitrate was reported in October 1977 (Abu Ajamieh, 1980, 1989). We did not detect nitrate in the

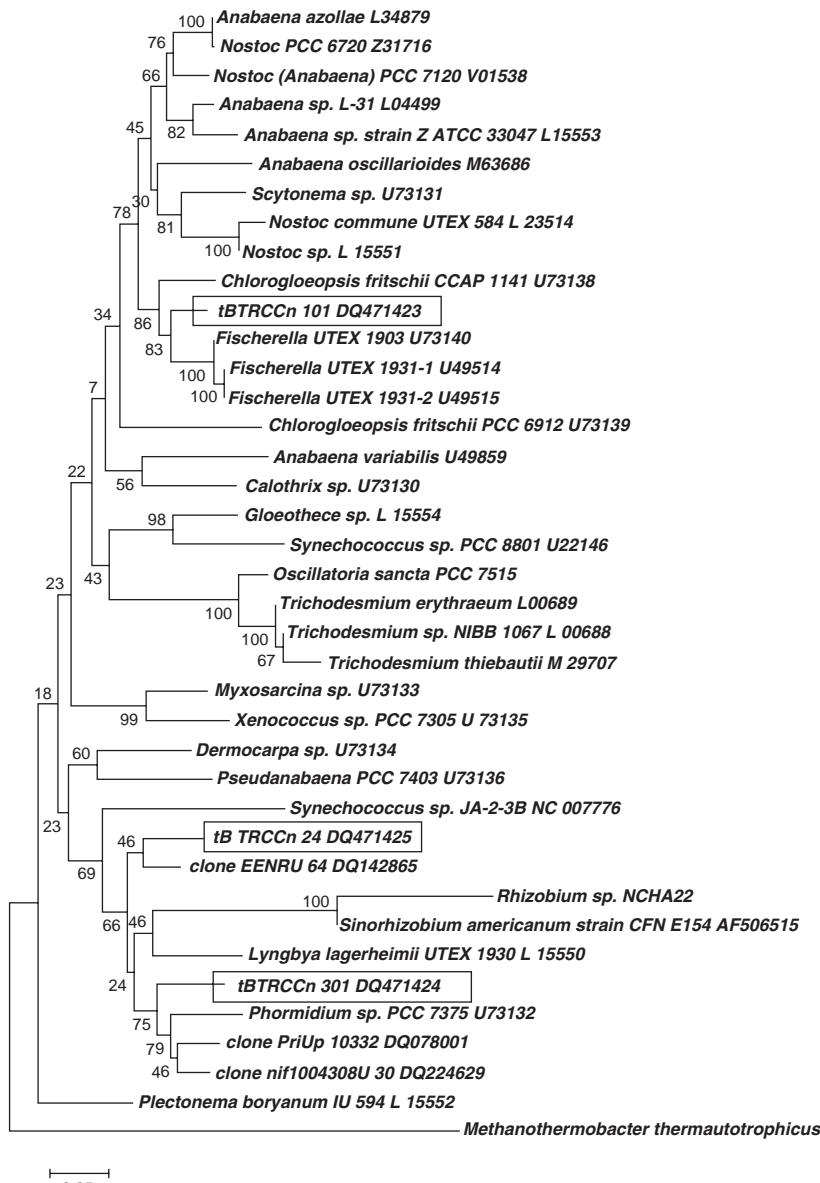


Figure 7. Phylogenetic tree showing the *nifH* gene sequences of several enrichment cultures from the Zerka Ma'in springs. The sequences were aligned using the ClustalW algorithm and were plotted as a tree using the Neighbor Joining algorithm. *Methanothermobacter thermautotrophicus* was used as the out-group. The enrichment cultures are marked as temporary Bridging The Rift Culture Collection number (tBTRCCn), and GenBank accession numbers are given after the strain designations.

December 2005 samples from sites 1A and 2B, and found 0.72 and 1.02 ppm in samples 1B and 2A, respectively (Table 1).

The isolation of the heterocystous *Fischerella/Mastigocladus*-like filamentous cyanobacteria from the Zerka Ma'in spring (Fig. 5) suggests that nitrogen fixation may occur in these springs. Studies of nitrogen fixation in *Mastigocladus*-containing microbial mats showed that this organism can fix nitrogen up to a temperature of 55°C (Fogg, 1952; Stewart, 1970; Wickstrom, 1980). In an attempt to further evaluate the nitrogen fixation potential of microbial community in the Zerka Ma'in thermal springs, we screened our enrichment cultures for the presence of the gene for dinitrogenase reductase, *nifH*, known as a good phylogenetic marker for nitrogen fixing prokaryotes (Zehr et al., 1997). We obtained three different *nifH* sequences (Fig. 7). The sequence obtained from strain tBTRCCn 101 and similar isolates showed 94% similarity with the *nifH* of *F. major* (with which it shares 98% 16S rRNA gene sequence similarity, see Fig. 6). The other two *nifH* sequences recovered cluster together with *nifH* of *Phormidium* sp., *Lyngbya* sp., and a number of environmental samples from the marine environment.

Whether or not the genes are expressed in the low-nitrogen, high temperature waters of the Zerka Ma'in remains to be determined.

6. Final Comments

The data showed above provide the first information on the nature of the cyanobacterial community in the Zerka Ma'in springs since its existence was indicated by Seetzen nearly 200 years ago.

The study presented here will form the basis for a more comprehensive study of the microbial community of the Zerka Ma'in springs and other unexplored hot springs in Jordan, in the framework of the Bridging the Rift Foundation, which aims at using science to improve the relationships between countries and fellow scientists in the Middle East.

7. Acknowledgments

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IRON-TOLERANT CYANOBACTERIA: *Implications for Astrobiology*

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1. Introduction

Iron is the fourth most abundant element in the Earth's crust and the most abundant element in the Earth as a whole (Ehrlich, 2002). General agreement is that life evolved in the presence of soluble iron concentrations much greater than those typical today (MacLeod et al., 1994; Emerson and Moyer, 2002) and that life is based, therefore, on redox processes mediated by iron compounds (Beinert et al., 1997).

The importance of cyanobacteria (CB) to the geophysical evolution of life on Earth cannot be overstated. In addition to contributions to Earth's biomass and poikilothermy¹ (Levit et al., 1999), cyanobacterial phototrophic activity from the Archean to present times is thought to have contributed significantly to present-day oxygen and carbon dioxide concentrations of our atmosphere (*ibid.*), thus driving the evolution of modern oxygen-dependent organisms. Even though the evolutionary path of oxygenic photosynthesis is well studied (Xiong et al., 2000; Xiong and Bauer, 2002; Olson and Blankenship, 2004; Iverson, 2006), what triggered the transition from anoxygenic to oxygenic photosynthesis is still not clear. The answer to this question might be obtained by the search for coincidences between the remarkable events in Earth and life's evolution.

Of all extant environments, iron-depositing hot springs may constitute the most appropriate natural models for analysis of the transition of ancestral CB [or protocyanobacteria (PCB) (Olson, 2001)] from anoxygenic photosynthesis to oxygenic ones and biogeochemical processes in the late Archean and early Paleoproterozoic eras.

¹ Poikilothermy is defined by Gorbushina and Krumbein (1999, 2000) as a special type of metabolic and morphological response to extreme spatial and temporal episodic changes in various environmental conditions, including temperature, humidity, solar irradiation, and nutrient supply.

The study of bacteria in iron-depositing hot springs may also be significant to the emerging field of astrobiology, particularly in the search for evidence of ancient life on the planet Mars (Allen et al., 2004). Research reviewed in this chapter details the chemical, mineralogical, and crystallographic properties of iron minerals associated with specific species of CB. If some of these minerals have unique properties that clearly identify them as biosignatures, they may prove to be valuable indicators of past life on the surface or in the rocks of Mars.

2. Ecology, Diversity, and Physiology of Iron-Tolerant CB

Because most iron-depositing hot springs are acidic, they do not support cyanobacterial growth (Brock, 1973, 1978; Amaral Zettler et al., 2002, 2003; Ferris et al., 2005); however, near-neutral springs do (Fig. 1).

The anoxic source waters in well characterized Chocolate Pots Hot Springs in Yellowstone National Park have temperatures of 51–54°C, pH of 5.8–6.0, and 45–200 µM dissolved Fe²⁺ (Pierson et al., 1999; Pierson and Parenteau, 2000; Trouwborst et al., 2004). The iron-depositing Bakreshwar hot spring (West Bengal, India) is characterized by temperatures of 66–69°C, alkaline pH, and a total iron concentration of approximately 1,000 µM (Ghosh et al., 2003). Those springs and other near-neutral iron-depositing hot springs are well populated with CB (Fig. 1a and b, green spots).

Very few studies dedicated to the diversity of CB inhabiting iron-depositing springs have been conducted despite insights into general cyanobacterial phylogeny and contemporary and ancient microbial iron transformations that such studies could potentially provide. Pierson and Parenteau (2000) used microscopy to identify several types of cyanobacterial mats in Chocolate Pots Hot Springs:



Figure 1. The iron formations being deposited at Chocolate Pots (left) and LaDuke (right) Hot Springs. The main source for Chocolate Pots is indicated by the white arrow head; CB mats by the white arrow. Physicochemical parameters for Chocolate Pots are in the text. The main source for LaDuke slope is about 25 m from the pictured frame. Parameters for this spot were temperature (62°C), pH (6.9), total iron (~7 µM). Warm water cascades over the mound faces. The colors are due to the mineral deposits and some mats (I. Brown).

Synechococcus sp.; *Cyanothece minnervae*, an olive mat consisting of a narrow *Oscillatoria* sp., which is the major mat that dominates the iron formation studied; and a *Pseudoanabaena* mat that was typical for the temperature range of 50–54°C. They also found *Chloroflexus*, an anoxygenic phototroph, in almost all mats. Wilson et al. (2000) revealed streamers of *Fischerella* in CB mats at ~50°C, and *Oscillatoria princeps* at lower temperature (~40°C). We microscopically verified *O. princeps* in LaDuke Hot Spring (slope side) at the same temperature (Brown, unpublished data) and confirmed the presence of *Fischerella* species in Chocolate Pots and LaDuke by molecular methods (clones JSC 3 and JSC 11 are a *Fischerella*, Fig. 2). Molecular phylogenetic exploration of Bakreshwar iron-depositing hot spring (West Bengal, India) revealed that all eight CB 16S rRNA clones examined exhibited 93–96% sequence homology to known species (Ghosh et al., 2003).

In 2002, Brown and collaborators began the polyphasic characterization of CB-inhabiting LaDuke (Montana) and Chocolate Pots iron-depositing hot springs (Yellowstone National Park). The work examined 15 unicellular isolates (Brown et al., 2005a, c, d; 2006a, c) and revealed the presence of 9 unknown species having 93–95% 16S rRNA gene similarity to published sequences (Fig. 2). Both phylogenetic and morphological analyses indicate that a number of these isolates, including *Chroogloeocystis siderophila*,² JSC 1 and JSC 10, represent new cyanobacterial genera (Brown et al., 2005a; 2006c), further suggesting that iron-depositing hot springs may select for novel CB lineages. Such cosmopolitan species as *Fischerella* (JSC 3 and JSC 11) and *O. princeps*, which are known to occur widely in different springs, however, were also isolated, suggesting potential adaptation to elevated iron concentrations. Further research is clearly needed to define relationships between cyanobacterial diversity of iron-depositing hot springs and iron tolerance.

Most research into the interactions between CB and iron is dedicated to the effects of iron limitation on different physiological and biochemical processes. Iron additions within the micromolar range were found to be insufficient, however, for optimal growth of some mesophilic, thermophilic, and marine CB (Gross and Martin, 1996; Benesova et al., 2000; Paczuska and Kosakowska 2003; Swingley et al., 2005). Pierson and coauthors (1999) found that ferrous iron concentrations up to 1 mM significantly stimulated light-dependent consumption of bicarbonate by CB isolated from iron-depositing hot springs, suggesting a specific role for elevated iron in photosynthesis by iron-tolerant CB.

Recent studies of the effect of elevated Fe³⁺ (added as FeCl₃) on iron-tolerant CB indicated that the maximal doubling times for four cultures occurred in the range of concentrations 0.4–1 mM Fe³⁺ while only 0.04 mM Fe³⁺ was sufficient for maximal doubling of *Synechocystis* PCC 6803 (Brown et al., 2005a; Brown and

² Recently, the group of A. Oren revealed a sister clone for *C. siderophila* – tBTRCCn 23 in the Zerka Ma'in springs (Jordan). Reference is in this book.

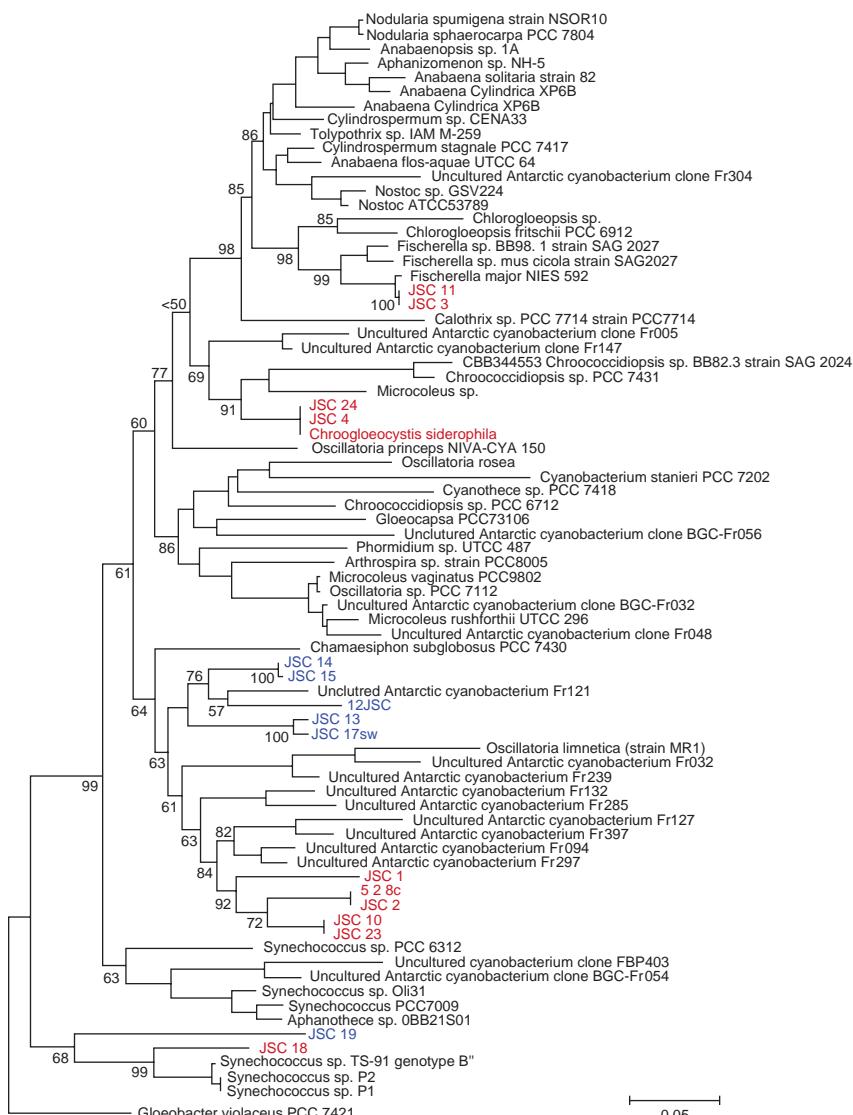


Figure 2. Neighbor-joining phylogenetic dendrogram depicting relationships between selected cyanobacterial species isolated from iron-depositing LaDuke and Chocolate Pots Hot Springs. Iron-tolerant isolates are depicted in color: red (LaDuke) and blue (Chocolate Pots). Phylogenetic analysis was provided by Dr. M. Podar. The tree is a maximum likelihood tree built with PHYLML using general time reversible model of evolution with gamma corrected rates (8 categories), parameter alpha estimated, and bootstrapping (100 replicates) under maximum likelihood with the same parameters.

Sarkisova, unpublished data). Concentrations of 5–10 mM Fe³⁺ completely inhibited iron-tolerant CB proliferation (Brown et al., 2006c). Pierson et al. (1999) showed the same range of toxicity for Fe²⁺.

The experiments of Brown et al. (2005a, b) were conducted under slightly alkaline conditions (pH 8), a pH at which nearly all iron would be expected to be in the form of Fe(OH)₃, a colloidal compound presumed to have a low ability to penetrate cyanobacterial cells. This is in agreement with the presence of an Fe³⁺ transport system in all sequenced CB, including *Thermosynechococcus elongatus*,³ although other mechanisms supporting biological availability of ferric iron for cyanobacterial metabolism may also be active.

In particular, Benderliev (1999) showed that the CB *Arthrospira* sp. and *Leptolingbia borianum* released chelators in the form of humic acids in response to increased Fe³⁺ concentration (54 µM). The release of Fe³⁺ chelators may be an adaptation for organisms living in oxygenated environments at neutral or alkaline pH with sporadic and irregular Fe supply, since chelator release results in a quick enhancement of Fe solubility and availability (*ibid.*).

Potential mechanisms by which CB may protect themselves from the negative effects of elevated intracellular Fe²⁺ include oxidation by photosynthetic O₂ and probably direct oxidation by either photosystem I (PS I) (Cohen, 1984, 1989) or photosystem II (PS II) (Pierson et al., 1999; Olson, 2006). Since CB are known to oxidize such different reduced inorganic elements and compounds such as H₂ and H₂S through PS I (Cohen et al., 1975; Belkin and Padan, 1978), PS I-facilitated oxidation of ferrous iron seems plausible though direct evidence is lacking.

Pierson et al. (1999) found that 3-(3', 4'-dichlorophenyl)-1,1-dimethylurea, an inhibitor of electron transfer between PS II and PS I, prevented stimulation of photosynthesis in iron-tolerant CB by 1 mM Fe²⁺. They interpreted this result as supporting the hypothesis that ferrous iron can be directly oxidized by PS II (RC2); however, these authors also observed a clear diurnal cycle for O₂ evolution in cyanobacterial mats of iron-depositing hot springs. Oxygen production in iron-tolerant cyanobacterial mats should be insensitive to light level if Fe²⁺ works as a donor for PS II. Reduced iron may thus have a secondary effect on PS II activity in iron-tolerant CB although there could be specific conditions for direct oxidation of Fe²⁺ in RC2 that do not affect the water-splitting complex.

Brown et al. (2005a; 2006c) reported thick polysaccharide sheaths around cells of iron-tolerant CB. In some cases, sheath thickness may reach 1 µm (Fig. 3). These sheaths serve as a repository for precipitated iron (Fig. 4) and might additionally serve to concentrate humic acids and moisture in cyanobacterial mats. Extracellular accumulation of iron by iron-resistant CB may potentially serve two functions: to decrease the chemical potential of active (accessible for cells) iron and to produce a pool of reserve iron for times of low iron availability (Brown et al., 2005a).

³ <http://www.kazusa.or.jp/cyano/Thermo/index.html>

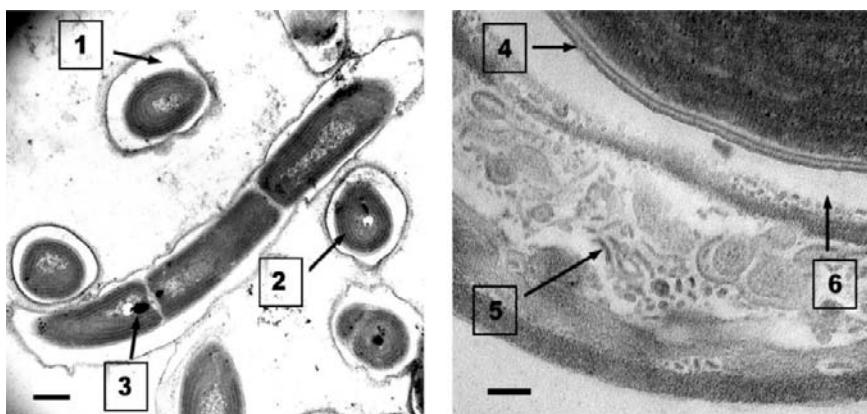


Figure 3. Transmission electron micrograph of the isolate JSC 1 s.c.1 cell cross sections: (1) thick sheath around a CB cell, (2) concentric thylakoids on cross section, (3) cyanophycin granules, (4) out membrane, (5) vacuole inside exopolymeric sheath inhabited with bacteria, (6) exopolymeric sheath. Bars: 500 (L), 100 (R) nm (I. Brown).

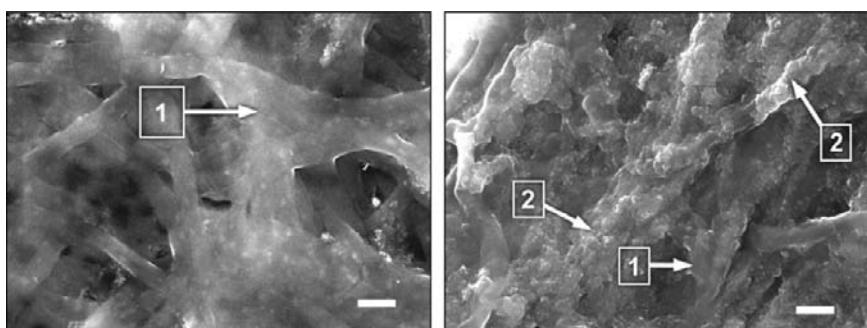


Figure 4. Scanning electron micrographs of *Phormidium* aa and isolate 14.3 fil.2 grown in DH medium supplemented with 0.6 mM FeCl₃. SEI mode, Bars = 2 µm (I. Brown).

Iron oxides precipitated on microalgal sheath were shown to be an efficient screen for ultraviolet light (Bishop et al., 2006a, b).

In summary, one may conclude that elevated concentrations (>40 µM) of both forms of environmental iron (Fe²⁺ and Fe³⁺) affect the metabolism and proliferation of iron-tolerant CB, although intimate mechanisms of this phenomenon are not clear yet. The same iron concentrations were found to be toxic for mesophilic CB (Brown, unpublished data), probably because of the loss of either detoxification or protective mechanisms, which may have been possessed by ancient CB and preserved in contemporary iron-tolerant CB.

3. Biogeochemical Activity of IT CB and Implications for Paleogeobiochemistry

The overarching assumption is that iron-tolerant CB were the most abundant organisms with the ability to oxidize iron in the relatively shallow areas of the warm, iron-saturated late Precambrian ocean. Understanding the patterns of iron oxidation by CB has great importance for paleogeobiochemistry since CB are presumed to have been involved in global oxidation of ferrous iron in the Precambrian era (Cloud, 1973).

Pierson et al. (1999) were the first to find that cyanobacterial members of iron-depositing bacterial mat communities might increase the rate of iron oxidation in situ. They noticed, however, that the activity of cyanobacterial mats does not lead to a significant decrease of Fe^{2+} since even in very active oxygenic cyanobacterial mats the oxygen is depleted within a few millimeters of the surface (Canfield and Des Marais, 1993; Little et al., 1997). Furthermore, in the absence of oxygen, anaerobic microbial activity can reduce $\text{Fe}(\text{III})$ to $\text{Fe}(\text{II})$ (Dobbin et al., 1996; Ghosh et al., 2003; Konhauser et al., 2005). Consequently, just beneath the surface of the microbial mat–water interface, the environment can be quite anoxic and rich in $\text{Fe}(\text{II})$. The suggestion that oxygenic photosynthesis led to global iron oxidation is, therefore, open to question.

Pierson and Parenteau (2000) initially identified fossil phototrophs in the lower depths of cyanobacterial mats in iron-depositing hot springs. Amorphous ferrihydrite was identified as the primary phase associated with the mats and encrusted cells. Deposition of colloidal silica and iron silicates on the cells was also detected. The same phenomenon was found in our studies (Fig. 5). Other mineral phases (goethite and siderite) were also identified in dry mineral samples on the surface of the main iron deposit. Parenteau et al. (2003, 2005) subsequently found that iron precipitation is a species-specific process although the effect of local pH should be considered .

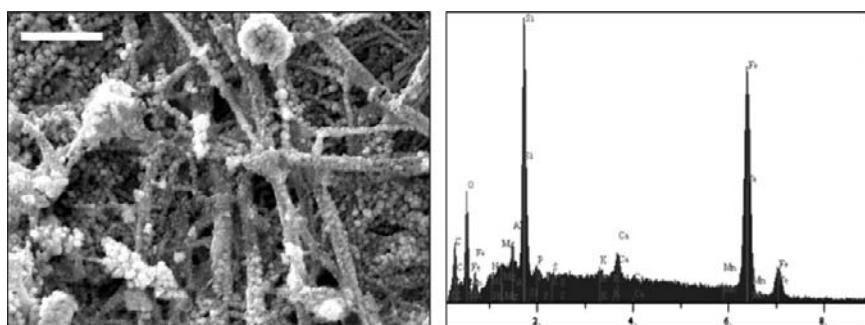


Figure 5. Scanning electron micrograph and elemental analysis of mineralized cyanobacterial mat (sample 14.3) isolated from main mound of Chocolate Pots Hot Springs. Picture in SEI mode, bar = 10 μm (left); energy dispersive x-ray spectroscopy spectrum (right) (I. Brown).

Experiments conducted by Brown et al. (2005a, b, c) have revealed that iron precipitation on cell sheaths⁴ is accompanied by simultaneous precipitation of P and Ca. Particles of colloidal iron apparently serve as nucleation centers for phosphate precipitation. Brown et al. (*ibid.*) also found that iron precipitation by CB might be an exclusive property of iron-tolerant species. This assertion is based on the observation that the growth of the mesophilic CB *Phromidium* aa (generous gift of Dr. K. Palinska) in iron-saturated (0.6 mM) DH medium did not lead either to the inhibition of CB growth or to iron precipitation on its filament surfaces (Fig. 4, left) while 14.3 fil.2 culture, isolated from Chocolate Pots Hot Springs and incubated under the same conditions, was covered with strong layer of precipitated iron (Fig. 4, right). These findings suggest that mineral precipitation by CB might be a genetically determined phenomenon rather than a simple physicochemical process; however, different interpretations are possible.

Recent studies (Brown et al., 2006b) have revealed that iron-tolerant CB are also capable of biodeterioration (the etching of minerals), in particular glasses enriched with Fe, Al, Ti, O, and Si (Fig. 6). These bacteria likely leach rocks by

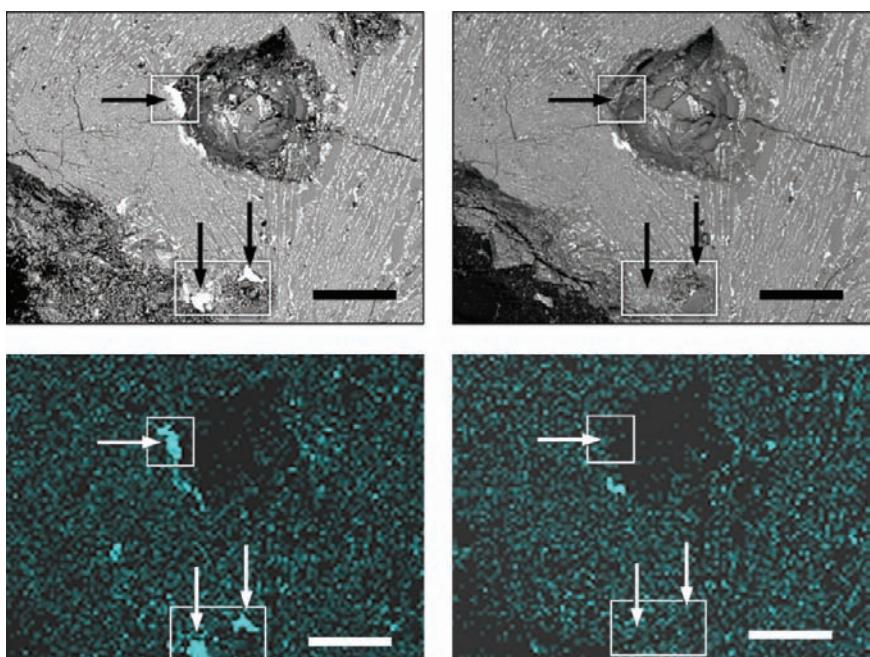


Figure 6. Deterioration activity of siderophilic CB, isolate JSC 1. SEM micrographs, magnification 5,000X, bar size – 100 µm (top left and right). EDS elemental maps. Arrows indicate areas and mineral grains before (bottom left) and after (bottom right) leaching by CB.

⁴ Cyanobacteria were cultivated in DH medium <http://www.atcc.org/common/documents/mmediapdfs/2453.pdf>.

the production of organic acids although siderophores, which are organic Fe^{2+} metal chelating molecules, solubilizing Fe^{3+} from the environment (Gress et al., 2004), might be responsible, also. This observation suggests that iron-tolerant CB are able to participate in the turnover of Fe, Al, O, Ti, Si, and other rock-forming elements. Thus Precambrian iron-tolerant CB and their predecessors could have been involved not only in iron deposition but also in the global release of elements. The abundance and wide distribution of iron-bearing minerals on Earth and in the solar system make bioweathering features in these minerals potentially important new biosignatures.

Prior to 2.4 Ga, global oceans were likely significantly enriched in soluble iron (Roussel et al., 2005), a condition that is not conducive to the growth of most contemporary mesophilic CB. Recent studies of the mechanisms of iron-deficiency stress in CB suggest that contemporary mesophilic freshwater and marine CB underwent long-term adaptation to a permanent decrease in soluble iron in the ocean environment (Boyer et al., 1987; Braun et al., 1998). The characteristics of these organisms are not, therefore, appropriate to model global CB evolution (Blank, 2004; Sanchez-Baracaldo et al., 2005), and these papers may actually describe the postoxygénéation history of CB rather than the history of the predecessors of “true” CB.

The dating of Earth’s oxygenation is tightly bound to parallel questions about the time sequence of the evolution of CB, carbonates sedimentation, and the generation of banded iron formations. The data obtained for iron-tolerant CB may help to model the principal features of the predecessors of contemporary CB living in the iron-saturated Precambrian ocean and the patterns of their evolution.

In particular, Olson (2001) proposed the definition for PCB and postulated that the common ancestor of PCB and CB might well have used Fe(OH)^+ as the principal electron donor for CO_2 fixation (Pierson and Olson, 1989; Widdel et al., 1993; Ehrenreich and Widdel, 1994; Olson, 2006). Olson (2001) proposed that the driving force for the evolution of RC2, in addition to RC1, was the necessity to use Fe(OH)^+ effectively for CO_2 fixation in the absence of reduced sulfur compounds while the driving force for the transition from anoxygenic to oxygenic photosynthesis was the global decrease of environmental reduced iron.

PCB could have an advantage over iron-oxidizing proteobacteria because the combination of both photosystems in the same organism yielded a significant gain for ferrous iron oxidation since homodimeric RC1 and homodimeric RC2 functioned in series to deliver electrons from Fe(OH)^+ to NADP^+ while RC1 and/or RC2 separately drove cyclic electron flow for the production of ATP (Olson, 2001). Correspondingly, PCB could theoretically have stronger abilities to participate in the generation of banded iron formations than anoxygenic phototrophs.

Some authors propose that the strong decrease of environmental ferrous iron during the late Precambrian era is connected with the efficient oxidation of iron by anoxygenic phototrophic organisms (Ehrenreich and Widdel, 1994; Konhauser et al., 2002; Croal et al., 2004; Konhauser, 2004; Kappler et al., 2005), although the possible contribution of oxygenic phototrophs is also appreciated

(Stal, 2000). Konhauser et al. (2005) hypothesized that iron-reducing bacteria could support relatively high Fe^{2+} concentrations in the Precambrian ocean at about 70% saturation levels; therefore, it is unlikely that the exhaustion of reduced iron due to its oxidation was the cause of the decrease in banded iron formation activity and the transition from anoxygenic to oxygenic photosynthesis. The Precambrian period was characterized by enormous tectonic movements that led to continent generation and dispersal (Davies, 1995; Lindsay and Brasier, 2002a, b; 2004). Around 2.3 Ga terrestrial volcanism and associated banded iron formation production significantly waned (Kump, 2005). We hypothesize (Brown et al., 2006c), therefore, that both processes may have decreased the reduced iron supply from deep mantle layers and terrestrial volcanoes. As a result, PCB likely switched to the photosynthetic oxidation of such different potential reductants as H_2O_2 (Blankenship and Hartman, 1998), bicarbonate (Dismukes et al., 2001; Ananyev et al., 2001), and water. The last major switch is dated about 2.7 Ga and led not only to O_2 evolution but also to the ability to fix N_2 upon oxygenation in special cells called heterocysts (Tomitani et al., 2006).

4. Implications for Astrobiology

Could microorganisms resembling CB and/or their predecessors exist or have once existed on Mars? The present Martian surface is extremely hostile to all known forms of life; however, the discovery of ancient and perhaps even recent volcanism, coupled with evidence of episodic water flow and glaciations, suggests the possibility of local conditions that could support cyanobacterial proliferation (Horvath et al., 2004).

Key parameters in this discussion are the concentration of oxygen in the Martian atmosphere and iron in the Martian crust during the first billion years of the planet's history – a time when the Martian atmosphere was much different from the atmosphere today – and significantly earlier than the major oxygenation on Earth (Lindsay and Brasier, 2002b; Catling, 2006). The modern Martian crust has higher iron content than the terrestrial crust; however, the apparent discontinuance of global Martian tectonics around 3.0 to 3.5 billion years ago (Lindsay and Brasier, 2002b) could have led to a significant decrease in the Fe^{2+} supply. Any Martian PCB, like their Earth counterparts, might have had to switch from anoxygenic to oxygenic photosynthesis about that time. Nevertheless, they could remain in such an environment since Martian soils containing iron could establish a favorable radiation environment for photosynthesis (Cockell and Raven, 2004).

A restriction on oxygenation of the Martian atmosphere by organisms with the characteristics of CB could be the very significant acidity that led to formation of the sulfate minerals prevalent in many of the planet's surface rocks (Fairen et al., 2004) since terrestrial CB exhibit low tolerance to acidic conditions (Brock, 1973, 1978). These species have never been found in environments with pH below 4. CB arose on Earth several billions years ago, likely in near-neutral or alkaline

environments (Zavarzin, 1993; Brown, 1994). These organisms diversified to occupy many different environmental niches, but acid-tolerant CB never developed.

The apparent acidity of the present Martian surface, however, may not eliminate the possibility of PCB or their oxygenic descendants. Near-neutral wet environments prevailed approximately 4 Ga according to the alteration history of Mars derived from recent multispectral mapping (Bibring et al., 2006). Martian areas displaying high concentrations of phyllosilicates may be more likely to contain traces of life than areas dominated by acidified sulfates.

To summarize the case for CB on Mars, there may have been temporal and spatial niches for the existence of the early Martian equivalents of PCB and classical CB that could have participated in the weak oxygenation of Mars. In addition, acidification of the Martian surface (possibly associated with volcanism) could have prevented further activity and proliferation of CB and thus led to the loss of oxygen and the increase in the CO₂ concentration that characterize the modern Martian atmosphere.

The further evolution of Martian CB, if any, seems very unlikely under the present surface conditions; however, remnants of such organisms might be protected in the frozen subsurface or in iron-bearing mineral deposits. This scenario could form the basis for an exploration strategy in the search for evidence of ancient Martian life. Clearly some of the subsurface of Mars is not highly acidic because indigenous carbonates have been described in many Martian meteorites (Wentworth et al., 2005).

5. Conclusions

Analyses of published data, combined with our own recent work on the ecology, physiology, diversity, phylogeny, and biogeochemical activity of iron-tolerant CB, have led us to conclude that CB inhabiting iron-depositing springs appear to be a new group of extremophiles. This group of CB is highly diverse and consists of deeply rooted species (Fig. 2, JSC 18). Siderophilic CB possess such unique poky-lotrophic features as very thick extracellular sheaths, the ability to precipitate mineralized iron on sheath surfaces, and the ability to readily leach iron-bearing minerals and amorphous phases of iron.

The significant stimulating effect of iron on the photosynthesis of iron-tolerant CB, taken together with the apparent ability to oxidize reduced iron, makes this group of organisms the best current candidates to use in studying the evolution of CB per se as well as the evolution of oxygenic photosynthesis. Since the main course of life's evolution coincides with the significant decrease of reduced environmental iron, iron-tolerant CB might well be the oldest evolutionary group among their contemporary counterparts, most of which inhabit iron-deficient water bodies, including the world oceans.

This chapter supports the hypothesis that anoxygenic phototrophs rather than oxygenic ones were mainly responsible for banded iron formations (Kappler

et al., 2005). We do, however, acknowledge that PCB had express advantages in Fe^{2+} oxidation. This property, combined with the ability of CB to precipitate silica, allows speculation that the role of PCB and their closest descendants for banded iron formation generation was more significant than the role of proteobacteria for this process.

Do (did) iron-tolerant CB have counterparts elsewhere in the solar system? This could be possible. The photosynthetic model of Earth's oxygenation is a viable alternative to the chemical model (Beukes, 2004). Additionally, reduced iron was and is a key element for life's emergence. Life on Mars, if ever present, may not have been developed as extensively as life on Earth in part because the acidification of Martian crust (Navarro-Gonzalez et al., 2006) may have terminated further development of oxygenic photosynthetic organisms. Organisms resembling iron-tolerant CB may exist, or may have existed, however, on any planet with a significant concentration of iron in its crust and minimal oxygen in its atmosphere.

6. Summary

CB inhabiting iron-depositing springs represent a separate group of highly diverse and deeply rooted species of extremophiles that possess unique poky-lotrophic features. They require concentrations of dissolved iron at least one order of magnitude higher than the concentrations found in major terrestrial water bodies with the exception of iron-depositing hot springs.

The apparent ability of these organisms to conduct anoxygenic photosynthesis in the presence of elevated iron concentrations suggests that iron-tolerant CB could have been one of the "lost links" in the evolution of oxygenic photosynthesis. This chapter also supports the hypothesis that anoxygenic phototrophs, including PCB and their closest descendants rather than oxygenic ones, were mainly responsible for the generation of banded iron formations.

Analysis of cyanobacterial evolution and environmental requirements suggests that conditions allowing the development of organisms resembling iron-tolerant CB may exist or may have existed on any planet with a significant concentration of iron in its crust and minimal oxygen in its atmosphere.

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EXTREME ACIDOPHILES:

Freshwater Algae Associated with Acid Mine Drainage

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1. Introduction

1.1. DESCRIPTION OF THE HABITAT

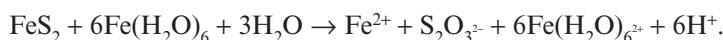
Acid mine drainage (AMD) is a phenomenon commonly associated with mining activities throughout the world. This acidification is the consequence of sulfides in rock strata becoming exposed to water and oxygen (see Section 1.2). The low pH of AMD-contaminated water bodies may resemble that of some naturally occurring freshwater systems that harbor acidophiles. For example, regions of the West Coast of the South Island of New Zealand have streams and rivers with naturally low pH produced by leaching of fumic and fluvic acids from podocarp rainforests, as well as artificially low pH systems caused by AMD (Collier et al., 1990). Ancient low-pH environments generated by volcanism may have been crucial for the origin of life on Earth (e.g., Holm and Andersson, 2005; Phoenix et al., 2006), and thus habitats resembling AMD have probably existed for billions of years. Distinctly, however, extremely acidic habitats from anthropogenic sources are associated with a massive burden of spoil and heavy metals. AMD began during the industrial revolution, and now accounts for most of the extremely acidic habitats worldwide (Johnson, 1998).

AMD can occur in both surface runoff (particularly in opencast mines) and in the groundwater associated with underground mines. Both surface runoff and groundwater frequently supply and contaminate streams (Harding and Boothroyd, 2004), and lakes within the catchment (Wicks et al., 1991; Niinioja et al., 2003; Tittel et al., 2005). Coastal mining may have significant local impacts on marine intertidal and coastal ecosystems. However, Johnson (1998) defined acidophiles as organisms with growth optima at or below pH 3.0, which would probably exclude much of the marine environment except at highly impacted river mouths and close intertidal zones. The impact of AMD in marine ecosystems has been the subject of much research that is beyond the scope of this chapter. Here, we focus on algal communities in freshwater systems impacted by AMD.

1.2. CHEMISTRY AND BIOCHEMISTRY OF AMD

Freshwater environments exposed to AMD typically have extremely low pH and high concentrations of dissolved metals (Table 1). In the following discussion we will refer to pH, which although not exactly the same as “acidity,” is frequently synomized (Johnson, 1998; Harding and Boothroyd, 2004). In freshwater ecosystems, AMD contamination frequently leads to the collapse of benthic communities through the elimination of sensitive taxa. A useful review of the environmental impacts of AMD, including an overview of chemical, physical, biological, and ecological impacts and details of the effects of metal toxicity, sedimentation, and breakdown of buffering systems in the context of community structure, is given in Gray (1997).

The processes generating acidity in AMD have been the subject of some debate (Evangelou, 1995; Sand et al., 1995; Johnson, 1998). However, consensus has emerged that the ferric ion acts as the major oxidant of the mineral according to the following equation:



The reactions described by Singer and Strumm (1970) show that pyrites can remain in their reduced state in undisturbed strata as long as they are not exposed to oxygen (Gray, 1997). Currently, mining is the most common means by which this exposure occurs. However, bacteria may also play a role, through subsequent

Table 1. A selection of reported values for pH and dissolved metals (mg L^{-1}) from AMD-impacted streams, and Maximum Contaminant Levels (MCLs) for drinking water according to the U.S. Environmental Protection Agency.

Country	pH	Fe	As	Cu	Zn	Al	Mn	Reference
USA	0.83	50 ^a	8.5	4.1	14	—	—	Baker et al. (2004)
Japan	2.3	276	1.67	—	—	69	—	Sasaki et al. (2005)
England	3.4	15.0	—	—	—	—	7.8	Singh et al. (1999)
England	—	140	2.5	0.4	75	50	23	Barley et al. (2005)
Germany	2.1—	390—	—	0.004—	1.69—	22—	1.3—	Lessmann et al. (1999)
(Lake)	2.5	670	—	0.047	2.91	60	43.5	
New Zealand	2.7—	0.2—	—	—	—	0.01—	—	Winterbourn et al. (2000)
	6.2	32.6	—	—	—	35.5	—	
New Zealand	2.8	36.5—	—	0.012—	0.53—	—	0.43—	Novis (2005)
		37.0	—	0.016	0.54	—	0.44	
New Zealand	2.7—	0.1—	0.18—	—	0.92—	159—	—	Harding (2006)
	7.7	84.5	0.001	—	0.001	0.04	—	
South Africa	1.88	98.95	—	3.49	7.16	—	—	Van Hille et al. (1999)
U.S. Recommended level	6.5—	<0.3	<0.01	<1.3	<5	<0.05	<0.05	USEPA (2006)
		6.8	—	—	—	—	—	

^a Fe^{3+} only.

biological oxidation of pyrites (Leduc and Ferroni, 1994; Marchand and Silverstein, 2002).

The buffering capacity of the receiving water, and the extent to which the water body can dilute the contamination, largely influence the impact of AMD (Gray, 1997). This is partially because the solubility of metal ions varies greatly with pH (Harding and Boothroyd, 2004) and also because dilution may reduce metal concentrations while not markedly influencing pH. At higher pH (>4.0), precipitation of metal hydroxides can smother biota with precipitates, whereas at lower pH the toxicity of dissolved metals, which can cross membranes (Van Ho et al., 2002), combined with acidity, may have the greatest impact. This complexity of impact has led to a view that AMD is “multifarious,” affecting organisms in “numerous interactive ways” (Gray, 1997).

The impact of strong acidity on biota is associated with the conversion of carbonate and bicarbonate to carbonic acid (Harding and Boothroyd, 2004). This has two effects. First, a low-pH system may be more sensitive to further addition of AMD, since buffering capacity to moderate the pH is lost. Second, the absence of bicarbonate deprives autotrophs of inorganic carbon (although dissolved CO₂ may be more rapidly replenished from the atmosphere at low pH; Gross, 2000).

Metals can be divided into those that are required by organisms in small quantities, such as copper and zinc which are essential in some biochemical reactions (e.g., Raven et al., 1999), and those of no known biological utility, such as mercury and cadmium (Pinto et al., 2003). The toxicity of heavy metals to algae and other organisms appears to be due to the generation of reactive oxygen species (ROS; Pinto et al., 2003). An example of this toxicity is the generation of peroxides, which can disrupt the plasma membrane by lipid peroxidation (Macfarlane and Burchett, 2001).

Most strongly acidic environments are also oligotrophic, being nutrient deficient (Johnson, 1998). Because sulfate is a product of the oxidation of metal sulfides (Singer and Strumm, 1970), it is invariably high in AMD-contaminated waters; however, other inorganic nutrients used by plants and algae may be very low, particularly inorganic nitrogen (<0.04 mg NH₄-N L⁻¹, Novis, 2005). Given that carbon limitation may also occur, nutrient limitation may be a significant additional problem for algae in AMD-contaminated waters.

The resistance of many algae to AMD is thought to be due to their ability to complex metals outside the cells, preventing entry to the cytoplasm. Laboratory studies showed an increase in extracellular polymeric substance (EPS) produced by *Chlorococcum* sp. and *Phormidium* sp. as a response to increased copper and zinc (Garcia-Meza et al., 2005). In seawater, *Nitzschia closterium* was also found to complex copper outside the cell membrane (Stauber et al., 2000). However, this mechanism is not universal: a study of *Oocystis nephrocytioides* indicated that resistance to copper was due to accumulation and sequestration in thylakoids, with adsorption to the cell surface being less important (Soldo et al., 2005).

2. Diversity of Algae Associated with AMD

Acidophilic bacteria (chemolitho-autotrophs) have been the focus of much attention in AMD-contaminated waters (see review by Johnson, 1998). However, a large number of algal taxa have been documented in AMD exposed to light (Table 2 and references therein). In severe AMD with stable flow conditions

Table 2. Freshwater algae associated with AMD.

Taxon ^a	pH range in study	Habitat (country)	Reference
Cyanobacteria			
<i>Phormidium</i> sp.	8.0	Lake (Mexico)	Garcia-Meza et al. (2005) ^b
<i>Hapalosiphon hibernicus</i> W. & G.S. West	2.5–7.7	Stream (Sarawak)	Douglas et al. (1998) ^c
Euglenids			
<i>Euglena mutabilis</i> Schmitz	≥1.41	Streams (multiple countries)	Whitton and Diaz (1981) ^d
	1.5–2.4	Stream (Spain)	Sabater et al. (2003)
	2.0–5.0	Streams (USA)	Brake et al. (2001a, b)
	2.5	Stream (Sarawak)	Douglas et al. (1998) ^c
	2.6–3.1	Lake (Germany)	Kapfer (1998)
	2.5–4.7	Stream (France)	Casiot et al. (2004)
	2.1–2.5	Lake (Germany)	Lessmann et al. (1999)
	2.3–2.9	Lake (Germany)	Lessmann et al. (2000)
<i>Euglena</i> sp.	2.5–7.3	Stream (Sarawak)	Douglas et al. (1998) ^c
<i>Phacus curvicauda</i> Svirensko	2.5–5.8	Stream (Sarawak)	Douglas et al. (1998) ^c
<i>Lepocinclis</i> sp.	2.3–2.9	Lake (Germany)	Lessmann et al. (2000)
Chromists: chrysophytes			
<i>Chromulina</i> sp.	2.3–2.9	Lake (Germany)	Lessmann et al. (2000)
<i>Ochromonas</i> sp.	2.6	Lake (Germany)	Kamjunke et al. (2004)
	2.3–2.9	Lake (Germany)	Lessmann et al. (2000)
	2.6–3.6	Lake (Germany)	Wollmann et al. (2000)
Chromists: cryptomonads			
<i>Cryptomonas ovata</i> Ehrenb.	2.5–5.7	Stream (Sarawak)	Douglas et al. (1998) ^c
<i>Cyathomonas</i> sp.	2.3–2.9	Lake (Germany)	Lessmann et al. (2000)
<i>Rhodomonas minuta</i> Skuja	2.3–2.9	Lake (Germany)	Lessmann et al. (2000)
Chromists: diatoms			
<i>Eunotia bilunaris</i> (Ehrenb.) Mills	2.5–7.7	Stream (Sarawak)	Douglas et al. (1998) ^c
<i>Eunotia exigua</i> (Bréb. Ex Kütz.) Rabenh.	2.3–2.9	Lake (Germany)	Lessmann et al. (2000)
<i>Eunotia hexaglyphis</i> Ehrenb.	2.5	Stream (Sarawak)	Douglas et al. (1998) ^c
<i>Eunotia incisa</i> Greg.	2.5–7.7	Stream (Sarawak)	Douglas et al. (1998) ^c
<i>Eunotia pectinalis</i> (Dillw.) Rabenh.	2.5–5.7	Stream (Sarawak)	Douglas et al. (1998) ^c

(Continued)

Table 2. Freshwater algae associated with AMD—cont'd.

Taxon ^a	pH range in study	Habitat (country)	Reference
<i>Eunotia sudetica</i> O. Müll.	2.5	Stream (Sarawak)	Douglas et al. (1998) ^c
<i>Frustulia rhombooides</i> (Ehrenb.) De Toni	2.5–7.6	Stream (Sarawak)	Douglas et al. (1998) ^c
<i>F. rhombooides</i> var. <i>crassinervia</i> Rabenh.	2.5–7.6	Stream (Sarawak)	Douglas et al. (1998) ^c
<i>Navicula mutica</i> Kütz.	2.5–7.3	Stream (Sarawak)	Douglas et al. (1998) ^c
<i>Nitzschia acicularioides</i> Hust.	2.5	Stream (Sarawak)	Douglas et al. (1998) ^c
<i>Nitzschia</i> sp.	2.3–2.9	Lake (Germany)	Lessmann et al. (2000)
<i>Nitzschia tubicola</i>	Not available	Stream (USA)	Brake et al. (2004)
<i>Pinnularia acoricola</i> Hust.	1.5–2.4	Stream (Spain)	Sabater et al. (2003)
	2.3–2.9	Lake (Germany)	Lessmann et al. (1999)
<i>Pinnularia braunii</i> (Grunow) Cleve	2.5–7.6	Stream (Sarawak)	Douglas et al., (1998) ^c
<i>Surirella tenuissima</i> Hust.	2.5	Stream (Sarawak)	Douglas et al. (1998) ^c
<i>Synedra</i> sp.	2.5	Stream (Sarawak)	Douglas et al. (1998) ^c
Chromists: dinoflagellates			
<i>Gymnodinium</i> sp.	2.6–3.6	Lake (Germany)	Wollmann et al. (2000)
Chromists: xanthophytes			
<i>Tribonema</i> sp.	3.1–3.4	Stream (New Zealand)	Winterbourn et al. (2000) ^e
Plantae: chlorophytes			
<i>Chlamydomonas acidophila</i> Negoro	2.5–3.3	Lake (Germany)	Tittel et al. (2005)
<i>Chlamydomonas botryopara</i> Rodhe & Skuja	2.5	Lake (Germany)	Woelfl et al. (2000)
<i>Chlamydomonas</i> sp.	2.6	Lake (Germany)	Kamjunke et al. (2004)
	2.3–2.9	Lake (Germany)	Lessmann et al. (2000)
	2.6–3.6	Lake (Germany)	Wollmann et al. (2000)
<i>Chlorococcum</i> sp.	8.0	Lake (Mexico)	Garcia-Meza et al. (2005) ^b
<i>Microspora tumidula</i> Hazen	2.6–3.3	Stream (USA)	Verb and Vis (2001) ^f
<i>Microspora</i> sp.	3.1–4.2	Stream (New Zealand)	Winterbourn et al. (2000) ^e
<i>Nanochlorum</i> sp.	2.3–2.9	Lake (Germany)	Lessmann et al. (2000)
<i>Oedogonium</i> sp.	2.5–7.9	Stream (Sarawak)	Douglas et al. (1998) ^c
<i>Pediastrum tetras</i> (Ehrenb.) Ralfs	2.5–5.8	Stream (Sarawak)	Douglas et al. (1998) ^c
<i>Scourfieldia cordiformis</i> Takeda	2.3–2.9	Lake (Germany)	Lessmann et al. (2000)
<i>Stigeoclonium</i> sp.	8.2	Stream (Poland)	Pawlak-Skowrońska (2001)
<i>Stigeoclonium tenue</i> Kütz.	8.2	Stream (Poland)	Pawlak-Skowrońska (2003) ^b
<i>Trentepohlia annulata</i> Brand	2.5	Stream (Sarawak)	Douglas et al. (1998) ^c
<i>Ulothrix</i> sp.	3.0–11.0	Stream (USA)	Rousch and Sommerfeld (1999)

(Continued)

Table 2. Freshwater algae associated with AMD—cont'd.

Taxon ^a	pH range in study	Habitat (country)	Reference
	3.7–5.4	Stream (USA)	Niyogi et al. (1999)
	n.d.	Stream (USA)	Tate et al. (1995)
	2.7–4.1	Stream (New Zealand)	Winterbourn et al. (2000) ^f
Plantae: charophyceans			
<i>Actinotaenium cucurbitinum</i> (Biss.) Teiling	2.5–5.7	Stream (Sarawak)	Douglas et al. (1998) ^e
<i>Klebsormidium acidophilum</i> Novis	2.8	Stream (New Zealand)	Novis (2006)
<i>Klebsormidium flaccidum</i> (Kütz.) P.C. Silva, Mattox & W.H. Blackw.	1.5–2.4	Stream (Spain)	Sabater et al. (2003)
<i>Klebsormidium rivulare</i> (Kütz.) M.O. Morison & Sheath	2.9–5.6	Stream (USA)	Stevens et al. (2001)
	≥2.30	Streams (multiple countries)	Whitton and Diaz (1981) ^d
	1.5–2.4	Stream (Spain)	Sabater et al. (2003)
	2.6–3.3	Stream (USA)	Verb and Vis (2001) ^f
<i>Klebsormidium</i> sp.	2.2–3.4	Stream (Australia)	Lottermoser et al. (1999)
<i>Mougeotia</i> sp.	1.5–2.4	Stream (Spain)	Sabater et al. (2003)
	2.5–7.9	Stream (Sarawak)	Douglas et al. (1998) ^e
<i>Spirogyra</i> sp.	2.5–7.9	Stream (Sarawak)	Douglas et al. (1998) ^e
Plantae: rhodophytes			
<i>Galdieria sulphuraria</i> (Galdieri) Merola	1.5–1.9	Stream (Spain)	Gross and Gross (2001)
Unidentified red alga	0.76–1.38	Stream (USA)	Baker et al. (2004)

^aMajor groups follow Keeling (2004); traditional names are used for subgroups

^bChemical analysis of the mine water in these studies indicates that bicarbonate was responsible for the high pH

^cAlthough Douglas et al. (1998) considered all their sites to be affected by (or at least associated with) mining activities, the range of pH of these sites was 2.5–8.7. Taxa included here were present in sites with pH < 3.0 (in fact no sites had a pH between 2.6 and 4.9, indicating that their sites J02 and J8 were much more heavily impacted than the others)

^dWhitton and Diaz (1981) list many taxa from a range of acidic environments. Unfortunately, for most of these it is not clear which come from AMD-affected sites, and which from geothermal acid streams

^eOnly taxa from Winterbourn et al. (2000) that occurred in streams of pH < 4.0 and Fe concentration of > 1.0 mg L⁻¹ are included

^fVerb and Vis (2001) noted 40 taxa, but regarded only the 2 included here as indicative of AMD contamination

acidophilic algae can dominate the streambed. These organisms do not appear to have received a synthesis previously. A selection of taxa is shown in Fig. 1.

Many groups of algae have been reported from AMD (Table 2), but some taxa are much more common than others, for example, *Euglena* and *Klebsormidium* spp. This suggests cosmopolitanism. Cyanobacteria, which commonly dominate natu-



Figure 1. A selection of filamentous algae collected from streams draining abandoned mine adits in Westland, New Zealand. From top to bottom *Klebsormidium acidophilum*, *Microspora* sp., *Mougeotia* sp., and *Tribonema* sp. Scale bar = 10 µm.

rally acidic thermal springs, are very poorly represented in AMD. Likewise, thermo-acidophilic red algae (the unicellular Cyanidiales) are well documented from natural acidic springs, yet rhodophytes are almost unknown from AMD-contaminated habitats (Barbier et al., 2005). One strain of *Galdieria sulphuraria* is known from mining environments (Gross and Gross, 2001). Environmental PCR of samples from AMD sites with water temperatures of 30–50°C revealed two clones with 75% sequence identity to the cyanidialean *Cyanidioschyzon merolae* (Baker et al., 2004). Therefore, it seems likely that a greater diversity of red algae may be present in mining environments than has been identified so far.

Together, members of the “green lineage” (as defined by Nozaki et al., 2003) are the most diverse and widely reported taxa growing in AMD. Two genera in particular, *Euglena* and *Klebsormidium*, seem almost ubiquitous and are frequently dominant where they occur. Other groups appear to be locally common, such as chrysophytes, all the records of which come from mining lakes in Germany. Undoubtedly, this is partly due to a lack of systematic surveys in many habitats, although the paucity of records of protists in AMD-contaminated habitats has perhaps been overstated by some authors (e.g., Brake et al., 2001b; Baker et al., 2004).

A number of diatom species are recorded from AMD-contaminated freshwaters. Given the advantages of easy preservation and identification to species level from vegetative material, some authors have advocated their potential as indicators of AMD contamination (Nakanishi et al., 2004). They also give a rare example of periodicity in the simplified AMD community: the dominance of *Nitzschia tubicola* under certain conditions leads to the development of stromatolites, marking layers of alternately dominant diatom and euglenoid biomass (Brake et al., 2004).

3. Taxonomic Issues and Evolution of AMD-Tolerant Taxa

The presence of algae in these extreme environments raises the issue of their evolution and dependence on these environments. Is growth by algae in AMD-contaminated habitats facultative or obligate? Floristic surveys rarely provide us with sufficient information to address this issue, but some evidence is available from more detailed studies on small groups of organisms. *Euglena mutabilis*, for example, is widely reported from AMD-contaminated habitats at low pH (Table 2). The growth of this species is optimal between pH 3.0 and 3.5, and it tolerates high sediment loading (Brake et al., 2001a, b), and yet organisms also ascribed to *E. mutabilis* are reported from environments of much higher pH. Comparison of *E. mutabilis* and *E. gracilis* has shown that both species are acid tolerant – in fact the latter outcompeted the former at pH values typical of AMD – but *E. mutabilis* was far more tolerant of several heavy metals including aluminum, nickel, iron, and cadmium, although not copper (Olaveson and Nalewajko, 2000). Therefore, the success of *E. mutabilis* in AMD systems may be due to recent acquisition of heavy metal tolerance rather than a species-specific ability to cope with low pH.

Perhaps the best documented example of preferential growth in AMD is that of *Klebsormidium rivulare*, which also illustrates the complexity of nomenclatural issues. The type specimen was collected from freshwater, apparently without influence of AMD, and named *Hormidium rivulare* (Kützing, 1845). However, Lokhorst (1996) identified this specimen as *Ulothrix implexa* (Kütz.) Kütz., suggesting that later transfer of *H. rivulare* to *K. rivulare* was inappropriate. The description provided by Kützing (1845) is also insufficient to distinguish *Klebsormidium* from several other genera, including *Ulothrix* and *Uronema*. In fact, ultrastructural study, requiring modern technology, is desirable in order to do this (e.g., Marchant et al., 1973; Lokhorst and Star, 1980, 1985; Lokhorst, 1996; Novis, 2006). The first “modern” study of *K. rivulare*, using ultrastructural data, was that of Morison and Sheath (1985). Their electron micrographs show that their specimens conform to the genus *Klebsormidium*, and hence differ from the *H. rivulare* of Kützing (1845). A number of subsequent studies (Table 2) from AMD-contaminated sites applied the name *K. rivulare* to similar taxa, perhaps because the material of Morison and Sheath (1985) was collected from an acidic stream (pH 4.3–5.8, Steinman and Sheath, 1984).

However, it is now clear that *Klebsormidium* from AMD-contaminated sites differs from the *K. rivulare* of Morison and Sheath (1985), at least in some cases. Comparative culturing of a species from Sullivan West Mine, New Zealand, with *Klebsormidium dissectum* isolated from higher-pH environments showed that the pH optimum for the acidophile was 2.4–3.4, whereas the optimum for *K. dissectum* was 4.8–6.2 (Fig. 2). This clearly differs from the situation in different species of *Euglena*. Furthermore, the acidophile differed consistently in diameter from *K. rivulare*, and lacked typical field morphology over the pH range specified by Steinman and Sheath (1984). The new species *Klebsormidium acidophilum* was justified by

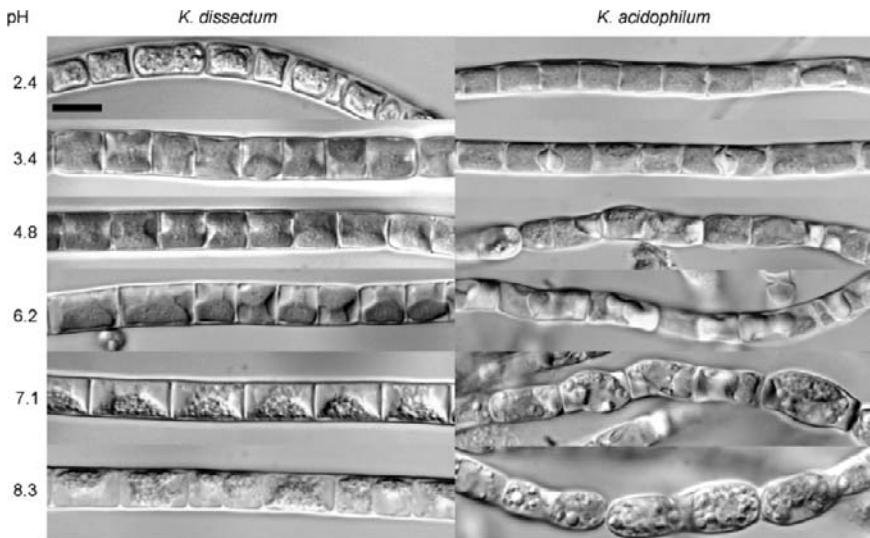


Figure 2. Morphology of two species of *Klebsormidium* grown for 3 weeks at different pH (left). *K. dissectum* was isolated from a stream of pH > 7.0; *K. acidophilum* from the stream from Sullivan West Mine, pH 2.8. Scale bar = 10 μm . Copyright (2006) International Phycological Society. From *Phycologia*, by P.M. Novis (2006). Reprinted by permission of Alliance Communications Group, a division of Allen Press, Inc.

these observations. *RbcL* gene sequences indicated that genetic variation of *K. acidophilum* was nested within the variation of *K. dissectum* from local pH-neutral habitats (Novis, 2006). Therefore, AMD-tolerant taxa may evolve from local, less extreme habitats, contrasting with a view that organisms that successfully colonize AMD, such as *K. rivulare* and *E. mutabilis*, are cosmopolitan taxa. Testing this idea, using molecular comparison of AMD and pH-neutral taxa from local areas in different parts of the globe, constitutes important future research.

4. Structure of Algal Communities in AMD-Impacted Ecosystems

For algae that are able to colonize these extreme environments, AMD-contaminated waterways may offer some unique advantages. In particular, streams that discharge from mine adits are often unaffected by the disturbance of high rainfall and flooding (e.g., Trumm et al., 2005), which might otherwise limit growth. Furthermore, the paucity of grazing benthic invertebrates and usually the total absence of fish, crayfish, and other possible benthic grazers may allow the development of very high biomass, despite slow growth rates due to low nutrients and toxic contaminants. This is particularly important in systems where growth rates must exceed the rate of iron precipitation onto biomass for sustained colonization of the site to be viable (Harding and Boothroyd, 2004).

4.1. INTERACTIONS BETWEEN PROTISTS AND BACTERIA

Studies of acidophilic flagellates (*Eutreptia/Bodo* spp.), a ciliate (*Cinetochilium* sp.), and an amoeba (*Vahlkampfia* sp.) from AMD confirmed that these grazed on acidophilic bacteria (Johnson and Rang, 1993). Although photosynthetic euglenoids are mixotrophic and also capable of grazing on bacteria, and depend on associated microbial floras for vitamins B₁ and B₁₂ (Graham and Wilcox, 2000), ecological relationships between the commonly reported AMD organism *E. mutabilis* and bacterial communities in these habitats are little studied. The AMD tolerance of *E. mutabilis* from Green Valley, Indiana, USA, falls within habitat preferences of sulfur- and iron-oxidizing bacteria published elsewhere (McIntosh et al., 1997; Brake et al., 2001b). Given the prolific growth of these bacteria at some sites (e.g., Johnson, 1998) and the carbon limitation for autotrophs at low pH (Tittel et al., 2005), this finding may partially explain the high frequency of colonization of AMD habitats by euglenoids.

Some bacteria are strongly implicated in the biological oxidation of pyrites, and hence their activities influence the function of AMD ecosystems. Surprisingly, interactions between bacteria and photosynthetic protists (i.e., eukaryotic algae) that are obligately autotrophic are poorly documented in AMD. Stable consortia have been formed between algae and bacteria in the treatment of industrial wastewater containing heavy metals and hydrocarbons, although the pH tolerance of these organisms was not reported (Safanova et al., 2006). Algal biomass may facilitate bacterial sulfate reduction in mine discharge in artificial wetlands (Russell et al., 2003; see Section 5.2).

4.2. INTERACTIONS BETWEEN ALGAE AND INVERTEBRATES

The diversity of benthic invertebrate taxa is frequently greatly reduced in AMD-impacted systems, and can be correlated with stream pH (Fig. 3). A number of benthic freshwater invertebrates feed either partially or wholly on epilithic algae. Within New Zealand, these grazing invertebrates are usually dominated by Mollusca (snails) and insects, for example, Ephemeroptera (mayflies), Trichoptera (caddisflies), and Chironomidae (midges). In North America, several fish species including freshwater crayfish may also consume algae; however, fish and crayfish are usually completely excluded from AMD waters.

Of all benthic invertebrates, snails may be among the most important obligate grazers. However, they are unable to tolerate low-pH waters. In a survey of 65 natural and AMD-impacted streams on the West Coast of the South Island of New Zealand, snails were not collected at any site with a pH less than 7 (Harding, unpublished data). In many New Zealand rivers, leptophlebiid mayflies, which also graze algae, may occur at high densities. However, Harding (2006) found that mayflies were almost entirely absent from AMD streams with pH < 4.2 in New Zealand. In contrast, several New Zealand invertebrate taxa are tolerant of

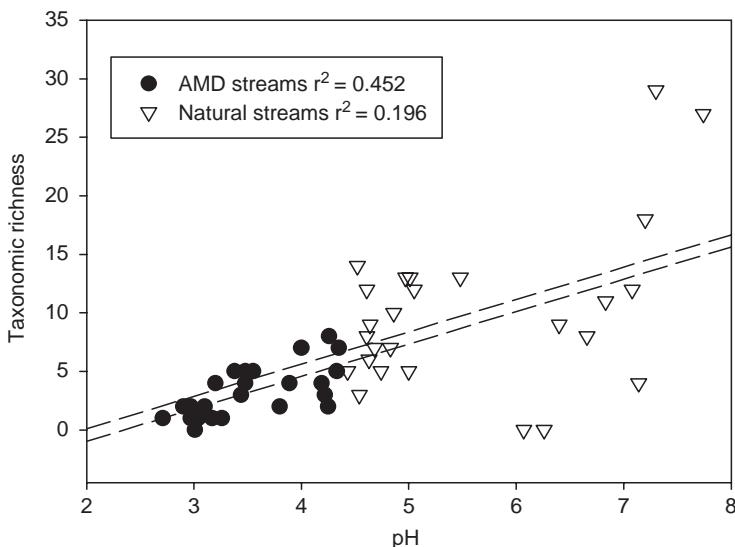


Figure 3. Correlations between benthic invertebrate taxonomic richness and pH in 50 streams on the West Coast of the South Island of New Zealand.

highly degraded AMD, including Chironomidae (nonbiting midges), which occur in streams of pH 2.9 dominated by *Klebsormidium*.

5. Use of Algae to Diagnose and Remediate AMD

5.1. ALGAE AS INDICATORS OF AMD CONTAMINATION

The use of algae as a diagnostic tool for the assessment of AMD requires that test species be sensitive to AMD contamination (e.g., *Pseudokirchneriella subcapitata*, Moreira-Santos et al., 2004; *Selenastrum capricornatum*, LeBlond and Duffy, 2001). The idea of using “indicator organisms” for AMD detection in the field has been advocated by Nakanishi et al. (2004). However, as AMD contamination is frequently obvious or easily measured, the value of these as tools may be limited.

Algae have been used in the assessment of AMD effects by using measures of diversity, abundance, and metal content (e.g., Perrin et al., 1992; Vinyard, 1996; Genter and Lehman, 2000; Winterbourn et al., 2000; Niyogi et al., 2002). These studies typically report an assemblage that is characteristic of AMD-contaminated habitats in the more extreme sites (see Section 2).

5.2. ALGAE AND REMEDIATION OF AMD

Remediation of AMD is extremely challenging (Nordstrom and Alpers, 1999), and usually involves either active or passive treatment systems. Active systems employ the addition of chemicals to contaminated water to reduce acidity and metals, while passive systems often involve the use of wetlands or treatment ponds. Although there is little evidence that algae can raise the pH of AMD, they can certainly remove metals from solution (Stevens et al., 2001). The adsorption of heavy metals is highly variable, depending on the metal, the taxon, age of material, and other conditions. Since inactivated algal biomass also adsorbs metals, a large component of uptake is a passive mechanism of adsorption to cell walls (Chu and Hashim, 2004).

Removal of heavy-metal contamination using algae has been at least partially successful in field trials (e.g., Gale and Wixson, 1979; Phillips et al., 1995; Xie et al., 1996; Van Hille et al., 1999; Kalin et al., 2005). The use of wetlands as an effective remediation solution for AMD is gaining acceptance (e.g., Howard et al., 1989; Dodds-Smith et al., 1995; Jones et al., 1996; Bernoth et al., 2000; Groudeva et al., 2004). The motivation for choosing such a system is often that active treatment such as lime addition, while efficient, becomes expensive over time (Van Hille et al., 1999). However, there is still considerable debate concerning the effectiveness of passive systems (e.g., see http://www.dartmouth.edu/~cehs/CAGsite/docs/report_6.html). Their effectiveness may be limited by topography (e.g., created wetlands require considerable land), the difficulty in optimizing uptake due to field conditions, and reclaiming metals from algal biomass.

In addition to field trials, laboratory experiments have examined the uptake of heavy metals by a number of algal strains. Some freshwater strains used in laboratory studies of metal uptake are given in Table 3. A comparison of these studies with those in Table 2 (taxa reported from AMD-contaminated habitats in the field) reveals that the two lists are almost mutually exclusive. The choice of test organisms in uptake studies may be largely influenced by the availability of cultured strains (many of those in Table 3 are well-known laboratory organisms). Because the pH of AMD may be considerably lower than other types of industrial effluent, it may be difficult to extrapolate the results of these uptake studies to AMD-impacted systems. Several workers reported an optimum pH for uptake of metals of greater than 5.0 (Chen et al., 2005; Gong et al., 2005; Gupta et al., 2006). The optimum pH range for growth of *Chlorella vulgaris* in mixed culture is 6.31–6.84 (Mayo, 1997), similar to that for metal uptake, and the toxicity of heavy metals to *Chlorella* may also be pH dependent (Franklin et al., 2000). Given that pH optima for growth of some acidophilic strains are much lower, further laboratory study of metal uptake by acidophiles is warranted.

Regardless of the limitation of remediation approaches, systems left to recover naturally from AMD contamination show extremely slow rates of rehabilitation. For example, Lake Orijarvi in Finland still has low productivity and lacks a planktonic diatom community, even though mining ceased in 1956

Table 3. Recent studies of heavy metal uptake by freshwater algae.

Algae	Metals	Reference
<i>Scenedesmus obliquus</i> (Turp.) Kütz.	Cr	Pellon et al. (2003)
<i>Microspora</i> sp.	Pb, Ni	Axtell et al. (2003)
<i>S. obliquus</i> , <i>Cyclotella meneghiniana</i> Kütz.	Ag, Co, Cs, Mn	Adam and Garnier-Laplace (2003)
<i>Spirogyra</i> sp.	U	Aleissa et al. (2004)
<i>Anacystis nidulans</i> Gard., <i>Chlorella vulgaris</i> Beij., <i>Scenedesmus quadricauda</i> (Turp.) Bréb.	Ni, Zn, Cd	Awasthi and Rai (2004)
<i>Spirulina maxima</i> (Setch. & Gard.) Geitler	Pb	Gong et al. (2005)
<i>Nostoc muscorum</i> Ag., <i>Anabaena subcylindrica</i> Borge	Cu, Co, Pb, Mn	El-Sheekh et al. (2005)
<i>Chlamydomonas reinhardtii</i> Dang.	Pb, Cd, Hg	Tuzun et al. (2005)
<i>Microcystis aeruginosa</i> (Kütz.) Elenkin	Pb, Cd, Hg	Chen et al. (2005)
<i>Pseudokirchneriella subcapitata</i> (Korshikov) Hindák	Cd	Casiraghi et al. (2005)
<i>Spirogyra</i> sp.	Cu	Gupta et al. (2006)
<i>Synechocystis aquatilis</i> Sauv.	Cu	Ergene et al. (2006)

(Salonen et al., 2006). Streams flowing from mine adits will continue to release AMD into the environment after the mine is abandoned, due to continued oxidation of exposed pyrites (Johnson, 1998).

Studies to date suggest higher potential for remediation of these systems if we can more effectively enlist the help of acidophilic microalgae. Possible strategies include laboratory studies of metal uptake by acidophilic strains to determine optimal conditions, low-cost technology to maintain these conditions in field sites, and techniques to efficiently harvest algal biomass and recover the metals.

6. Summary

AMD creates an extreme water-chemistry environment by drastically lowering the pH of waterways and exposing them to high concentrations of heavy metals. These conditions are most commonly derived from the oxidation of pyrites as a result of mining activities.

A number of algal taxa have been reported from these environments, but a few (e.g., species of *Euglena* and *Klebsormidium*) are far more common than others. These taxa form assemblages that are characteristic of AMD-contaminated ecosystems. However, our understanding of the role of algae in AMD systems is still hindered by issues of nomenclature, uncertain species concepts, and a lack of understanding of the evolution of AMD-tolerant taxa. Despite these issues, molecular studies indicate that AMD-tolerant taxa arise through colonization of AMD-contaminated habitats by local pH-neutral populations, rather than through long-distance dispersal from other areas of the globe. The mechanism of evolutionary change appears to differ between taxa, for example, *E. mutabilis* has

acquired greater heavy metal tolerance than related species, whereas *K. acidophilum* has reduced its pH optimum.

Interactions between different trophic levels in AMD-contaminated systems have been poorly studied. However, biological oxidation of pyrites by bacteria means that the interactions of these organisms affect all other components of the food web. The phagocytotic *E. mutabilis* presumably grazes bacteria, and this may be a key reason for its success in these environments. In turn, bacteria may use algal biomass as a carbon source to power sulfate reduction. In natural stream and river systems, algae form a basal resource for ecosystem food webs; in AMD systems, many benthic invertebrates that normally graze on these algae are absent. Severely impacted AMD systems have highly truncated food webs with few invertebrates, and no higher invertebrates (e.g., crayfish) or vertebrates (e.g., fish).

Several studies have shown that algae can adsorb heavy metals, suggesting their possible use in remediation. However, attempts to turn this observation into remediation technologies have met with mixed success, and require a greater understanding of AMD effects on biota.

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Eukaryotic Community Structure from Río Tinto (SW, Spain), a Highly Acidic River

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EUKARYOTIC COMMUNITY STRUCTURE FROM RÍO TINTO (SW, SPAIN), A HIGHLY ACIDIC RIVER

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1. Introduction

A major question in microbial ecology is to identify the limits of life for growth and survival, and to understand the molecular mechanisms that define these limits. Our ongoing exploration of the Earth has led to continued discoveries of life in environments that have been previously considered uninhabitable. Thus, interest in the biodiversity and ecology of extreme environments has grown in recent years for several reasons: some of these are scientific and related to the idea that extreme environments are believed to reflect early Earth conditions; conditions that persisted for most of the time that life has been on the Earth and to which prokaryotes originally evolved and adapted (Schopf and Walter, 1982). Other reasons are more commercial, such as the use of the metabolic properties of some microorganisms for metal extraction.

The discovery and study of extremophiles also contribute to the search for life beyond Earth. Thus, according to the NASA Astrobiology Roadmap (<http://astrobiology.arc.nasa.gov>), one of the main goals of astrobiological research is to understand life that survives or thrives under the most extreme conditions on Earth in order to characterize the biochemistry that defines the limits for cellular life. In this regard, the study of extremely acidic environments ($\text{pH} < 3$) is becoming increasingly important since the environmental acidity is often caused by microbial activity (Hallberg and Johnson, 2001).

Highly acidic environments are relatively scarce worldwide and are generally associated with volcanic activity and mining operation (Baffico et al., 2004). The natural oxidation and dissolution of the sulfidic minerals exposed to oxygen and water results in acid production, and the process can be greatly enhanced by microbial metabolism (Nordstrom and Southam, 1997; González-Toril et al., 2001). At the same time, low pH facilitates metal solubility in water, particularly cationic metals (such as aluminum and many heavy metals), and

therefore acidic water tends to have high concentrations of heavy metals (Johnson, 1998).

Río Tinto, a 92-km river located in south-west Spain, is one of the most unusual acidic ecosystems due to its size, rather constant low pH, high concentrations of heavy metals, and a high level of mainly eukaryotic microbial diversity (López-Archilla et al., 2001; Amaral-Zettler et al., 2002).

2. The River and Its Setting

The river raises in Peña del Hierro, in the core of the Iberian Pyritic Belt, flows through it and reaches the Atlantic Ocean at Huelva (Fig. 1). The Iberian Pyritic Belt is one of the richest metal sulfide ore deposits on Earth formed by massive bodies of iron and copper sulfides, as well as minor quantities of lead and zinc (Fernández-Remolar et al., 2003). Consequently, the area has been mined for millenia since the Phoenician and Roman era (Davis et al., 2000).

The river basin can be divided into three main zones on the basis of topological, geological, and geochemical characteristics: the northern, the transitional



Figure 1. Geographical map of the Tinto River showing the three domains, the north area, the transitional area and the estuary. (A) The river at the origin in Peña del Hierro. (B) The river at La Palma del Condado.

and the estuarine, which have been differentiated by a Pliocene half-graven activity with NE-SW direction (Flores, 1996). The northern zone includes the towns Riotinto, Nerva, Berrocal La Palma del Condado and Niebla and their surroundings (Fig. 1). The area is characterized by highlands (100–660 m above sea level), stable of the hydrochemical parameters such as pH, which remains between 0.9 and 3 (mean value of 2.3) and a high concentration of iron in solution (between 1.5 and 20 g L⁻¹). The transitional area is located between Niebla and San Juan del Puerto, although its position varies depending on the tidal regime and seasonal changes of the river flow.

Thus, the hydrochemical parameters experience periodic variations due to the seawater neutralization including a pH increase (higher than 3) and a decrease in the ferric iron concentration (lower than 0.2 g L⁻¹). The third zone is the estuary near Palos de la Frontera, where the seawater causes ferric iron precipitation as sulfides (Fernández-Remolar et al., 2003).

The hydrological characteristics of Río Tinto are typical of a semi-arid, Mediterranean-type climate. The mean water discharge is ca. 3 m³ sec⁻¹, although important variations have been observed throughout the year (Elbaz-Poulichet et al., 2000). Rainfall discharge reaches up to 10–70 mm day⁻¹ in winter and approaches 0 mm day⁻¹ in summer. Evapotranspiration is ca. <1 mm day⁻¹ in winter and up to 10 mm day⁻¹ during the summer (Sanchez-España et al., 2005). Under these hydrological regime, the river shows a clear bimodality in the annual water availability with a humid and temperate season in winter, followed by a dry and warm summer. This seasonality strongly affects the eukaryotic microbial communities by changing important hydrochemical characteristic such us water flow, water temperature, and element composition.

The northern part of Río Tinto maintains a constant low pH (ranging from 0.9 to 3, mean 2.3), and high concentrations of heavy metals (López-Archilla et al., 2001; Fernández-Remolar et al., 2004). Since pyrite (FeS₂) is the dominant sulfide in the Iberian Pyritic Belt, ferric ion is the dominant oxidant in the river and mainly responsible for the constant pH due to its buffering capacity (Gómez et al., 2004). These extreme conditions are the product of the metabolic activity of microorganisms, including iron- and sulfur-oxidizing bacteria, which can be found in high numbers in its waters. Most of these prokaryotes are autotrophic. Thus, in addition to promoting the extreme conditions of the habitat, they are also primary producers (González-Toril et al., 2001). Recent microbial studies have shown that ca. 80% of the prokaryotic diversity in the water column is explained by three chemolithotrophic bacteria, *Acidithiobacillus ferrooxidans*, *Leptospirillum* spp. and *Acidiphilium* spp., all of them involved in the iron cycle (González-Toril et al., 2003). Although some other species related to the iron cycle have also been identified (i.e., *Ferroplasma acidophilum* or *Thermoplasma acidophilum*), their low number in the water column suggests that they could play a minor role in the function of this cycle (Amils et al., 2004, 2006).

Ferric iron is produced by the metabolism of these iron-oxidizing microorganisms, which are very active in the aerobic part of the river. Sulfuric acid originates

Table 1. Physicochemical parameters at some of the most extreme sampling sites (Mean ± SD).

Location	pH	Conductivity (mS cm ⁻¹)	Redox		Cu (mg L ⁻¹)	As (mg L ⁻¹)	Cd (mg L ⁻¹)	Zn (mg L ⁻¹)
			potential (mV)	Fe (g L ⁻¹)				
Iz-Iz	1.8 ± 0.2	25.7 ± 2.3	569 ± 22	17 ± 4	12 ± 3	16 ± 4	43 ± 16	14 ± 3
Angeles	1.7 ± 0.2	30.8 ± 3.4	471 ± 16	16 ± 3	132 ± 43	24 ± 3	30 ± 12	162 ± 5
UMA	1.7 ± 0.3	40.2 ± 8.3	473 ± 10	18 ± 7	85 ± 36	32 ± 5	40 ± 18	118 ± 4
Richi	1.2 ± 0.3	38.9 ± 16	460 ± 30	22 ± 5	100 ± 36	48 ± 7	34 ± 11	94 ± 31
La Palma	2.5 ± 0.3	3.70 ± 1.1	548 ± 70	0.2 ± 0.1	19 ± 7	0.2 ± 0.1	0.7 ± 0.1	50 ± 10

from sulfides by chemical oxidation or the activity of the sulfur-oxidizing microorganisms, depending on the sulfide mineral substrate (González-Toril et al., 2003). The result is a strongly acidic solution of ferric iron which brings into solution other heavy metals, increasing their concentrations relative to neighboring rivers with higher pH. Thus, Rio Tinto heavy metal concentrations, of Fe at ca. 22 g L⁻¹, Zn at ca. 0.5 g L⁻¹ or Cd at ca. 70 mg L⁻¹, can be found (Table 1) (López-Archilla and Amils, 1999).

Comparison of physical conditions and concentrations of heavy metals ions in winter, summer, and late summer denote a clear bimodality in their annual distribution. Oxygen and pH generally show higher values in winter, while heavy metals reach their peaks in summer and late summer.

This fact is coincident with the bimodal pattern of annual water availability reported in previous work on Río Tinto area (Fernández-Remolar et al., 2003).

The climograms of this area showed a clear bimodality in the pluviosity and water availability consisting of a humid and temperate season alternating with a warm and dry season. The high phreatic level maintains the river flow during the summer, although a high rate of evaporation induces high concentrations of heavy metals due to concentration processes.

However, what makes the Río Tinto a unique acidic extreme environment is that eukaryotic organisms are the principal contributors of biomass in the river as well as the unexpected degree of eukaryotic diversity found in its waters (López-Archilla et al., 2001, Amaral-Zettler et al., 2002, 2003).

3. Eukaryotic Biodiversity

It is usually assumed that high metal concentrations in acidic habitats limit eukaryotic growth and diversity due to their toxicity (Gross, 2000). It has been also proposed that metal hydroxide deposition could change the physicochemical conditions of surfaces resulting in a reduction of epilithic growth on rocks. However, colorful biofilms covering large surfaces of the river basin as well as filamentous microbial communities and macroscopic algae are common features of the acidic streams at the Río Tinto. In fact, eukaryotic algae contribute at least 60% of the river biomass (López-Archilla et al., 2001).

The eukaryotic biodiversity in this ecosystem includes species of most of the major eukaryotic lineages (Fig. 2) (López-Archilla et al., 2001; Amaral-Zettler et al., 2002, 2003; Sabater et al., 2003). Most of the eukaryotic species thriving in

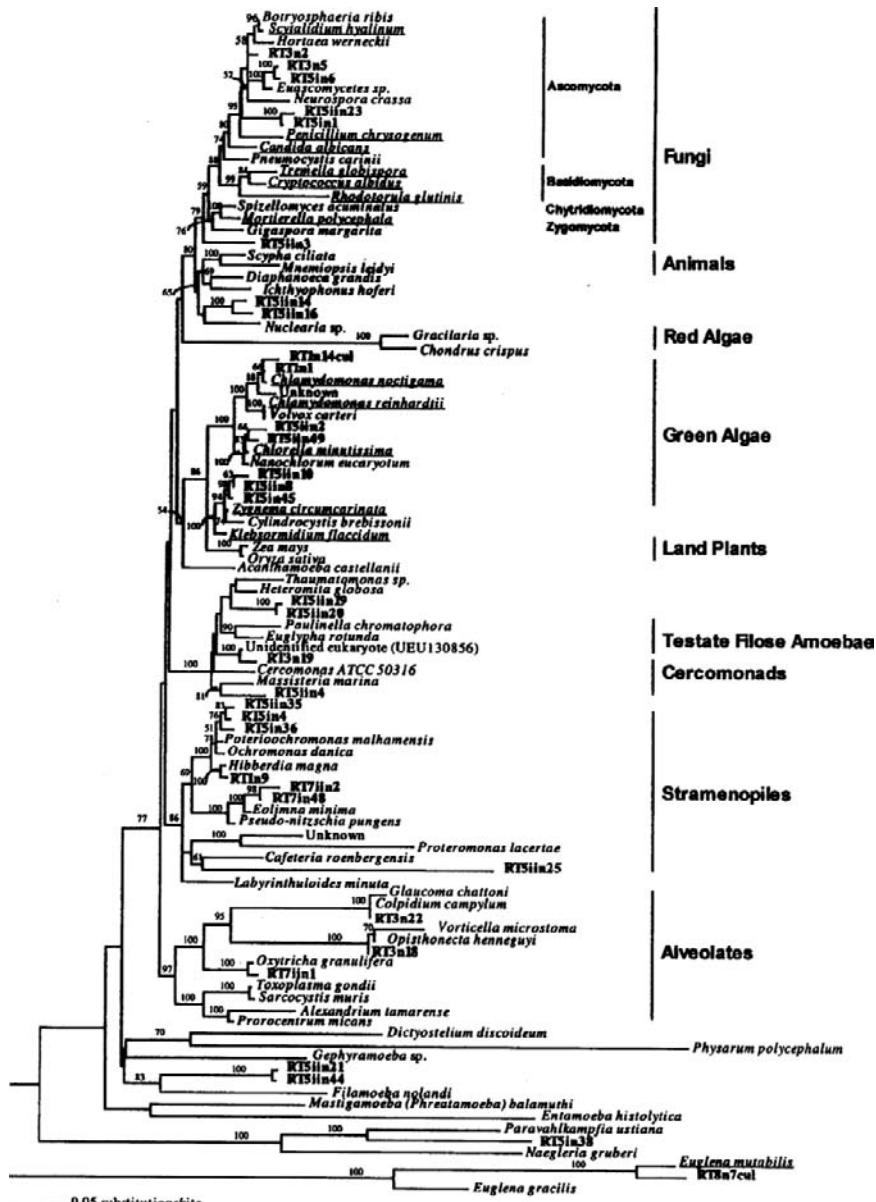


Figure 2. Phylogeny based on minimum evolution analyses of ribosomal RNAs using a likelihood method. Published by Amaral et al. Biological Bulletin (2003) 204: 205–209.

Río Tinto are photosynthetic species. Within them, chlorophytes related to different genera such as *Chlamydomonas*, *Dunaliella*, *Chlorella*, as well as *Euglena mutabilis* are the dominant eukaryotic microorganisms present in the river, forming large green patches along the river bed. These species are known for their high metal and acid tolerance from several investigations in extremely acidic lakes (Olaveson and Nalewajko, 1994; Fisher et al., 1998) and showed the same patchy distribution as in other acidic environments. Filamentous algae, represented by the genera *Zygnemopsis* and *Klebsormidium* have been also found. The occurrence of both filamentous species is highest during the dry summer months, when ion concentrations were highest and most of the physicochemical parameters are most extreme. These species are usually attached to the sediments forming long filamentous biofilms on the water surface that could exceed 1 m in length. During the winter, they almost disappear, probably due to an increase in the water current velocity which makes maintaining these structures difficult. Other chlorophytes, such as species belonging to the genera *Mesotaenium* and *Stichococcus*, have also been found, although at low numbers.

The most acidic part of the river is inhabited by an eukaryotic community dominated by two species related to the genera *Dunaliella* (*Chlorophyta*) and *Cyanidium* (*Rhodophyta*). The genus *Dunaliella* includes some of the most extreme acidophiles reported until now, surviving at a restricted pH between 0 and 3 (Gimmler and Weis, 1992). The Cyanidiaceae group is also exceptionally well adapted to warm (ca. 45°C) and acidic (ca. pH 2) habitats (Seckbach, 1999).

Pennate diatoms are also present in the river forming large brown biofilms. These biofilms are usually clearly dominated by only one species related to the genus *Pinnularia*, although some other minoritary genera have been identified, including *Nitzschia* or *Cyclotella* (Sabater et al., 2003). Species belonging to these genera, especially *Pinnularia*, are fairly widespread at environments with pH values around 3.0 (DeNicola, 2000). From all the environmental variables that affect freshwater diatoms, pH seems to be the most important and, most taxa show a preference for a narrow pH range (Battarbee et al., 1986). The low diversity of diatoms present in Río Tinto in comparison with the diversity found in neighboring freshwaters, supports the idea that there is a threshold between pH 4.5 and 3.5 in which many species of diatoms are eliminated (DeNicola, 2000).

In addition to photosynthetic species, heterotrophic protists are also widely distributed throughout the river. The mixotrophic flagellates are dominated by members of the genera *Bodo* and *Ochromonas*. These organisms show the same ecological strategies as the phytoflagellates to overcome limitation in nutrient supply such as mixotrophy and mobility which results in an important advantage in these environments (Lessmann et al., 2000). Phagotrophic species such as ciliates, cercomonads, amoebae, and heliozoans have been also found in Río Tinto.

At least two species of ciliates are members of the community. The dominant ciliate taxa belongs to the order Hypotrichida. Although two different species have been microscopically observed, only clones related to *Oxytrichia granulifera* have been molecularly identified. The other morphotype could be

tentatively assigned to the genus *Euplates*. The reduction of species diversity and ciliate abundance with increasing acidity is well documented (Beaver and Crisman, 1981; Bienert et al., 1991). Hypotrichida species predominantly thrive in soils or benthos usually associated with algal clumps and they can be found in almost all sampling sites in the river. They are relatively large and slow-swimming, and this may contribute to the lack of predatory impact. Similarly, amoebas are found frequent, even in the most acidic parts of the river, eating large diatoms. *Vahlkampfia* species have been identified microscopically as well as other species, including lobosea-like amoebas and acanthamoeba-like amoebas.

One species of heliozoan belonging to the genus *Actinophrys* is also present in the river. Heliozoa seem to be characteristic top predators of the benthic food chain in the river. They are omnivorous and can consume organisms larger than themselves, including rotifers, algae and ciliates that get stuck at their adhesive podiae. In Río Tinto, we have observed them ingesting algae, mainly *Chlorellas*, *Chlamydomonas*, and *Euglena*. The only animal found in the river is a species of bdelloid rotifer related to the genus *Rotifera* (Amaral-Zettler et al., 2003). This low diversity of rotifers is consistent with prior observations that few species of rotifers have been found in waters of pH < 3.0 (Deneke, 2000). This pioneer rotifer species can persist because of their high physiological tolerance of severe acidic stress and the lack of other more efficient competitors.

Among decomposer fungi are the most abundant, and both unicellular and filamentous forms are present (López-Archilla et al., 2001; López-Archilla et al., 2005). While many species of fungi have been isolated from the river, one fungus (related to *Hobsonia*) has been identified in many parts of the river where it forms thick dendritic macrofilaments closely associated with other protists. When the fungus is present, a whole community forms embedded in a mucilaginous substance that could protect the inner microbial community from the external conditions by creating differential physicochemical conditions.

Although all these species have been previously described in other acidic environments, recent studies using molecular techniques showed the presence of novel eukaryotic lineages closely related to the base of the animal-fungal radiation (Amaral-Zettler et al., 2003). Further studies regarding eukaryotic biodiversity are underway in order to further identify additional novel lineages in the river.

4. Dynamics of Eukaryotic Communities in the River

The seasonal bimodality of physical conditions and levels of heavy metals ions (Fig. 3) greatly influences the eukaryotic community biomass (Aguilera et al., 2006). In winter the eukaryotic biomass is usually lower than in summer. The increase in the eukaryote population in the summer and late summer is mainly due to the occurrence of two species of filamentous green algae (*Klebsormidium* sp. and *Zygnemopsis* sp.) as well as to the presence of higher amounts of *E. mutabilis*. In general, green algae are responsible for nearly the total eukaryotic biomass increase

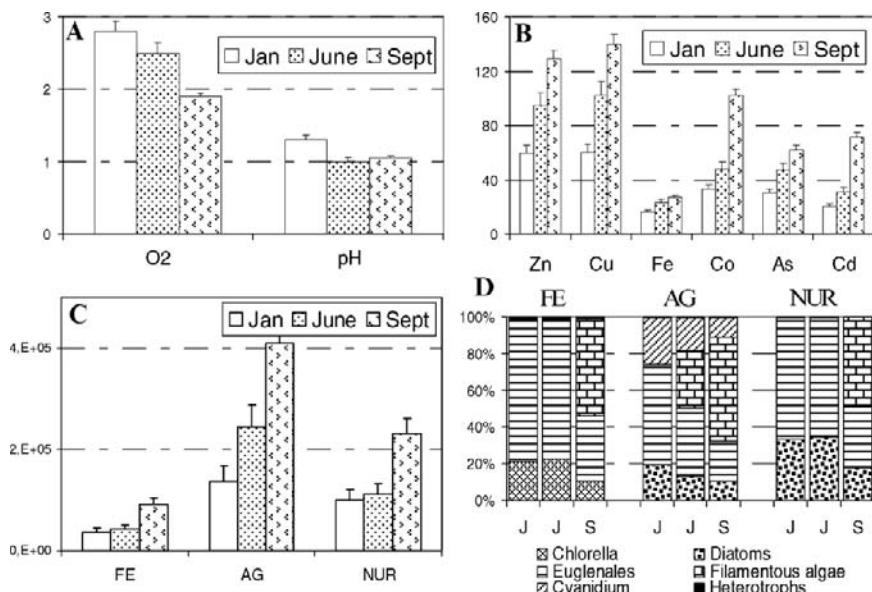


Figure 3. (A) Means and standard errors of water dissolved oxygen (mg L^{-1}) and pH at RI, one of the most extreme sampling stations of Río Tinto. (B) Heavy metal concentrations (mg l^{-1} except for Fe in g L^{-1}) at the same sampling point. (C) Benthic eukaryotic cell densities (cells cm^{-2}) at three different sampling sites (FE, AG and NUR). (D) Percentages of seasonal distribution of dominant taxa at three different sampling sites (FE, AG, and NUR).

during summer, which agrees with other studies in extremely acidic environments (Walton and Johnson, 1992; Nixdorf et al., 1998; Nordstrom and Alpers, 1999).

This fact is closely related to the significant increase in temperature values and daylight, as well as to the decrease in water flow. These factors facilitate cell deposition and biofilm formation.

There is a strong indication that sites with lower pH show lower species diversity. This finding agrees with other studies conducted in acidic lakes in which increases in acidity produces a reduction in species richness (Niederlehner and Cairns, 1990; Locke, 1992; Packroff, 2000). In the same manner, data from sampling stations located along the river show that the highest diversity index appeared at the stations located closer to the mouth of the river where the physicochemical water conditions are less severe.

5. Biofilm Formation as a Mechanism for Surviving Extreme Conditions

In aquatic ecosystems eukaryotic biofilms are diverse species communities. In Río Tinto, these are typically dominated by micro-algal consortia that form thick biofilms all over the riverbed (Fig. 4). Microscopic observations of the biofilms revealed a variety of prokaryotic morphotypes, algae, protozoa, or fungi. The

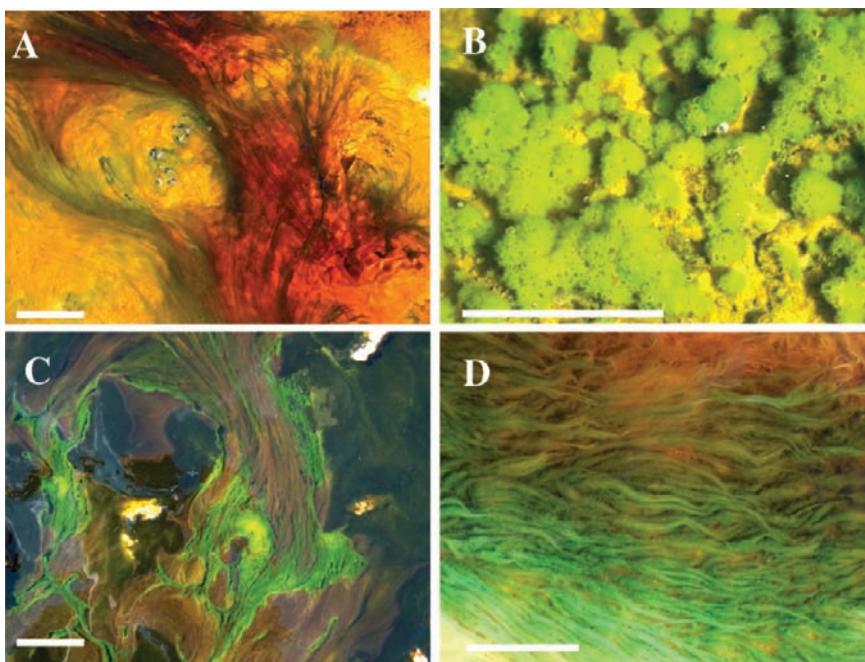


Figure 4. Microbial eukaryotic biofilms founded in Río Tinto. Scale bar = 5 cm. (A) Green filaments formed mainly by *Zygnemopsis* and *Klebsormidium* located at the origin. (B) Biofilms of *Chlamydomonas* and fungi. (C) Euglenas and diatoms are the main components of these biofilms. (D) Biofilms of *Chlorella* and fungi.

whole community is usually embedded in a thick mucilaginous coating that could protect the inner microbial community from the external water conditions.

In situ colonization studies have shown that the microbial colonization sequence starts with the fixation of an organic conditioning biofilm formed mainly by fungal hyphae, and bacterial and detrital mineral particles that will remain permanently attached to the substrate (Aguilera et al., 2006). Pioneer motile eukaryotic species, such as amoeba or heterotrophic flagellates then colonize, and this is followed by the establishment of an increasing number of sessile eukaryotes such as *Chlorella* or diatoms. Finally, biofilm formation involves the incorporation of filamentous algae. A similar pattern of biofilm growth has previously been observed in other freshwater aquatic environments, and the nonspecific, permanent adhesion of bacteria and fungi to inert surfaces has been thoroughly described (Tolker-Nielsen and Molin, 2000). Thus, the development of these preconditioned substrata is of major importance to ensure colonization by autotrophs, especially in lotic systems (Korte and Blinn, 1983).

An earlier study of metal-exposed diatom biofilms has shown that the extracellular mucous matrix could be partially responsible for an increase in biofilm tolerance (Barranguet et al., 2000). This matrix is mainly composed of

extracellular polymeric substances (EPS) which provide a suitable microenvironment for microbial development. EPSs could act as detoxification agents against heavy metals because they contain high amounts of negatively charged functional groups that could act as metal-binding sites (Sutherland, 1999). In this regard, the capacity of the acidic biofilms to accumulate metals was recently reported by García-Meza et al. (2005). Their results showed that EPS production was a critical feature for the survival of algal biofilms on mining tails. In addition, EPS synthesis increased when the biofilms were exposed to different heavy metals.

Many studies have investigated the physiology and structure of bacterial biofilms in order to understand the underlying processes of their formation. Analysis of the internal structure of biofilms by confocal laser scanning microscopy (Norton et al. 1998) have shown that biofilm communities form highly structured microbial assemblies (Moller et al. 1998). For eukaryotic biofilms, the internal structure has been less studied; however scanning electron microscopy in backscattered mode has permitted the observation of transversal sections of the biofilms still attached to the substrate (Aguilera et al. 2006). Microscale structural differences among naturally grown biofilms have been observed in different localities of Río Tinto. Although some of the biofilms formed a well defined layered structure (Fig. 5A), others showed several layers of cells loosely packed between minerals or decaying organic matter (Fig. 5B). Many biofilms were dominated by a specific photosynthetic microorganism bacteria were conspicuous throughout them (Fig. 5C). Some biofilms such as the ones formed by *Dunaliella* (*Chlorophyta*), only appear in the most acidic localities, and have a clear association with fungal communities (Fig. 5D). In addition to the vertical distribution of the same organism, different biofilms are distributed horizontally under the same environmental conditions. In general the surface is heterogeneously colonized, the factors responsible for this distributional pattern are still not well known, but might be related with specific growth of different microorganisms and surface characteristics (Hutchinson et al., 2006). The structural study has shown the importance of metabolic interactions within the microbial communities themselves, and also between different biofilms.

Different factors may be responsible for these structural differences. Several layers of sediment indicate fluctuations of the flow and rapid recolonization of the new surfaces. Several studies have demonstrated that age is a determining factor of biofilm structure with the more loosely structured mats representing a younger state in biofilm development (Horodyski et al., 1977; Heinen and Lauwers, 1985). In our case, most of the biofilms are dramatically reduced every year during the rainy season. Thus age is not the only parameter that increases structure. Differences in water velocity may also play a significant role in determining biofilm structure (Battin et al., 2003) and the amount of material accumulated on the sediments, allowing a higher density of material to accumulate. It has been demonstrated that some biofilm-forming microorganisms can produce sticky excretions that are able to trap allochthonous mineral particles

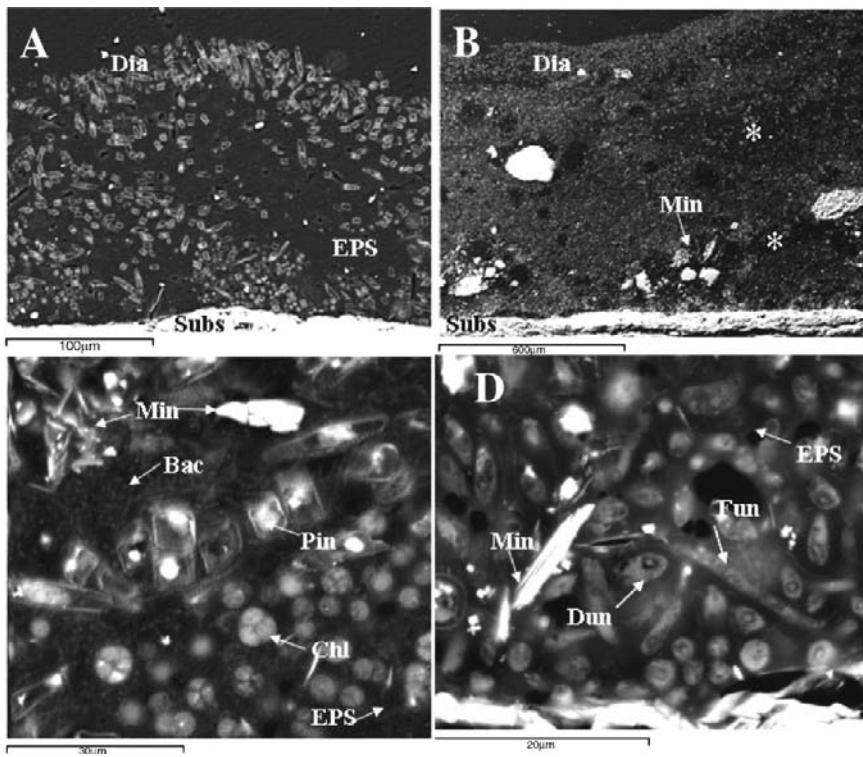


Figure 5. SEM-BSE images of transversal sections of microbial biofilms found in Río Tinto. (A) Diatom biofilm showing distribution of cells. The upper and lower part in contact with the substrate (Subs) are dense areas whereas the inner part is less compact and has more extracellular polymeric substances (EPS) between the cells. (B) Biofilms of diatoms can reach thickness of several mm over the surfaces (Subs). Some areas marked with asterisks are filled with decaying organic matter. (C) The areas where two photosynthetic biofilms contact (*Pinnularia* and *Chlorella*) shows also important bacteria communities (Bac) surrounded by extracellular polymeric substances (EPS). (D) Biofilms of *Dunaliella* within a matrix of extracellular polymeric substances (EPS) are present in the most extreme conditions and show a clear relation with fungi.

within the mats. This may be an adaptation to prevent the mats being swept away by the current (Golubic et al., 2000).

Finally, intrinsic physiological features of the microorganisms forming the biofilm can be responsible for specific internal, lateral structure, and distribution on the riverbed.

In biofilms, the matrix around the cells is a critical feature associated with physiology and environmental conditions. Some studies have stated that diatoms in marine habitats produce variable amounts of extracellular polymeric substances under different environmental conditions (Taylor et al., 1999). In the case of bacteria, the complex regulation of surface attachment, surface binding, biofilm

maturity and ultimately, biofilm detachment, is affected by the physiology of the cells and by the physicochemical parameters of solid surfaces and environments (Davies et al., 1998). However, in eukaryotic biofilms, data regarding intercellular regulation in the formation of the biofilms is scarce. Although the development of microorganisms forming biofilms in the presence of a variety of stressful environmental conditions has been well documented (Allan, 1995), specific adaptation that explain the mechanisms why biofilm survives are not yet well understood.

Thus, biofilm formation and structure reflect the adaptation of the microorganisms to different environmental conditions of Río Tinto. Periphytic algae when forming biofilms with other microorganisms might have nutritional advantages or specific microenvironmental conditions that allow them to be exposed to much less severe physical and chemical conditions than those of the external habitat. Further *in situ* microsensor techniques studies and controlled mesocosmos experiments will be necessary to fully understand the factors controlling biofilm formation and the association among different microorganisms.

6. Eukaryotic Isolates and Their Properties

Culturing of extremophilic organisms has received an increasing attention due to economic importance in industries for agricultural, chemical, and pharmaceutical applications. Thus far, enzymes from extremophiles are the most useful molecules commercialized (i.e., Taq polymerase), although some organisms can be used directly in bioremediation or as nutritional supplement (Hough and Danson, 1999). In the same manner, interest in organisms that thrive in extreme environments has grown because they can provide important insights into molecular biology such as information on protein folding, membrane properties, or cellular repair mechanisms.

Río Tinto has yielded a number of species of eukaryotes that can be grown in culture. Thus, different types of chlorophytes (*Chlorella* sp. and *Dunaliella* sp.), euglenids (*E. mutabilis*), rhodophytes (*Cyanidium* sp.) or amoebae (*Vahlkampfia* sp.) have been isolated. However, most of the physiological studies regarding acidophilic eukaryotes from Río Tinto have been performed with different strains of one species of *Chlamydomonas* (*Chlorophyta*) closely related to *Chlamydomonas pitschmanii*.

Messerli et al. (2005) have characterized the electrochemical H⁺ gradient that exists across the plasma membrane in a clonal isolate of *Chlamydomonas* from Río Tinto and compared it with *Chlamydomonas reinhardtii*. The optimal pH for growth of the acidophilic strain occurred between pH 2 and 6, while *C. reinhardtii* showed optimal growth between pH 5.5 and 8.5. The acidophilic isolate maintained an average cytosolic pH of 6.6 in culture media at both pH 2 and pH 7, while *C. reinhardtii* showed an internal pH of 7.1 in pH 7 culture. This indicates that these acidophilic algae are maintaining a slightly acidic cytosol even

under more neutral conditions, reversing the electrochemical force on H⁺. Furthermore, the transmembrane electric potential difference of the acidophilic *Chlamydomonas* strain was close to 0 mV, a rare value for plants and protists. At the same time, these acidophilic strains consumed about 7% more ATP per second at pH 2 than at pH 7. Due to these facts, the protection mechanism proposed is the active extrusion of H⁺ into a cytosolic vacuoles. These vacuoles would help to maintain neutral cytosolic pH without H⁺ membrane transporters exposed to the extreme acidic environment (Messerli et al., 2005).

The effect of cadmium on the growth and ultrastructure of three strains of *Chlamydomonas* isolated from Río Tinto has been studied (Aguilera and Amils, 2005). Most research on heavy metals and phytoplankton physiology are related to environments polluted by industrial and domestic wastes. However, little is known about the toxicity of these substances in environments with a natural, nonanthropogenic source of high levels of heavy metals. Acidic environments are the ideal ecosystems in which these studies could be carried out. pH has a considerable effect on the availability and, as a consequence, the toxicity of heavy metals (Mason and Jenkins, 1995). As previously mentioned, acidic environments tend to contain unusually high concentrations of heavy metals because their solubility increases markedly as the pH decreases (Nordstrom and Alpers, 1999).

Acidophilic *Chlamydomonas* strains show an unusual tolerance to cadmium (EC₅₀ 0.2 mM of cadmium), being resistant to up to two orders of magnitude more Cd than other phytoplanktonic species analyzed to date grown under similar conditions except for pH. Light microscopy shows that cytological changes induced by Cd are evident even at low concentration (0.1 mM Cd) (Fig. 6). A significant reduction of the chlorophyll content was observed in all the concentrations assayed from the first day. Cultures grown under 0.4 and 0.8 mM Cd remained bleached throughout, and the cells became nonmotile, even in cultures at 0.2 mM Cd. TEM analysis confirmed these results, and Cd impacts include changes to chloroplasts, pyrenoids, starch granules, and vacuoles. The chloroplasts of *Chlamydomonas* sp. exposed to Cd were significantly smaller than controls, indicating a decrease in the photosynthetic activity of this alga. This reduction has been widely reported for other species of microalgae growing under Cd exposure, and it has been suggested that these changes could be due to loss of chlorophyll a and b and/or carotenoids (Rai et al., 1990).

Exposure to 0.1 and 0.2 mM Cd resulted in a significant increase in the number and relative volume of starch granules. The pyrenoid was also affected with a reduction in volume (Fig. 6). These results are consistent with observations on *Dunaliella minuta* and *Chlamydomonas bullosa* (Visviki and Rachlin, 1993). TEM microanalysis highlighted the accumulation of condensed electron-opaque material, within vacuoles. Similar dense granules have been visualized in other microalgae species treated with heavy metals (Nassiri et al. 1997). EDXA analyses have shown that these dense bodies contain Cd, P, and Fe. Metal deposition in vacuoles is a mechanism that may contribute to heavy metal tolerance, indeed,

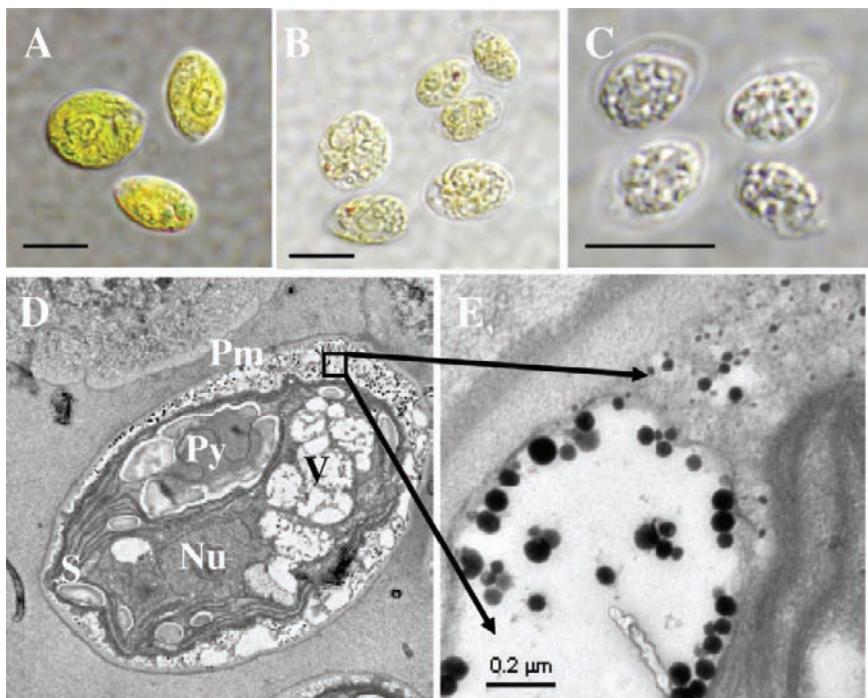


Figure 6. Light microscopy photographs of acidophilic *Chlamydomonas* sp. growing under different amounts of cadmium: (A) controls; (B) 0.2 mM Cd; (C) 0.4 mM Cd. Scale bar 10 μ m. Electron micrographs of ultrathin sections: (D) cells grown under 0.4 mM Cd, the periplasmalemma space (Pm) surrounds the cell is increased, as well as the number and volume of cytoplasmic vacuoles (V) and starch granules (S). The nucleolus (Nu) is seen within the nucleus (Nu) as well as the pyrenoid (Py); (E) Dense precipitates are now located in the cytoplasmic vacuoles.

the accumulation of Cd in the vacuoles seems to be the most effective system for maintaining a low cytoplasmic Cd elsewhere in the cell.

In addition to algae, fungi are the major organisms isolated from Río Tinto. These fungi have been used as models for their tolerance to different heavy metals, as well as their capacity to remove metals from the environment. Thus, these organisms may have important applications in bioremediation (Durán et al., 2001).

Canovas et al. (2003) reported the isolation of a filamentous fungus that was *Aspergillus* sp. from Río Tinto, able to grow at 200 mM arsenic. Further, this strain showed a polyresistant phenotype, being able to grow at concentrations of As, Cu, and Cr at least 50-fold higher than other strains of *Aspergillus*. Similar to *Chlamydomonas*, this fungus grows at heavy metals concentrations over an order of magnitude higher than those found in the river. At the same time, the strain sequestered ca. 50%, of these heavy metals. Vacuolar compartmentalization has been suggested to play an important role in the regulation of metal concentrations in the cytosol (Gadd, 1993). In this acidophilic strain, this mechanism might partly

explain the resistance to low concentrations, but it cannot account for the whole phenomenon.

Although further studies on the specific mechanisms of heavy metal resistance should be performed, interest in organisms that thrive in extreme environments can provide important data regarding the molecular biology and genetic of metal tolerance and detoxification processes.

7. Río Tinto as a Model of Astrobiological Interest

The discovery by the MER Opportunity Rover of the extensive *Terra Meridiani* iron formation on Mars has led to Río Tinto being recognized as an important mineralogical analog to the Martian site (Fernández-Remolar et al., 2004, 2005). Although thermal oxidation mechanisms have been proposed for its origin on Mars, geomorphology and IR spectroscopy support an aqueous environment as host to its secondary iron-enriched mineralogy. It has also been suggested that such environments are acidic due to sulfur complexation and iron buffering of aqueous solutions (Fairén et al., 2004). Although other terrestrial hydrothermal systems have been proposed as analogs to understand these interesting Martian environments, different studies found hematite as a mineral phase of Mars's iron rocks, not the silica that frequently appears in hydrothermal systems. However, the acidic waters from Rio Tinto produce ferric iron-enriched sediments from which silicates are absent. These features make the river an interesting analog for the Mars hematite site if an aqueous origin of these oxides is considered.

The existence of high numbers of eukaryotic organisms thriving in the extreme conditions of Río Tinto poses interesting questions of astrobiological interest. First, that complex eukaryotic systems, like those described in this chapter, can rather easily develop in the extreme acidic conditions and high concentration of heavy metals present in the Tinto ecosystem. Furthermore, the level of eukaryotic diversity found in this environment is several orders of magnitude higher than the prokaryotic diversity. This contradicts our current notions of biology and ecology. Although we do not understand at present the reasons for this adaptation (probably related to the availability of iron) (Amils et al., 2004; Gómez et al., 2004), it is clear that complex and evolved organisms can develop on other planets in conditions that had been thought to be incapable of sustaining life only a few years ago. Obviously this observation has to have an important impact on the design of future astrobiological exploration missions.

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SPECIES COMPOSITION OF CYANIDIALES ASSEMBLAGES IN PISCIARELLI (CAMPI FLEGREI, ITALY) AND DESCRIPTION OF *GALDIERIA PHLEGREA* SP. NOV.

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1. Introduction

In his landmarking “Thermophilic organisms and life at high temperature,” Brock (1978) wrote about the biogeography of *Cyanidium* that virtually every acidic hot springs in the world had been colonized by this organism. This was the end-point of a series of geographical expeditions which had been carried out since the mid 1920s of the past century, when Molisch (1926) visited numerous Japanese thermal springs, recording the ubiquitous presence in sulphuric acid rich waters of a microalga initially identified as a *Chroococcus* species. In the same years a team of German scientists was studying the algal flora of Java, Bali and Sumatra (Deutsche Sunda expedition 1928–1929), isolating from different volcanic sites a microalga quite similar to that observed by Molisch (1926). Geitler and Ruttner (1936) classified this species as *Cyanidium caldarium* and placed it within the Cyanophyceae.

During the 1930s the occurrence of *C. caldarium* in Japanese hot springs was extensively recorded by Negoro, who later (1944) published a detailed account of his field studies. Further Japanese thermal springs were visited approximately in the same years by Schwabe (1944), who confirmed the ubiquitous presence of *C. caldarium* in these habitats. Schwabe in the same paper also described the results of his explorations in Chile, presenting a description of another organism, *C. caldarium* forma *chilense* previously classified as *C. caldarium* var. *chilensis* (1936). The main diacritical character of this organism was its occurrence in non-thermal and non-acidic habitats (caves located along the shores of Central and South Chile).

The modern exploration of *Cyanidium* begins with T.D. Brock, who carried out a broad survey of more than 200 thermal areas in North and Central America, Europe (Iceland and Italy), Japan, Hawaii and New Zealand (Brock, 1978). The exploration covered aquatic and terrestrial stations of each thermal spring, documenting the presence of *Cyanidium* in both kinds of habitat and in all the thermal sites. According to Doemel and Brock (1971) the occurrence of *C. caldarium* was recorded in aquatic habitats between 20°C and 55°C, whereas

on soils the temperature range was between 10°C and 55–57°C. In both habitats *C. caldarium* was always found at pH values below 5.0.

During the 1970s two other extensive explorations on the algae living in acidic habitats were performed in Europe. Hargreaves and Whitton (1976) carried out a comprehensive survey of the algal flora of highly acidic streams of England, which later was extended to the rest of UK and to several sites in Belgium and in the USA (Whitton and Diaz, 1981). The selected sites were mainly associated to mine drainage, and to a minor extent, with the cooler part of geothermal springs and with several industrial effluents. According to the authors *C. caldarium* occurred in some of these non-thermal habitats, even though the site in which it was found was not indicated.

In the same years Pinto and Taddei (1978) visited up to 120 acidic sites of Italy, reporting the presence of *C. caldarium* not only in acidic hot springs, but also in acidic non-thermal sites, such as the sulphur mines. The authors documented for the first time that almost all the sites investigated were characterized by the presence of mixed populations in which not only *C. caldarium* was found but also two other very similar unicellular algae, which were subsequently classified as *Cyanidioschyzon merolae* and *Galdieria sulphuraria* (De Luca et al., 1978; Merola et al., 1981). Pinto and Taddei (1978) did not give an account of the relative abundance of these three microalgae in natural assemblages, but stressed that *Cy. merolae* was less frequent than the other two species, and scarcely present in all the samples in which it had been found.

The coexistence of up to three thermo-acidophilic unicellular algae was subsequently confirmed in American thermal sites (De Luca et al., 1979; Gambardella et al., 1980) and in Asia (De Luca et al., 1981). In more recent times a similar situation was also found in the Kamchatka peninsula (Russia) and on Kunashir Island, where in addition to the three Cyanidiales species worldwide distributed, three new *Galdieria* species were for the first time observed (Sentsova, 1994).

All the data so far collected suggest that thermoacidic environments throughout the world are inhabited by mixed populations of Cyanidiales, but composition and patterns of distribution of each single species in these natural assemblages remain unknown.

To shed light on the influence that physical and chemical variations exert on Cyanidiales assemblages we have examined the distribution and variability of their natural assemblages at Pisciarelli, a hydrothermal system located on the eastern edge of the Solfatara crater in the central part of the Campi Flegrei Caldera (Napoli, Italy) (Fig. 1). Pisciarelli is a liquid-dominant system, with spring water temperatures of up to 92°C (Valentino and Stanzione, 2003). Thermal pools are fed by meteoric waters intersected by fumarolic gases rising from a deep boiling aquifer. The gaseous sulphur in the fumarolic fluids is totally represented by H_2S .

The microbial community is composed of different groups of prokaryotes, such as Archaea (Kvist et al., 2005), chemotrophic sulphur bacteria, anoxygenic



Figure 1. The site of Pisciarelli, Campi Flegrei (Naples, Italy).

phototrophic bacteria and eukaryotic algae (Brock, 1978); these latter are mainly represented by Cyanidiales assemblages, growing diffusely over the entire site, and other acid-tolerant belonging to Chlorophyceae and Bacillariophyceae (Huss et al., 2002; Ciniglia et al., 2005; Pollio et al., 2005).

The coexistence of several ecological conditions (hot springs, streams, mud, rock walls), characterized by different pH, temperature values, water potential and mineralogical parageneses makes Pisciarelli as an ideal site for analysing the Cyanidiales assemblages and their relationships with the different microhabitats occurring in the site.

2. Description of the Site

For the purposes of this study, the site of Pisciarelli, whose dimensions are approximately 40×20 m, has been divided into five stations characterized by different environmental conditions:

Station A: This area is delimited by a rock wall of irregular outline, 1.50 m wide \times 6 m long; surrounding a hot sulphur spring where strong fumarolic emissions continuously blow against the rocks. The temperature of the rock walls ranges from 33°C to 45°C. Cyanidiales form a soft-green mat which completely covers the wall surface (Fig. 2, station A).

Station B: It corresponds to the soils covering a large part of the site, starting 10 m from the hot pools. Station B is covered by sandy soils, irregularly covered by opal and/or alunite layers generated by the hydrothermal activity. Here, Cyanidiales assemblages lie superficially as a thin stratum

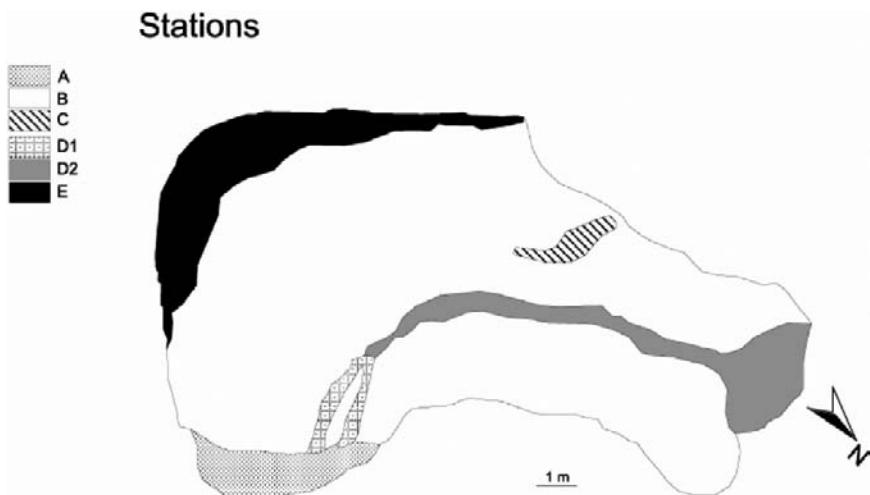


Figure 2. Map of Pisciarelli, with locations of five sampling stations: (A) rocks around hot boiling pools; (B) altered soil surrounding hot pools; (C) fissure in the rock wall; (D1 and D2) muddy soil and border of the rivulet; (E) endolithic community.

between the crushed stones, forming clearly identifiable emerald-green patches. The temperature variation depends on the presence of numerous small fractures in the soil, scattered all over this station, from which hot vapours are liberated. Soil temperature is 37–45°C across a round area of about 20–30 cm in diameter encircling each fracture. In some spots of station B, where fractures are absent, temperature ranges from 18°C to 30°C from winter to spring. Cyanidiales assemblages are largely present, forming deep-green patches diffused all over the station, and particularly in the vicinity of small fractures (Fig. 2, station B).

Station C: This station is a fissure of approximately 2 × 1.2 m, enveloped in sulphur emissions, and covered by a thin and crumbly sulphate crystal layer. It is an interlithic site in which the Cyanidiales grow at temperature ranging from about 35°C to 43°C (Fig. 2, station C).

Station D: Two rivulets rise respectively from the main pool and from a secondary hot spring, and flow across a slope of siliceous sinter into channels about 10 cm deep, for a length of 10 m (Fig. 2 station D1), after which they become a single stream, running for 10 m and opening out at the end into a small pond, which dries up during summer. The temperature of the two bubbling rivulets ranges from 60°C to 65°C, both in winter and in spring, because of fumarolic emissions. Downstream, when the rivulets flow together, gaseous emissions were never observed and temperatures ranged from 32°C to 45°C (Fig. 2, station D2). Cyanidiales assemblages occur along the border of the streamlets, often intermingled with dense local aggregations of diatoms, which could be macroscopically recognized by their green-brown colour.

Station E: A rock wall situated in the south-western part of the area of Pisciarelli. It is a dry, crypto-endolithic site, in which the Cyanidiales occur beneath the superficial layers of alunite. Temperature ranges from 18°C to 22°C in winter and from 24°C to 30°C in summer, due to the scarce influence of the gaseous emissions (Fig. 2, station E).

3. Results

3.1. CHEMICAL ANALYSES OF ROCKS AND MINERALS

Al, Si, P, S and K are the main chemical elements constituting rocks in Pisciarelli; Cl and Ti are scarcely represented, and Mg is totally absent. Sulphates are very widespread in this environment, as are sulphur, alunite and other soluble sulphate minerals. The main product of the alteration of the volcanic cover produced by acidic fluids is opal, spread on the surface of the trachitic rocks and among fractures. Alunite is the predominant supergenic sulphate phase at Pisciarelli, mainly represented by the potassic term $KAl_3(OH)_6(SO_4)_2$. The sulphate mineral paragenesis includes Al-sulphate hydrate minerals, letovicite, alunogen, as well as terms of the alum group like tschermigite; native sulphur also occurs.

In station A the sulphur fumes are extremely powerful, providing constantly high temperatures and high humidity rates; here, rocks around the pool are mainly composed by Si; all the other chemical elements (P, K, Ti, Al, S, Cl) are scarcely represented.

Chemical analyses of mats collected in the altered soil of station B revealed a high content of Si (65%), K (30.8%) and S (8.04%), likely to be related to the presence of opal and scarce microcrystalline alunite. No significant mineralogical differences have been detected between stations B and C.

The altered soil surrounding rivulets and stream (station D) results from trachytic rock alteration by hot acid fluids. It is rich in opal, while sulphate minerals represent only a minor component. The muddy bed of the rivulets was made up of silica deriving from volcanic rocks.

The rock walls of the endolithic sites at Pisciarelli (station E, Fig. 2) are strongly leached and appear white due to the presence of alunite; here gases and vapours come from surrounding fumaroles and pools with minor flow.

4. Algal Distribution

4.1. STATION A – ROCKS AROUND HOT BOILING POOLS: THE CYANIDIALES COMMUNITY

The sulphur fumes in station A (Fig. 2) are extremely powerful, favoring the development of a large, thick algal mat characterized by algal communities in which only members of the Cyanidiales were observed; green patches, formed

exclusively by Cyanidiales, were distributed along the station at temperatures ranging from 33°C to 45°C and at very low pH values (0.5–1.5).

The population density of Cyanidiales assemblages at the five sample points of this station ranged from 11 to 65×10^6 cells/g of sample (fresh weight). The relative abundance of *G. sulphuraria* varied from about 20% to 50%, whereas *C. caldarium* was the dominant species, occurring at a relative percentage of 48–67%. Finally *Cy. merolae* was the least represented species in the Cyanidiales assemblages: its abundance did not exceed 15% (Table 2).

4.2. STATION B – ALTERED SOIL SURROUNDING HOT POOLS: THE CYANIDIALES-PINNULARIA-CHLAMYDOMONAS COMMUNITY

At station B sites pH values ranged from 1.0 to 1.8 and temperature was 37–43°C. The algal communities were composed of Cyanidiales, *Chlamydomonas pitschmannii* and *Pinnularia* sp. Cyanidiales accounted for about 60% of the assemblages, whereas *Ch. pitschmannii* and *Pinnularia* sp. amounted to 40% of the population. The total cell number of Cyanidiales in the sampling sites was in the range between 34 and 59×10^6 cells/mg of fresh weight (Table 2). The relative abundance of *C. caldarium* in the Cyanidiales assemblages was between 38% and 84%, whereas *G. sulphuraria* was present at 9–60% and *Cy. merolae* represented not more than 7% of the total Cyanidiales population.

4.3. STATION C – FISSURE IN THE ROCK WALL

This station is characterized by the presence of a fracture in the rock wall, 2 m high and 1.5 m wide. Here the temperature is constantly high, ranging from 35°C to 55°C, and the pH is between 0.5 and 1.5 (Table 1). The algal community detected in station C was dominated by Cyanidiales. The total cell number of Cyanidiales in the algal assemblages of station C was in the range between 3.9 and 79×10^6 cells/g fresh weight (Table 2). In station C *G. sulphuraria* numerically predominant (60–95%), whereas *C. caldarium* in one sampling point reached 40% of relative abundance in the Cyanidiales assemblages, being limited in the other sampling points at 30% or less. *Cy. merolae* was not present in this station.

4.4. STATIONS D1 AND D2 – MUDDY SOIL AND BORDER OF THE RIVULET

In station D1 (two rivulets flowing separately) temperature was higher than 60°C and no algal mat was found along the border of rivulets.

In station D2, a single rivulet flows that originated from the confluence of the two rivulets, a photosynthetic community composed of Cyanidiales and

Table 1. Main chemical elements detected for each station. Lowest and highest pH and temperature values during the samples collection are also reported.

Station	Chemical elements	pH		T	
		Min	Max	Min	Max
A – rocks surrounding hot pools	Al 0.89%, Si 73.26%, P 5.05%, S 0.61%, Cl 0.65%, K 3.52%, Ti 1.32%, Fe 0.00%	0.5	1.5	33	45
B – altered soil surrounding hot pools	Al 10.02%, Si 65.0%, P 10.32%, S 8.04%, Cl 0.50%, K 25.08%, Ti 0.05%, Fe 3.84%	1.0	1.8	37	43
C – fissure in the rock wall	Al 9.89%, Si 67.08%, P 9.48%, S 9.81%, Cl 0.00%, K 20.72%, Ti 0.9%, Fe 2.78%	0.5	1.5	35	55
D – muddy soil of the rivulet					
D1	Al 5.63%, Si 57.5, P 4.5%, S 18.05%, Cl 0.00%, K 15.5%, Ti 0.50%, Fe 0.5%	1.0	3.0	60	65
D2	Al 5.63%, Si 57.5, P 4.5%, S 18.05%, Cl 0.00%, K 15.5%, Ti 0.50%, Fe 0.5%	2.0	3.0	32	42
E – altered rock walls	Al 10.64%, Si 20.02, P 2.03%, S 43.45%, Cl 0.00%, K 2.09%, Ti 0.00%, Fe 0.2%	0.5	1.0	18	30

Pinnularia sp. was detected underneath a thin slimy layer (1–3 mm), only on the borders and on the muddy bed of the single stream (Fig. 2, D2) formed by the confluence of the two rivulets. Here temperature and pH were respectively in the range 32–42°C and 2.0–3.0 (Table 1). Cyanidiales and *Pinnularia* sp. were present and the diatom was the dominant species. Cyanidiales in this assemblages represented less than 20% of total cell number. *C. caldarium* was also in this station the dominant species, amounting to 43–92% of the total cell number, whereas *G. sulphuraria* occurred in a range between 5% and 55%, and *Cy. merolae* was always below 11% (Table 2).

4.5. STATION E – ENDOLITHIC COMMUNITY

The endolithic algal population had a density ranging from 23 to 30×10^6 cells/g of fresh soil (Table 2) and was exclusively represented by *Galdieria* at 0.5 mm depth, under a more external sulphur–sulphate layer exposed to the air, in a low altered rock matrix lying on opal resulting from rock alteration. Temperature was not high ($T_{\max} = 30^\circ\text{C}$), and pH very low (0.5–1.5) (Table 1). The algal layer was around 0.2 mm thick. In one case, an endolithic population of *Galdieria* cells was found at 10 cm depth inside the white and crumbly wall.

Table 2. Total cells number and % of each genera of Cyanidiales in the investigated stations. The values are the average of three counts. The standard error was never higher than 5%.

Station	Total cell number	% <i>Galdieria</i>	% <i>Cyanidium</i>	% <i>Cyanidioschyzon</i>
A1	118,000,000	42	53	5
A2	33,300,000	35	50	15
A3	31,200,000	20	67	13
A4	56,300,000	38	58	4
A5	65,400,000	50	48	2
B1	58,400,000	9	84	7
B2	59,500,000	39	59	2
B3	57,600,000	32	65	3
B4	46,500,000	35	61	4
B5	34,300,000	60	38	2
C1	14,300,000	69	31	0
C2	3,900,000	80	20	0
C3	31,400,000	60	40	0
C4	13,800,000	95	5	0
C5	79,000,000	85	15	0
D2.1	25,400,000	5	92	3
D2.2	6,900,000	55	43	2
D2.3	4,000	22	67	11
D2.4	8,000	50	45	5
D2.5	20,000	18	78	4
E1	25,000,000	100	0	0
E2	23,000,000	100	0	0
E3	27,000,000	100	0	0
E4	30,000,000	100	0	0
E5	28,000,000	100	0	0

The data so far presented represent the results of two field campaigns carried out in late winter and spring of 2005. Cyanidiales assemblages present a composition of species which can be highly different in the stations considered: as a general rule, *Cy. merolae* is always a minor component of these assemblages, being limited to about 10% of the Cyanidiales populations, whereas *C. caldarium* or *G. sulphuraria* alternatively dominate in the different stations. To explore differences in abundance of these two species between pH or temperature classes, data on the numerical composition of natural assemblages were examined with one-way ANOVA, when they met the assumption of normality or homogeneity of variance, and with ANOVA Kruskall–Wallis when groups were not homogeneous. Statistical analyses have revealed that the relative abundance of *C. caldarium* and *G. sulphuraria* in the assemblages is not significantly different between pH or temperature classes (not shown).

Doemel and Brock (1971) suggested that soil water content is one of the major forces driving the composition of Cyanidiales assemblages. As can be seen in Fig. 3, laboratory experiments indicate that there is a clear difference in desiccation

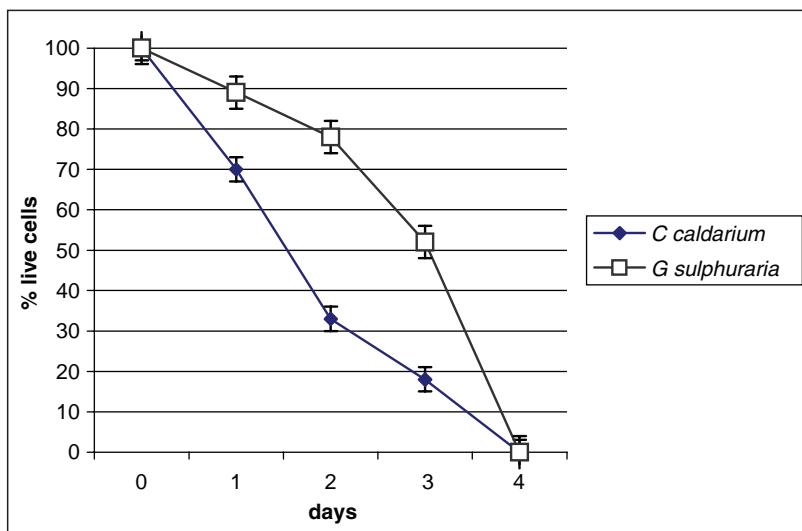


Figure 3. Desiccation tolerance between *C. caldarium* and *G. sulphuraria*. The algae, collected in the exponential phase of growth (500,000 cells/mL), were dehydrated by mild heating in a convection oven at 40°C for 1–4 days. A sample of each strain was stained daily with the vital stain neutral red to estimate the number of dead cells.

tolerance between *C. caldarium* and *G. sulphuraria*, this latter species being much more resistant to loss of water. From these results it could be possible to predict that in humid stations the dominant species of Cyanidiales assemblages could be *C. caldarium*, whereas in relatively dry habitats *G. sulphuraria* should prevail.

The population structure of the Cyanidiales assemblages sampled in the five station of Pisciarelli was also investigated by analysing a three-gene data set of plastid sequences: *psaA*, *psb A* and *rbcL*. Molecular analyses of environmental samples were performed by extracting DNA from each natural sample, and subsequently by amplifying the *rbcL* gene using PCR. The results have confirmed the relative composition of Cyanidiales assemblages, as obtained from the counts with the optical microscope. Moreover, two phylogenetic analyses were performed: in the first one were partial sequences of the three concatenated plastid genes *psaA* (1,395 nt), *psbA* (957 nt) and *rbcL* (1,215 nt) found in 17 Cyanidiales, 15 non-Cyanidiales red algae and two green algae and a glaucophyte as the out-group (Fig. 4). In the second analyses *rbcL* data-sets representative of Cyanidiales strains from different geographical locations were compared: four Asian, three Russian, three American and 17 local populations from Italy (Fig. 5). Both trees documented that the *Galdieria* lineage is divided in two subgroups, defined as *Galdieria A* and *Galdieria B*. The former includes strains with a worldwide distribution, whereas members of *Galdieria B* lineage are restricted to Italy. All the samples from the endolithic station (E) of Pisciarelli belong to the *Galdieria B* lineage,

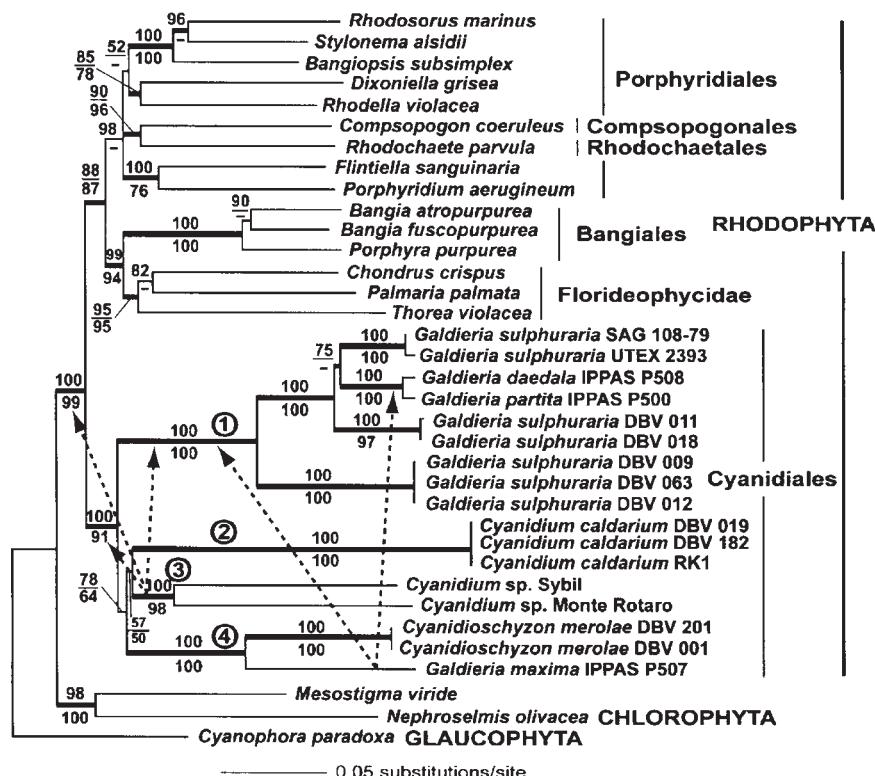


Figure 4. Phylogeny of the Cyanidiales inferred from minimum evolution (ME) analysis using the logDet transformation of the combined plastid DNA sequences of *psaA*, *psbA* and *rbcL*. Results of a ME-logDet bootstrap analysis are shown above the branches, whereas the bootstrap values from a protein maximum likelihood analysis using the JTT evolutionary model are shown below the branches. Only bootstrap values >60% are shown. The thick nodes represent >95% Bayesian posterior probability for clades using the site-specific GTR model (from Ciniglia et al., 2004).

which was also found in few other natural samples of station C, where light intensity was much reduced.

The differences between the two *Galdieria* lineages have also been confirmed by ecophysiological experiments. Two strains respectively belonging to *G. sulphuraria* A and *G. sulphuraria* B have been isolated from Pisciarelli natural samples. *G. sulphuraria* A cultivated at 25 and 38°C exhibited higher growth rates than *G. sulphuraria* B. Moreover this latter strain showed the best growth at 25°C (Fig. 6). The photosynthetic rates versus light intensity of *G. sulphuraria* A and B are shown in Fig. 7. *G. sulphuraria* B has documented a very low P_{max} , confirming that it is a shape-adapted strain.

The *G. sulphuraria* strains so far isolated from Pisciarelli revealed no morphological traits that could be used to distinguish between them, but molecular

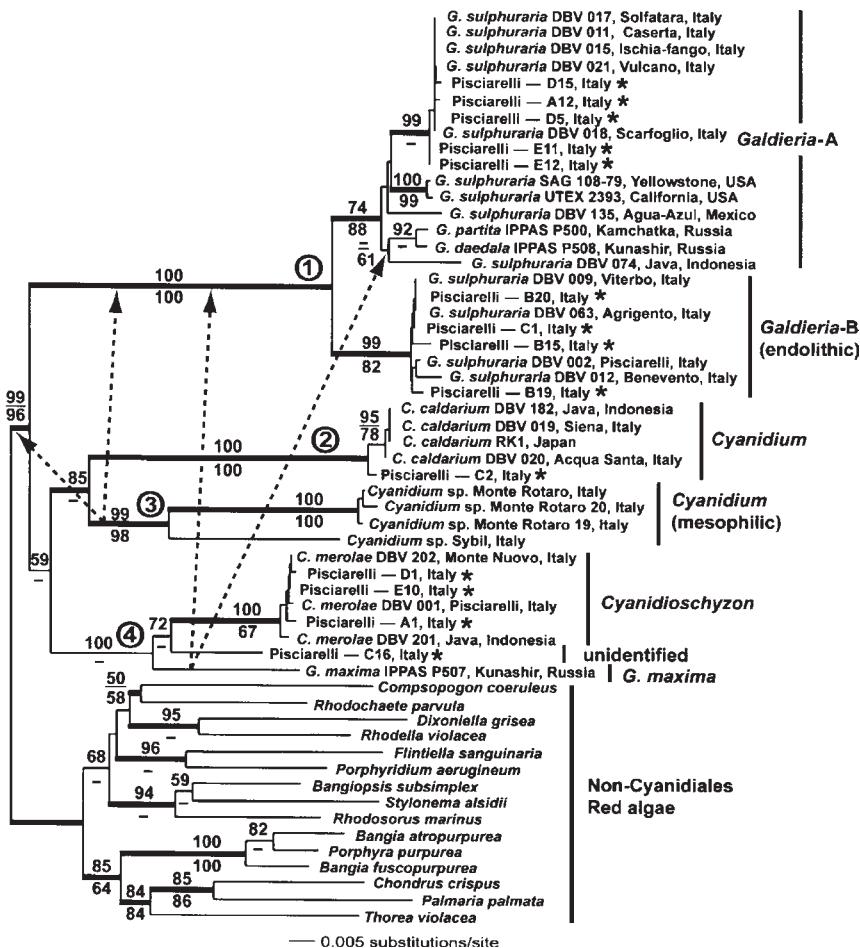


Figure 5. Phylogeny of the Cyanidiales inferred from a minimum evolution (ME) analysis using the LogDet transformation of the *rbcL* sequences: Results of a ME-LogDet bootstrap analysis are shown above the branches, whereas the bootstrap values from a protein maximum likelihood analysis using the JTT evolutionary model are shown below the branches. Only bootstrap values >60% are shown. The thick nodes represent >95% Bayesian posterior probability for clades using the site-specific GTR model. Sequences from the environmental survey are represented by Pisciarelli [followed by] collection site and are marked with asterisks (from Ciniglia et al., 2004).

analyses have shown that the endolithic strain is clearly distinct from the *G. sulphuraria* typical species. Similar situations have already been described for unicellular green algae: Suda et al. (2002) proposed the species *Nannochloropsis oceanica* on the basis of *rbcL* sequence differences, and Krienitz et al. (2004) have instituted the new genus *Parachlorella*, based on molecular data, such as the complete sequences of both the 18S rRNA gene and the ITS2 region.

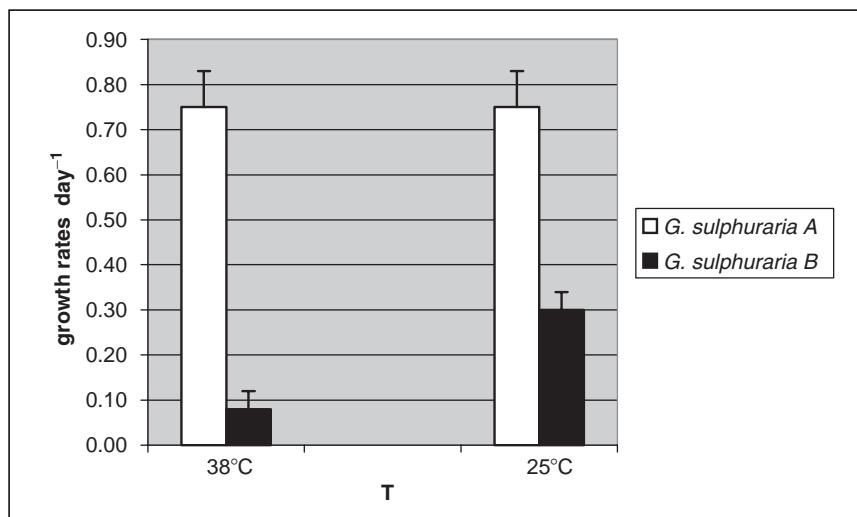


Figure 6. Growth rates of *G. sulphuraria* A and *G. sulphuraria* B at 38°C and 25°C. The two strains were grown on liquid medium (Allen) and the growth was followed for 15 days by colorimeter readings at 550 nm.

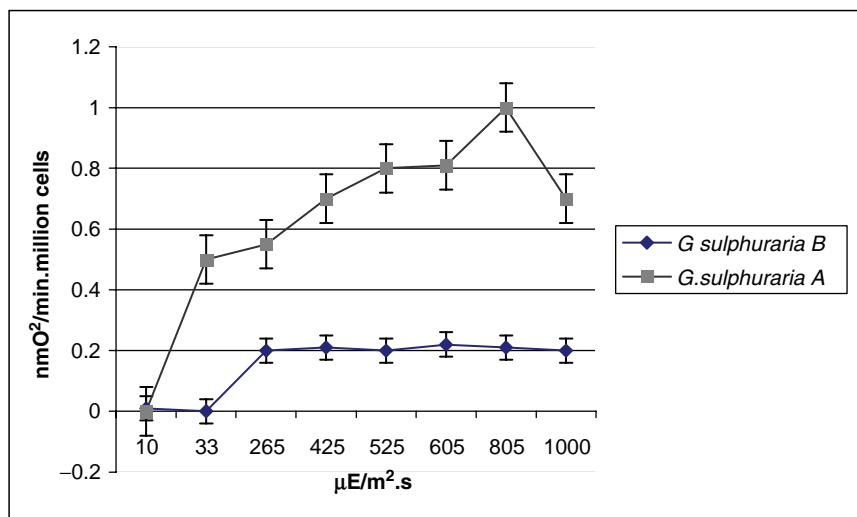


Figure 7. Photosynthetic rates of *G. sulphuraria* A and *G. sulphuraria* B as a function of light intensity. The experimental temperature was 25°C.

In our opinion, the molecular differences observed between the strain *G. sulphuraria* A and *G. sulphuraria* B support the institution of a new *Galdieria* species, which has been named as *G. phlegrea* and includes the *Galdieria* B lineage. Further tests should be performed on other isolates of the same lineage to confirm

that also ecophysiological traits, such as the best growth at relatively low temperature (25°C), and the low P_{\max} value, can be considered as diagnostic characters of this species.

Galdieria phlegrea G. Pinto, C. Ciniglia, C. Cascone, A. Pollio sp. nov.
Etymology from the latin *phlegrea* = volcanic. The specific epithet refers to Campi Flegrei (Naples, Italy), the site where the alga was found.

Proprietas morphologicae et propagatio similis sed haec species ordine nucleotidorum in rbcL cetera specie generis differt. In Campi Phlegrei, Neapoli segregata.

Morphological characters and reproduction are the same, but this species differs from the other species of the genus by the order of nucleotides in *rbcL*. Isolated from Campi Flegrei, Naples, Italy.

Authentic strain: deposited in the Algal Collection of Dipartimento delle Scienze Biologiche, Section of Plant Biology, University “Federico II” of Naples, Italy with strain number 291.

Type Locality: Pisciarelli, a branch of Campi Flegrei Caldera, Naples, Italy.

5. Acknowledgements

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A GENOMICS APPROACH TO UNDERSTANDING THE BIOLOGY OF THERMO-ACIDOPHILIC RED ALGAE

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1. Introduction

While members of the archaea rule at the high end of the temperature spectrum of life, members of the bacteria and eukaryotes thrive in a wide range of extreme conditions, including low temperatures, high and low pH-values, high salinity, and desiccation. In this context, it is important to note that the definition of extreme (and thus extremophilic) is anthropocentric, defining those environments as extreme that are hostile to human life. Photosynthetic protists are particularly versatile when it comes to occupying extreme habitats and thriving under extreme conditions. Protists thrive in saturated salt solutions, in hot acid, in extreme cold, and at high pH. This chapter deals with a small group of thermo-acidophilic unicellular red algae, called the *Cyanidiophyceae*.

Cyanidiophyceae (Cyanidiales) are evolutionary ancient extremophilic red microalgae. They are obligate acidophiles (pH 0–4); only a single *Cyanidium*-like strain has been reported from a nonacidic habitat (Hoffmann, 1994). The *Cyanidiophyceae* are also facultative meso-thermophiles, with optimal growth temperatures of approximately 42°C and an upper temperature limit of 56°C (Doemel and Brock, 1970; Deluca and Taddei, 1976; Gross et al., 2002). Some strains have also colonized habitats with moderate to cold temperatures (Gross and Gross, 2001; Gross et al., 2002). They thrive in pure CO₂ (Seckbach et al., 1970; Seckbach and Libby, 1970) and can withstand toxic concentrations of heavy metals (Albertano and Pinto, 1986; Nagasaka et al., 2004) and high amounts of salt (Gross et al., 2002) (Horst and Weber, unpublished results).

Earlier hypotheses on a bridging function of Cyanidiales between prokaryotic blue-green cyanobacteria and the eukaryotic algae could not be verified by recent molecular evidence. Based on molecular clock estimates, Cyanidiales are probably more than 1.5 billion years old. They are thus likely representing some of the oldest extant eukaryotic organisms. Yoon et al. (2002, 2004) have recently shown that the *Cyanidiophyceae* form a sister group to other rhodophytes and are located at the root of secondary endosymbiosis. The phylogenetic position of the *Cyanidiophyceae* is also supported by detailed studies on the evolution of starch

metabolism in Apicomplexa, rhodophytes, and chlorophytes (Coppin et al., 2005; Patron and Keeling, 2005). Yoon et al. (2006) recently classified the *Cyanidiophyceae* as a single class of the subphylum Cyanidiophytina, whereas the rest of the red algae was placed under the subphylum Rhodophytina.

Currently, six species are classified as *Cyanidiophyceae*: *Cyanidium caldarium*, *Cyanidioschyzon merolae*, *Galdieria partita*, *Galdieria daedala*, *Galdieria maxima*, and *Galdieria sulphuraria* (Albertano et al., 2000; Ciniglia et al., 2004). Differentiation between the genera assembled in the *Cyanidiophyceae* is difficult and their systematics and taxonomy has long been a matter of debate (Deluca and Taddei, 1976; Merola et al., 1981; Seckbach, 1991). *Cyanidioschyzon* is quite easily distinguished by its oval shape, the lack of a cell wall and binary fission as mode of replication. Due to a very similar morphology, differentiating between *Galdieria* and *Cyanidium* is not straightforward. Biochemical and physiological features such as the utilization of nitrate, carbon heterotrophy, and the occurrence of linolenic acid have been proposed as markers (Boenzi et al., 1977; Merola et al., 1981; Nagashima et al., 1986). However, only the latter two criteria hold up to scrutiny (Gross, 1999).

Despite the interesting biological features of the *Cyanidiophyceae*, progress in gaining a molecular understanding of the biology of these organisms has been relatively slow. Recent progress in genomics opens exciting new avenues to the understanding of the biology of thermo-acidophilic rhodophytes. This chapter will provide an overview of genomics technologies and their application to *Cyanidiophyceae*.

2. The Dawn of the Genomics Area

The genomics revolution in biology started a decade ago with the completion of the genome sequence of *Haemophilus influenzae* in 1995 (Fleischmann et al., 1995), followed by that of *Escherichia coli* K-12 in 1997 (Blattner et al., 1997). Shortly after, the complete genome sequences of the unicellular eukaryote *Saccharomyces cerevisiae* (Goffeau et al., 1996), the multicellular eukaryotes *Caenorhabditis elegans* (Chervitz et al., 1998) and *Drosophila melanogaster* (Adams et al., 2000) followed, and the human genome was completed in 2001 (Lander et al., 2001; Venter et al., 2001). The first genome of a land plant (*Arabidopsis thaliana*) was completed in 2000 (TAGI, 2000), more than 3 years ahead of the original timetable. The rice genome followed in 2002 (Sasaki et al., 2002; Yu et al., 2002), and several other plant genomes are currently being sequenced. As of May 2006, more than 373 complete genome sequences are listed in the Genomes On Line Database (<http://www.genomesonline.org/>) (Liolios et al., 2006) and thousands of genome projects are ongoing, with the number of genome projects growing exponentially. The knowledge of complete DNA sequences for a variety of organisms has already led to remarkable insights and discoveries, such as fundamental understanding of evolutionary processes and

ecological systems. Comparative genomics analyses across species have revealed novel insights into the structure and function of genomes. Using high-throughput sequencing technology, it became possible to characterize the genetic and taxonomic diversity of entire microbial communities, even though most of the species present in these communities have never been cultured. For example, using a whole-genome shotgun approach, 148 previously unknown bacterial species were found in seawater samples from the Sargasso Sea (Venter et al., 2004). In another example, nearly complete genomes of two microorganisms and partial genomes of three other organisms have been reconstructed by sequencing total DNA from a natural acidophilic biofilm (Tyson et al., 2004), emphasizing the power of metagenomics approaches (Allen and Banfield, 2005).

Technological advances, including the high-throughput sequencing technology, have fueled the genomic revolution. Over the course of a decade, through the parallelization, automation, and refinement of established sequencing methods, the Human Genome Project (HGP) motivated a 100-fold reduction in sequencing costs, from \$10 per finished base to 10 finished bases per \$1 (Collins et al., 2003).

2.1. CYANIDIALES GENOMICS

A milestone in the genomics of Cyanidiales occurred in 2004 with the publication of the complete sequence of *C. merolae* (Matsuzaki et al., 2004). Before 2004, the plastid (Ohta et al., 2003) and mitochondrial genomes (Ohta et al., 1998) of *C. merolae* and the plastid genome of *Cyanidium caldarium* had been characterized (Glöckner et al., 2000). The genome of *C. merolae* 10D is 16.5 Mbp in size and is organized on 20 chromosomes (Matsuzaki et al., 2004). Only 26 genes (0.5%) were found to contain introns and it was shown that at least 86% of the 5,331 genes are expressed. For comparison, 5% of all yeast genes and 79% of *Arabidopsis* genes contain introns (Matsuzaki et al., 2004). The overall G + C content of the *C. merolae* genome is 55%, significantly higher than that in *G. sulphuraria*, which has a G + C composition of 37% (Barbier and Weber, unpublished result).

Genomic analysis of *G. sulphuraria* was initiated in 2004 by sequencing of some 6,000 randomly selected cDNAs (expressed sequence tags, ESTs) from two different libraries that were constructed from cells grown under autotrophic or heterotrophic conditions, respectively. Clustering of the ESTs yielded 3,047 contigs that contained 1.7 Mbp of nonredundant sequence (Weber et al., 2004). This study revealed for the first time a complete biosynthetic pathway for lipid A in a eukaryotic organism. In addition, it was found that Cyanidiales genomes do not encode typical eukaryotic hexokinases but prokaryotic glucokinases. Many genes potentially involved in carbohydrate and starch metabolism were identified and several genes of the photorespiratory pathway could be recognized. A relatively large number of carbohydrate and other transporters were detected, several of these for the first time in eukaryotes, such as the magnesium transport system mgtE and a Na⁺/H⁺ exchanger. Furthermore, it was found that the red algae most

likely use a triose phosphate/phosphate antiporter for export of triose phosphates from the chloroplasts to the cytosol (Weber et al., 2004). A recent phylogenetic study revealed that these plastidic phosphate translocators are ubiquitously distributed throughout all plastid-containing organisms, including apicomplexa (Weber et al., 2006). It can therefore be assumed that plastidic phosphate translocators were crucial for the establishment of plastids in photosynthetic eukaryotes via endosymbiosis by connecting the metabolisms of host cell and plastid (Weber et al., 2006). The translocators most likely originated from nucleotide–sugar transporters of the endomembrane system, which must have evolved shortly after the first eukaryotic mitochondrial cell had evolved (Weber et al., 2006).

Also in 2004, sequencing of the *G. sulphuraria* genome was initiated, using a whole-genome shotgun sequencing approach. This is the method of choice for small genomes containing only a limited number of repetitive sequences such as the *Galdieria* genome. It is particularly useful if a clone-based physical map of the genome has not yet been established, as it was the case for *G. sulphuraria* (Fraser and Fleischmann, 1997; Fraser et al., 2000; Sterky and Lundeberg, 2000; Green, 2001; Wendl et al., 2001). The whole-genome shotgun sequencing strategy has been successfully used to sequence the genomes of prokaryotes such as *H. influenzae* (Fleischmann et al., 1995) or *Halobacterium* (Ng et al., 2000), but also for large and complex eukaryotic genomes such as the human (Venter et al., 2001; Waterston et al., 2002a) and the mouse genomes (Waterston et al., 2002b). Crucial requirements for this sequencing approach are: (i) narrow insert size distribution of representative shotgun libraries; (ii) adequate sequence coverage generated by paired-end sequence reads; (iii) paired-end sequences from larger insert clones to serve as additional landmarks in the assembly process; (iv) algorithms and processing capacity to assemble the sequence fragments; and (v) independent methods to verify the quality of the computer generated assembly. As of March 2006, 238,136 random sequence reads have been obtained from small-insert plasmid libraries, and from cosmid and BAC ends. These sequences equal 147 Mb of Q20 bases. The assembly program Arachne, which was developed at the Whitehead Institute/MIT Center for Genome Research (Batzoglou et al., 2002) and has been successfully used to assemble the sequence of the mouse genome (Waterston et al., 2002b) was used for assembling the *Galdieria* reads. From 147-Mb high-throughput reads, 14 Mb of nonredundant sequence was obtained, indicating >10-fold coverage of the genome sequence by shotgun reads. Currently, the genome sequence data is contained in 962 contigs that assemble into 894 scaffolds. The N50 length of the scaffolds is 173 kbp, and >80% of the cumulative scaffold length is contained within the 68 largest scaffolds. Based on the scaffold derived from BAC and cosmid end sequencing, we estimate a final genome size of approximately 15 Mbp, similar to the genome of *C. merolae*. Different genome sizes have been reported for *G. sulphuraria* in the literature. For example, using microspectrophotometry, a genome size of 10.8 Mbp was estimated for *G. sulphuraria* 074W (Muravenko et al., 2001) and genome sizes ranging between 9.8 (*G. sulphuraria* 19.71) and 14.2 Mbp (*Galdieria spec.* isolates, Rio Tinto, Spain) were determined using pulsed-field gel electrophoresis (Moreira

et al., 1994). Further, chromosome numbers between 2 (Muravenko et al., 2001) and over 40 (Moreira et al., 1994) have been reported. Our own karyotyping data indicate that the latter figure is more realistic; using pulsed-field gel electrophoresis, we found approximately 42 chromosomes sized between 100 kbp and 1 Mbp (Fig. 1). The genome size determined from karyotyping data is approximately 12 Mbp, close to the estimate derived from genome sequencing data.

Mapping ESTs to the genomic sequence revealed that many *G. sulphuraria* genes contain introns, frequently even several introns per gene (Barbier et al., 2005a, b). This is a marked difference to *C. merolae*, which contains introns in only 26 genes (Matsuzaki et al., 2004). *G. sulphuraria* introns are short (45–60 bp) but the borders of the introns display typical spliceosomal features as previously described for a LHC gene from *G. sulphuraria* (Marquardt et al., 2000).

In addition to the very small introns, the intergenic regions are also small (see Fig. 2). These specific features of the *Galdieria* genome hamper ab initio gene

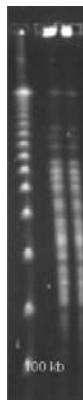


Figure 1. High-resolution pulsed-field separation of *G. sulphuraria* chromosomes. Two independent DNA preparations have been loaded. The banding pattern is highly reproducible between independent preparations. Please note the intense staining of chromosomes between 150 (second marker from bottom of lane 1) and 400 kbp (band 7 of marker, counted from the bottom of lane 1), indicating multiple chromosomes per band.

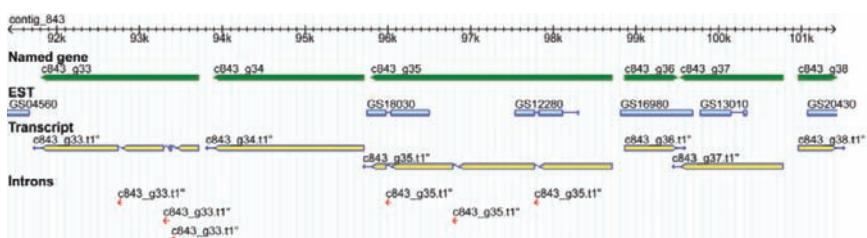


Figure 2. Very high gene density in the *G. sulphuraria* genome. Predicted genes are shown in green color, ESTs mapping to these genes are shown in light blue. Predicted transcripts are shown in yellow. Five of the six genes contained in a 9-kbp region of contig 843 are supported by cDNAs. The intergenic regions in this area are less than 200 bp in all cases.

predictions using programs such as GenScan (Burge and Karlin, 1997) or GeneMark.hmm (Lukashin and Borodovsky, 1998). None of the programs tested (GenScan, GlimmerR, FGENESH, GeneMark.hmm) precisely predicted more than 60% of gene structures. In particular, the small introns (<100 bp) proved to be problematic and frequently adjacent genes were spliced together, leading to gene fusions (Barbier and Weber, unpublished results). Although the accuracy of gene predictions can be improved by combining several programs (Mathe et al., 2002), or by training predictors with genomes showing similar characteristics (Korf, 2004), it becomes clear that reliable gene models require experimental support from cDNAs and ab initio gene prediction programs that are specifically trained for detecting *G. sulphuraria* genes.

2.2. COMPARATIVE GENOMICS

Comparative genomics, for example, the comparison of whole genomes, is a powerful approach to gain insight into the evolution of species and genomes (O'Brien et al., 1999; Wei et al., 2002). The genome sequences of the red alga *C. merolae* and the diatom *Thalassiosira pseudonana* have already yielded many new insights (Armbrust et al., 2004; Matsuzaki et al., 2004; Misumi et al., 2005), and further genome sequences, such as that of *G. sulphuraria*, will be extraordinarily useful to the study of, for example, the evolution of plant genomes, the study of genome reduction in the nucleomorph of cryptomonads (Gilson et al., 1997; Gilson and McFadden, 1997, 2002; McFadden et al., 1997; McFadden, 1999; Douglas et al., 2001; Cavalier-Smith, 2002b, 2003), the phylogeny of plastids (Bhattacharya and Medlin, 1995, 1998; Stiller and Hall, 1997, 1998, 2002; Martin et al., 1998, 2002; Palmer, 2000, 2003; Stiller et al., 2001, 2003; Cavalier-Smith, 2002a), and gene transfer between plastid and the nuclear genome (Keeling and Palmer, 2001; Martin et al., 2002). In particular, the latter two aspects will especially benefit from the availability of the full genome sequences of additional photosynthetic protists.

C. merolae and *G. sulphuraria* represent particularly interesting objects for comparative genomics analyses. Although *Cyanidioschyzon* and *Galdieria* are members of the same family, a comparison of 18S rRNA sequences suggests an early divergence of the two genera in evolution (Gross et al., 2001; Yoon et al., 2002). Both species show many biochemical, cytomorphological, and physiological differences (Albertano et al., 2000). The comparison of two Cyanidiales genomes (e.g., *G. sulphuraria* and *C. merolae*) will certainly lead to the discovery of conserved regulatory elements, conserved syntenic regions, and genome rearrangement events. In addition, we expect to uncover the molecular mechanisms of physiological differences between *G. sulphuraria* and *C. merolae*, for example, the lack of heterotrophy, the lack of a cell wall, the lower salt tolerance, and the different mode of replication in *C. merolae*. Initial comparative analysis of the *C. merolae* genome with ESTs and genomic fragments of *G. sulphuraria*

showed that the *G. sulphuraria* genome encodes a much larger repertoire of carbohydrate uptake systems than that of *C. merolae*. Both algal genomes encode a similar set of carbohydrate-metabolizing enzymes, indicating that the number and variety of carbohydrate transporters in *Galdieria* are key to its metabolic flexibility (Barbier et al., 2005a). More than 30% of *G. sulphuraria* genes do not have orthologs in *C. merolae* (and vice versa), emphasizing the large evolutionary distance between both algae and reflecting the adaptation of both species to different niches within their extreme habitats (Ciniglia et al., 2004; Barbier et al., 2005a).

3. Outlook and Future Challenges

While many speak of the “post-genomic era,” we feel that the genomic age has just begun. Genomics is an essential cornerstone of systems biology, and genome sequencing is prerequisite for other “omics”-technologies, such as transcriptomics and proteomics. Exciting novel technological developments will increase throughput and decrease cost of genomic sequencing, thus allowing for genomes to be sequenced not just from model organisms but also from any source from which DNA can be isolated. This sequence information will enhance our knowledge on the fundamental genotypic differences and commonalities between organisms, it will support the development of novel biomedical and agricultural applications, and it will generate new understanding in fundamental aspects of biology and evolution.

Several academic and commercial efforts are developing new ultra-low-cost sequencing (ULCS) technologies that aim at reducing the cost of DNA sequencing by several orders of magnitude (Shendure et al., 2004). Currently, a novel massive-parallel sequencing technology developed by 454 Life Sciences (Branford, CT) is the only commercially available ULCS instrumentation capable of generating whole genome assemblies at very low cost. The instrument (GS20) uses micro-fabricated high-density picoliter reactors that can generate >20 million sequence reads in 4.5 hours with 99% accuracy (Margulies et al., 2005). To generate a similar amount of sequence information, either 40 conventional sequencing machines (e.g., the 96 lane ABI Prism 3730 xl DNA Analyzer currently used in many genome sequencing centers) are required for simultaneous 4-h runs or 160-h continuous operation time is needed if only one sequencing machine is available. Using a GS20, typical microbial genomes with a 2 million base genome can be sequenced and assembled within days by a single operator. Thus, the amount of sequencing time and the cost with GS20 can both be reduced by at least one order of magnitude as compared with conventional sequencing. In addition, GS20 sequencing requires no cloning. Therefore, this technology can be used to sequence unclonable DNAs with a strong GC content bias that are invisible to conventional sequencing approach and/or also samples that are too degraded (e.g., fossil or ancient DNA) to be reasonably sequenced by

traditional approaches. The power of the novel technology has already been demonstrated in several applications. For example, one 4.5-h run generated >33 million bases of sequence information from >300,000 high-quality sequence reads that cover the genome of *Mycoplasma genitalium* (the causal agent of non-gonococcal urethritis) 40 times (Margulies et al., 2005). In other examples, genome sequences of extinct species were used to answer long-standing questions in molecular evolution and to tackle the molecular basis of speciation, gene evolution, and selection during domestication. Earlier this year, researchers used the novel massive-parallel pyro-sequencing technology to generate sequences from a wooly mammoth that perished 300,000 years ago (Poinar et al., 2006) and at a recent Biology of Genomes meeting in New York Svante Pääbo reported 1 million base pairs of *Homo neanderthalensis* nuclear DNA, the first nuclear DNA sequences from a Neanderthal (<http://www.nature.com/news/2006/060515/full/441260b.html>). Sequencing at even lower cost and throughput may become possible by application of multiplex polony sequencing (Shendure et al., 2005; Church et al., 2006).

So, what can we do with all that genome information, basically a huge mass (or mess, if you will) of As, Cs, Gs, and Ts? Of course, there is the obvious – annotation, comparative genomics, designing mRNA profiling and whole-genome tiling microarrays, single gene and multi-gene phylogenies, etc. A higher-order goal would be to deduce the metabolic network of a recently sequenced organism from its genome sequence, that is, ab initio metabolic reconstruction. Naturally, this includes functional annotation of gene products based on sequence homology. More importantly, however, this also addresses assigning functions to unknown genes and this includes assigning genes to metabolic functions, for which no gene has been identified yet (orphan metabolic activities) (Osterman and Overbeek, 2003; Chen and Vitkup, 2006). There are many exciting developments in this area and it is to be expected that these will lead to major breakthroughs in genome sequence analysis very soon. A detailed discussion of these aspects is beyond the scope of this chapter, and the reader is referred to the recent literature for further information (Bono et al., 1998; Pellegrini et al., 1999; Bowers et al., 2004b; Kharchenko et al., 2004; Vitkup, 2004; Bowers et al., 2005; Sato et al., 2005; Chen and Vitkup, 2006; Kharchenko et al., 2006; Yamada et al., 2006). The power of these higher-order data analysis increases with the depth of the sequence space, allowing to generate phylogenetic profiles (cooccurrence profiles across multiple species) (Pellegrini et al., 1999) and logical relationships (Bowers et al., 2004a, 2005), and to deduce information about genomic neighborhoods (Overbeek et al., 1999). Overlaying and integrating these data with transcript coexpression data, protein–protein interaction and protein fusion data, and with metabolomics data (i.e., all small molecules present in an extract of a tissue or organism) will be the next challenge. This will require close interaction of experimental biologists with bioinformaticians, computational biologists, and mathematicians.

What is required for further progress with understanding the biology of Cyanidiales is additional sequence information from all species of this group, and

from other red algae. Furthermore, we will need to gather transcriptome and metabolome information and we need proteomics to decipher the cellular localization of currently unknown proteins. Excitingly, essential functional genomics tools such as targeted gene disruption technology have already been established for *C. merolae* (Minoda et al., 2004), and microarray profiling of chloroplast gene expression has already yielded significant new insights into transcriptional regulation of plastid-encoded genes in red algae (Minoda et al., 2005).

To make Cyandiales broadly accepted model systems, it will require free exchange of biological material, sequence information, and research tools. It will require a multi-national concerted effort. The exciting biology of these extremophilic eukaryotes certainly deserves more attention and more resources devoted to their study.

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ENIGMATIC ARCHAEL AND EUKARYOTIC LIFE AT HYDROTHERMAL VENTS AND IN MARINE SUBSURFACE SEDIMENTS

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1. Introduction: Hydrothermal Vents and Subsurface Habitats

This chapter is intended as a brief introduction on specific, novel aspects of archaeal and eukaryotic biodiversity in two extreme marine environments, hydrothermal vents and deep subsurface sediments: deeply-branching, uncultured archaea occurring in both environments that in some cases do not fit into the well-established crenarchaeota–euryarchaeota dichotomy; the partial overlap in the archaeal community structure of hydrothermal vents and deep subsurface sediments; new developments to decode the physiology and carbon sources of subsurface and vent archaea; and the unexpected diversity of enigmatic protists at hydrothermal vents.

With a deepening understanding of hydrothermal vent geology, chemistry and microbiology came a stronger emphasis on hydrothermal vents as a “window” to the deep subsurface (Deming and Baross, 1993). The conspicuous surface features of deep-sea hydrothermal vents, the distinctive chimney-like structures (e.g., black smokers), are formed when sulfates and metal sulfides precipitate from hot anaerobic fluids upon contact with cold, oxic seawater. The metal-sulfide minerals that constitute hydrothermal vent chimneys are characterized by considerable porosity and thus provide a suitable habitat for substantial accumulations of hyperthermophilic bacteria and archaea. Due to the enormous physical and geochemical differences of the hydrothermal fluids and the surrounding seawater and sediments, these structures are characterized by steep environmental gradients of temperature, pH, redox potential and metals, thus providing diverse niches for microbial communities (McCollom and Shock, 1997). In general, the inner parts of chimneys are very hot ($>250^{\circ}\text{C}$) due to close proximity to the vent fluids; middle layers of chimneys are cooler, and can harbor a thermophilic and hyperthermophilic prokaryotic community consisting of methanogens, anaerobic hydrogen oxidizers, $\text{NO}_3^-/\text{Fe}/\text{S}^\circ/\text{SO}_4^{2-}$ reducers and fermentative heterotrophs. The outer chimney walls are more strongly influenced by cooling and oxygenation via the surrounding seawater, and are dominated by a mesophilic community, consisting mostly of H_2S , Fe, Mn oxidizers, oxygen- and nitrate-respiring hydrogen oxidizers, and aerobic methanotrophs (Jeannotton, 2000; Reysenbach et al., 2001; Corre et al., 2001; Takai et al., 2003; Hoek et al., 2003;

Nercessian et al., 2003; López-Garcia et al., 2003a; Kormas et al., 2006). As their most characteristic aspect, hydrothermal vents have yielded a treasure trove of archaeal hyperthermophiles representing a wide range of predominantly anaerobic metabolisms. For an adequate overview of currently recognized species, the first volume of Bergey's *Manual of Systematic Bacteriology* (second edition, 2001, Springer-Verlag) should be consulted. The discoveries of the most thermophilic life form, a dissimilatory Fe-III reducing archaeon capable of growth at 121°C (Kashefi and Lovley, 2003), and of obligately symbiotic nanoarchaea associated with a sulfur-reducing archaeal host (Huber, H. et al., 2002) illustrate the unexhausted potential of hydrothermal vents to yield microbiological surprises.

Hydrothermal circulation at mid-ocean ridges brings subsurface fluids and, by the same token, potentially subsurface-derived bacteria and archaea to the vent surface (Holden et al., 1998; Summit and Baross, 2001; Huber, J.A. et al., 2002; Takai et al., 2004a, b). The presently unknown depth range and point of origin of these vent subsurface microbiota is physically limited by porosity and permeability of the basaltic ocean crust at mid-ocean ridges, which would allow subsurface life within a few hundred meters of sufficiently porous near-surface lava and hydrothermal deposits (Alt, 1995). In contrast to their limited depth range, lateral dispersal of vent prokaryotes via hydrothermal circulation within the porous, basaltic ocean crust appears to be possible over at least 50 km, based on hydrological studies of the young, sediment-capped Juan de Fuca ridge flanks (Fisher et al., 2003). By subsurface transport from active hydrothermal vent areas and mid-ocean ridges via subsurface aquifers of the basalt ocean crust, hydrothermal microbial populations could gradually transition into the microbial populations of an adjacent extreme habitat, deep marine sediments.

Marine sediments cover more than 2/3 of the Earth's surface. Microbial cells and prokaryotic activity appear to be widespread in those sediments. Total counts prokaryotic cells (Parkes et al., 2000), rRNA-dependent counts of bacterial and archaeal cells (Schippers et al., 2005; Biddle et al., 2006) and analysis of intact membrane lipids (Sturt et al., 2004; Biddle et al., 2006) provide multi-faceted evidence of active prokaryotic populations in deep subsurface sediments on a global scale. Prokaryotic activity, in the form of sulfate reduction and/or methanogenesis, occurs in sediments throughout the world's oceans (D'Hondt et al., 2002). The prokaryotes of subseafloor sediments have been estimated to constitute 1/2 to 5/6 of Earth's prokaryotic biomass (Whitman et al., 1998) and 1/10 to 1/3 of Earth's total living biomass (Whitman et al., 1998; Parkes et al., 2000). The redox gradients within a few mm or cm of hot hydrothermal vent chimney walls are found in different form, extended over scales of tens and hundreds of meters, in the cool sediment layers that cover the ocean floor world-wide (D'Hondt et al., 2002, 2004). Prokaryotic cell density in subsurface sediments decreases exponentially with depth, not as a function of temperature (with the exception of hydrothermal sediments, temperatures remain moderate even in very deep sediment layers), but more likely as a result of decreasing organic carbon quality and availability in aged, deeply buried sediments (Parkes et al., 2000). Analyzing the community

composition and biogeochemical activities of these subsurface microbial communities is currently a highly active research field (Teske, 2006).

2. Enigmatic Archaea in Hydrothermal Vents and Subsurface Sediments

One of the surprising aspects of hydrothermal vents and subsurface sediments are their partially overlapping microbial community compositions; some archaeal lineages are consistently recovered from both environments. Some of these lineages do not fit into the phylogenetic scheme of crenarchaeota and euryarchaeota, which constitute the two major branches in the archaeal tree of life; they await recognition as separate phylogenetic lineages within the archaeal domain, analogous to the Korarchaeota (Barns et al., 1996). These deeply-branching archaea should be of great evolutionary interest to explore the phylogenetic link of the archaeal and the eukaryotic domains.

Members of the Deep-Sea Archaeal group (DSAG), synonymous with Marine Benthic Group B (MBG-B), provide a good example for such a widespread, novel archaeal lineage; DSAG/MBG-B archaea are well represented in clone libraries of archaeal 16S rRNA genes from hydrothermal vents and sediments (Fig. 1).

DSAG/MBG-B archaea have been reported simultaneously from hydrothermal vent sites near Japan (Takai and Horikoshi, 1999) and from cold benthic marine sediments in the Northwest Atlantic (Vetriani et al., 1999). Since then, DSAG/MBG-B archaea appear in a growing number of molecular surveys of deep subsurface sediments (Reed et al., 2002; Inagaki et al., 2003a; Newberry et al., 2004; Inagaki et al., 2006; Biddle et al., 2006), in methane seeps and seep-associated carbonate mounds and reefs (Knittel et al., 2005), in hydrothermal vent sites such as Guaymas Basin sediments (Teske et al., 2002) and Mid-Atlantic Ridge vent growth chambers (Reysenbach et al., 2000).

The small-subunit rRNA of these archaea has been isolated and reverse-transcribed from subsurface sediments, demonstrating that DSAG/MBG-B archaea have a detectable ribosome and rRNA content and thus are metabolically active in the deep subsurface (Biddle et al., 2006; Sørensen and Teske, 2006). Published phylogenies show the DSAG/MBG-B archaea as a basal group next to euryarchaeota (Reed et al., 2002) or more frequently to the crenarchaeota (Takai and Horikoshi, 1999; Vetriani et al., 1999; Takai et al., 2001b; Inagaki et al., 2003a, b; Sørensen et al., 2004; Knittel et al., 2005). Highly conserved, phylum-specific “signature” nucleotides show a mix of eury- and crenarchaeotal features (Vetriani et al. 1999). Most deeply-branching DSAG/MBG-B lineages were found in hydrothermal vents, whereas the uppermost branches (the “crown”) of the tree consists of sediment- and carbonate-associated phylotypes (Fig. 1). If this phylogenetic pattern holds up with time and further molecular surveys of the marine environment, it may indicate hydrothermal vent ancestry for this group, and later evolutionary diversification into nonhydrothermal, anaerobic sediment habitats.

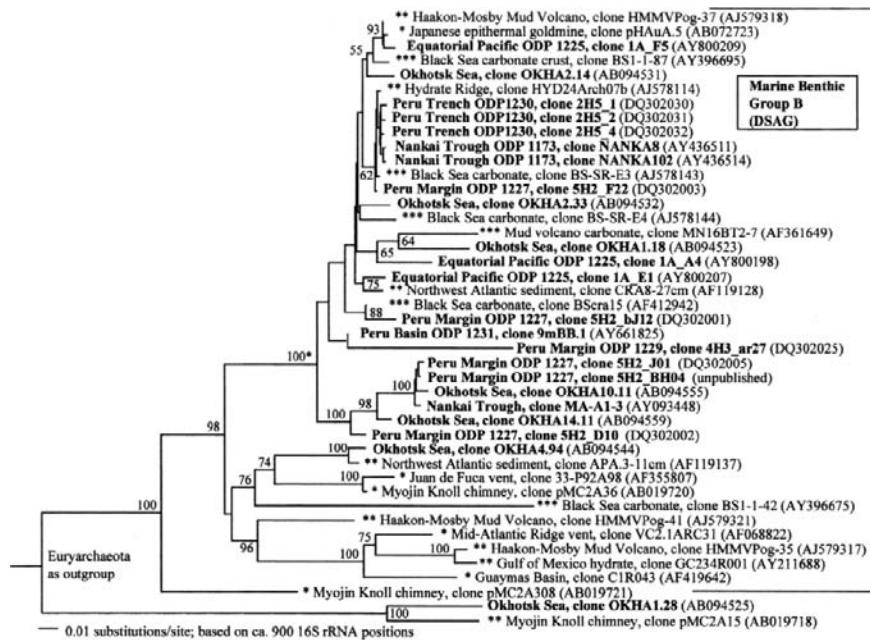


Figure 1. 16S Phylogeny (minimum evolution) of the DSAG/MBG-B archaea. 16S rDNA and rRNA phylotypes from marine sediments and carbonates constitute a well-supported large cluster (100% bootstrap; marked with asterisk); hydrothermal phylotypes occur mostly in deeper, basal lineages. GenBank numbers are given in parentheses. *, clones from hydrothermal or geothermal habitats; **, from surficial marine sediment; ***, from marine carbonate reefs and crusts. Bold print indicates subsurface sediment clones.

Habitat and physiology of DSAG/MBG-B archaea are currently under study. Recently, active (rRNA-detected) DSAG/MBG-B archaea were found within a steep methane-sulfate gradient layer sandwiched between other archaeal populations (Fig. 2, next page), suggesting that these Archaea may benefit directly or indirectly from anaerobic methane oxidation (Sørensen et al., 2006). Members of the DSAG/MBG-B archaea have been detected in a wide range of anoxic, marine environments, including methane-consuming Black Sea microbial mats and carbonate reefs (Knittel et al., 2005), deep-sea sediments from the Okhotsk Sea (Inagaki et al., 2003a), hydrate-containing sediments of the Pacific Margin and in the Nankai Trough (Reed et al., 2002; Newberry et al., 2004; Inagaki et al., 2006), in surficial sediments of the Northwestern Atlantic Ocean (Vetriani et al., 1999), and in diverse hydrothermal vent sites (Takai and Horikoshi, 1999; Reysenbach et al., 2000; Teske et al., 2002). This diversified occurrence pattern shows that DSAG/MBG-B archaea are not limited to methane-sulfate transition layers, and do not rely on a sulfate-dependent methane-oxidizing type of metabolism. Carbon isotopic signatures of archaeal

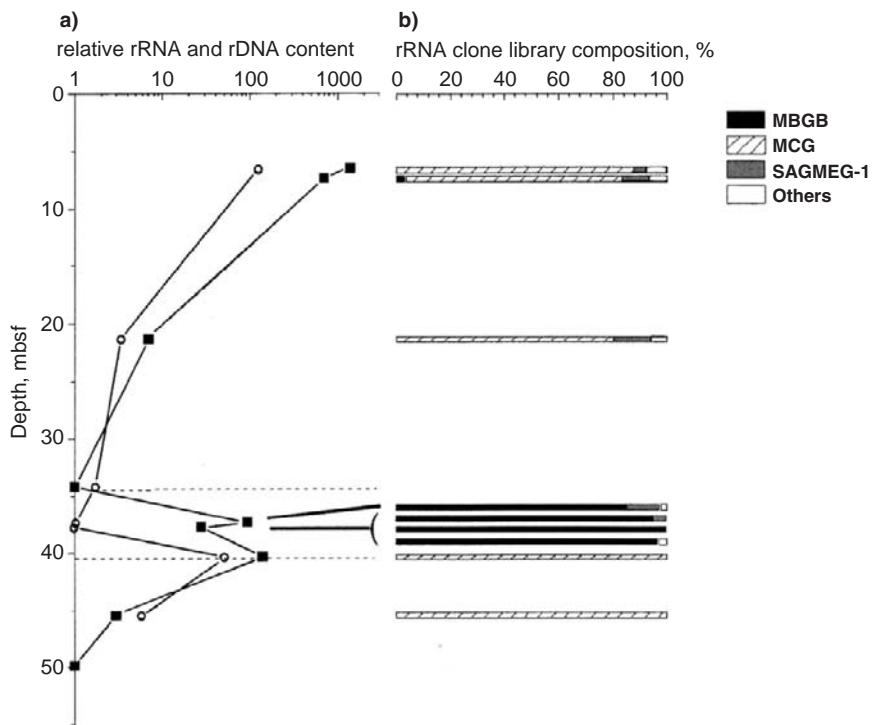


Figure 2. Stratified populations of active archaea in Peru Margin subsurface sediments (ODP Site 1227), by reverse transcription and sequencing of extracted 16S rRNA (Sørensen and Teske, 2006). *Left*, relative amount of 16S rRNA in different depths. The sulfate/methane transition zone is marked by punctuated lines. (N.D., rRNA below detection.) *Right*, phylogenetic composition of clone libraries from reverse-transcribed 16S rRNA from each depth. The sample from 37.75 mbsf was extracted twice and amplified using two primer sets. Figure modified from Sørensen and Teske (2006).

phospholipids and archaeal cell biomass from methane-sulfate interface sediments on the Peru Margin (ODP Sites 1230 and 1227) showed that buried organic carbon, not methane, is the primary carbon source for DSAG/MBG-B-dominated archaeal communities; therefore, a heterotrophic, potentially fermentative metabolism is most likely (Biddle et al., 2006). With the possible exception of hydrothermal vent phylotypes, the DSAG/MBG-B archaea are unlikely to be thermophiles; they are active in cold deep-sea sediments.

Other deeply-branching archaea share the vent and subsurface habitat of the DSAG/MBG-B archaea. The DSAG/MBG-B archaea have a sister group, the “Ancient Archaeal Group” (Takai and Horikoshi, 1999) which has not been subsumed under the eury- or crenarchaeotal kingdoms. These uncultured archaea have been detected only in hydrothermal vents and deep subsurface sediments. Phylotypes of the Ancient Archaea group were recently found in Peru Margin

subsurface sediments after reverse transcription of extracted rRNA, indicating that these archaea are metabolically active in the subsurface (Sørensen and Teske, 2006). Interestingly, these archaea were only detected with alternate PCR primers that avoided mismatch problems of a commonly used reverse primer; otherwise, deeply-branching lineages with insufficiently conserved primer target sequences escape PCR detection (Sørensen and Teske, 2006).

Similar cases of phylogenetic lineages consisting only of hydrothermal vent and sediment phylotypes include distinct lineages within the euryarchaeota, such as the “Peru Basin cluster” (Sørensen et al., 2004) and the Deep-Sea Hydrothermal Vent Euryarchaeota Group 6 (Takai and Horikoshi, 1999) within the Euryarchaeota, and the UCIIb cluster (Schrenk et al., 2003) within the Crenarchaeota. In all cases, these rare hydrothermal vent clones formed well-supported phylogenetic clusters with related phylotypes from cold, oligotrophic deep-sea sediments (Sørensen et al., 2004). These phylogenetic patterns suggest links between deep subsurface sediments and hydrothermal vent habitats, for example by hydrothermal circulation of seawater near mid-ocean ridges and subsurface seawater flow within the basaltic oceanic crust (Fisher et al., 2003; DeLong, 2004; D’Hondt et al., 2004), allowing the exchange of subsurface and vent archaea (Sørensen et al., 2004).

In marked contrast to these specialized vent and subsurface lineages, other uncultured archaeal lineages appear to colonize a wide variety of terrestrial and marine, hot and cold, surface and subsurface habitats. A good example are the members of the awkwardly named Miscellaneous Crenarchaeotal Group (MCG) (Takai et al., 2001b). This diversified group has been found in the deep terrestrial subsurface, such as South African goldmines (Takai et al., 2001b), in terrestrial palaeosol (Chandler et al., 1998), in freshwater lakes (Stein et al., 2002), in surficial marine sediment (Vetriani et al., 1999), in a wide range of marine subsurface sediments (Coolen et al., 2002; Reed et al., 2002; Inagaki et al., 2003a; Newberry et al., 2004; Parkes et al., 2005; Inagaki et al., 2006; Biddle et al., 2006; Sørensen and Teske, 2006), in terrestrial hot springs (Yellowstone; Barns et al., 1996), and in marine hydrothermal vents (Guaymas Basin; Teske et al., 2002). The MCG archaea constitute a dominant group in surveys of reverse-transcribed rRNA in deep subsurface sediments (Biddle et al., 2006) and are therefore active in the subsurface. Carbon-isotopic signatures of archaeal cells and polar lipids from MCG-dominated sediment horizons indicate utilization of buried organic carbon by the archaeal community (Biddle et al., 2006).

3. Growth Patterns of Archaea at Hydrothermal Vents and in the Deep Subsurface

Microbial growth patterns in nature are often surface-associated, for example as ubiquitous biofilms and microbial mats (Teske and Stahl, 2002). Hydrothermal vents are no exception to this trend; the deep subsurface remains to be studied under this aspect.

Growth patterns of hyperthermophilic archaea within the porous chimney mineral matrix show cell aggregates and surface association, similar to biofilm formation and early growth stages of microbial mats (Schrenk et al., 2003). Due to temperature constraints, total cell counts of bacteria and archaea tend to be two to three orders of magnitude lower in the hot chimney interior, compared to the outer chimney layers (Harmsen et al., 1997a, b; Takai et al., 2001a; Kelley et al., 2002; Schrenk et al., 2003). Biofilm formation could be a major mode of life in hydrothermal microbial ecosystems, and enable microorganisms to tolerate harsh environmental conditions, such as temperature and chemical extremes in the inner chimney matrix, that they could not survive as individual cells (Reysenbach and Shock, 2002). For several hydrothermal vent thermophiles, biofilm formation has been studied in pure culture. In the sulfate-reducing archaeon *Archaeoglobus fulgidus*, biofilm formation appears to be a response to a wide range of chemical stress factors, such as high metal concentrations, non-physiological pH, xenobiotics or antibiotics, and oxygen exposure (LaPaglia and Hartzell 1997). The fermentative, sulfur-reducing archaeon *Thermococcus litoralis* readily formed biofilms on hydrophilic surfaces after addition of complex carbon sources to the growth medium (Rinker and Kelly, 1996). Gene expression patterns of biofilm-forming cells of the fermentative anaerobic bacterium *Thermotoga maritima* revealed increased transcription of genes involved in iron and sulfur transport, and biosynthetic functions that point to up-regulation of iron–sulfur cluster assembly and repair functions (Pysz et al., 2004).

So far, microscopy of bacterial and archaeal cells in deep subsurface sediments using Fluorescence in situ hybridization techniques has detected individual cells distributed in the sediment, but no biofilms or cell clusters (Mauclaire et al., 2004; Schippers et al., 2005; Biddle et al., 2006). Specifically, DSAG/MBG-B archaea have been visualized by rRNA-targeted FISH hybridization in Black Sea microbial mats growing on carbonate reefs; they appear as small (<1 µm), coccoid or slightly elongated cells growing in clusters (Knittel et al., 2005).

4. Protists at Hydrothermal Vents and in the Subsurface

The search for deeply-branching, ancestral protist lineages by small subunit rRNA gene surveys is expanding the known diversity of life significantly, in parallel to ongoing surveys for deeply-branching bacteria and archaea (López-Garcia et al., 2001; Moon van der Staay et al., 2001; Dawson and Pace, 2002; Edgcomb et al., 2002; Stoeck and Epstein, 2003; Stoeck et al., 2003). Deeply-branching lineages near the bases of the archaeal and the eukaryotic domains in the tree of life would represent the closest relatives across the prokaryotic/eukaryotic divide. In addition to the phylogenetic rationale, there are ecological reasons to examine protist diversity in hydrothermal and subsurface habitats. The abundant microbial biomass at hydrothermal vents, free-living or biofilm-associated, constitutes a rich food source for the next trophic layer, the microscopic, single-celled eukaryotes or protists. In pelagic marine environments,

cycling and recycling of microbial cells into higher trophic levels proceeds through the microbial loop, with heterotrophic nanoflagellates as a central component (Azam, 1998). Whether this concept retains some relevance at hydrothermal vents is an open and intriguing research question. Protist diversity and trophic ecology at vents have remained understudied, especially in comparison to the numerous vent-typical symbiotic associations of bacteria with marine invertebrates that transfer chemoautotrophically produced microbial biomass directly to host animals.

Hydrothermal vents harbor diverse populations of cultivable and not-yet-cultured protists, as shown by pure culture isolations (Atkins et al., 2000) and rRNA gene studies (Edgcomb et al., 2002; López-Garcia et al., 2003b; Takishita et al., 2005). Sediment, water and mineral samples from four hydrothermal vent areas along a North-South transect in the eastern Pacific Ocean (Juan de Fuca; Guaymas Basin; 21°N and 9°N East Pacific Rise, depth range 2,000–2,500 m) yielded pure cultures of heterotrophic flagellates belonging to six phylogenetically distinct orders, the Ancyromonadida, Bicosocida, Cercomonadida, Choanoflagellates, Chrysomonads, and Kinetoplastids (Atkins et al., 2000). Members of these orders have a cosmopolitan distribution range and occur in diverse marine and terrestrial habitats, which now extends to hydrothermal vents (Atkins et al., 2000). Some vent isolates (a *Caecitellus parvulus* strain and a *Rynchomonas nasuta* strain) showed barotolerant growth under simulated in situ pressure (up to 250 atm) in the laboratory, indicating that they are indeed active in the deep-sea vent environment (Atkins et al., 1998). Other vent strains had only limited barotolerance, indicating that they are of shallow-water origin and do not grow under in situ pressure at the vent sites (Atkins et al. 1998). Interestingly, the diversity of hydrothermal vent isolates appeared similar to heterotrophic flagellates isolated from nonhydrothermal deep-sea sediments (Scheckenbach et al., 2005), demonstrating that cold deep-sea sediments and hydrothermal vents share mutually closely related members of the same protist orders and genera.

In contrast to the limited spectrum of heterotrophic flagellates that were isolated in pure culture, molecular studies based on small-subunit rRNA gene amplification, cloning and sequencing have detected highly diverse protist populations on different levels: novel intragroup diversity within known eukaryotic phylogenetic lineages such as the Alveolates, Stramenopiles, or Cercozoa, and novel deeply-branching eukaryotic phyla that emerge as basal nodes below the eukaryotic crown group (Edgcomb et al., 2002; López-Garcia et al., 2003b; Takishita et al., 2005). Reliably distinguishing between autochthonous vent protists and introduced shallow-water or pelagic eukaryotes emerges as an interesting challenge. At this early stage it can be hypothesized that phylotypes of phototrophic eukaryotes (diatoms and green algae) that occasionally appear in Guaymas Basin clone libraries are almost certainly vestiges of locally high surface water productivity and high sedimentation rates in the Gulf of California; these factors, in combination, create the thick, organic-rich sediment cover of the Guaymas vents (Edgcomb et al., 2002). Conceptually, sedimentation maintains

a steady influx of photosynthetic eukaryotic phytoplankton (or its remnants) into hydrothermal vent sites, as shown by coccoliths accumulating in young, hydrothermally influenced Mid-Atlantic Ridge sediments (López-Garcia et al., 2003b). However, small-subunit rRNA genes of photoautotrophic eukaryotes are conspicuously absent in clone libraries from organic-poor sediments and from water samples at the Mid-Atlantic Ridge; these are dominated by different Alveolate lineages, ciliates, kinetoplastids, and novel deeply-branching eukaryotic phylum-level lineages without cultured representatives (López-Garcia et al., 2003b). In the absence of a photosynthetic background, these lineages are candidates for authochthonous deep-sea hydrothermal vent protists; however, the possibility cannot be excluded that some of these lineages represent widespread deep-sea taxa that are not necessarily vent specialists (López-Garcia et al. 2003b). More surveys of specific environments and samples will allow a more precise identification of habitat-specific protists. As a note of caution, novel phylum-level eukaryotic lineages have to be checked for chimeric artifacts, phylogenetic misplacement of fast-evolving lineages, and incomplete sampling of described but yet unsequenced eukaryotes (Berney et al., 2004).

Eukaryotic communities in the deep subsurface remain understudied at present. Cultivations and microscope observations have shown that terrestrial subsurface aquifers harbor mostly heterotrophic nanoflagellates, plus amoebae and in lower numbers ciliates; autotrophic protists, such as autotrophic flagellates, euglenids, green algae, and diatoms, were found only in a few cases, and their presence was explained by surface recharge importation (Novarino et al., 1997, and references therein). Coastal marine tidal-flat sediments have yielded eukaryotic 18S rRNA gene sequences over the full length of the cored sediment horizons (3.6 m); the phylotypes were related to marine heterotrophic communities of Alveolates, Euglenozoa, Fungi, and Metazoa (Wilms et al., 2006). There is no principal reason to assume that eukaryotic protists cannot exist in deep marine subsurface sediments as well, as long as porosity permits passage of at least small eukaryotic cells (3–5 µm), and as long as food sources, in the form of subsurface bacteria and archaea, remain sufficiently available. In a recent study of Peru Margin subsurface sediments, eukaryotic 18S rRNA genes were quantified by q-PCR and occurred in decreasing numbers with depth; the authors concluded that their results most likely indicate a sedimentary reservoir of surface-derived, degraded eukaryotic DNA (Schippers and Neretin, 2006). Transcription studies and isolation of intact 18S rRNA might resolve this issue in the future.

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PART 6:

**PHOTOTROPHS UNDER WATER
STRESS: DRY AND HYPERSALINE
ENVIRONMENTS**

Flechtner

Grilli

Caiola

Billi

Lewis

Karsten

Schumann

Mostaert

Lopez-Bautista

Rindi

Casamatta

Rindi

Oren

Biodata of **Dr. Valerie R. Flechtner**, author of the chapter “*North American Microbiotic Soil Crust Communities: Diversity Despite Challenge.*”

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NORTH AMERICAN DESERT MICROBIOTIC SOIL CRUST COMMUNITIES:

Diversity Despite Challenge

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1. Introduction

Deserts are defined in a classic paper by Noy-Meir (1973) as “water-controlled ecosystems with infrequent, discrete, and largely unpredictable water inputs.” They are found to a greater or lesser extent on all six continents (including Antarctica). Based on the moisture index system of Thornthwaite (1948), Meigs (1953) divided deserts into three categories: extremely arid (less than 60–100 mm mean annual precipitation), arid (60–100 to 150–250 mm mean annual precipitation), and semiarid (150–250 to 250–500 mm mean annual precipitation). Using this criterion, most of the deserts of North America would be considered semiarid or arid environments.

2. The Challenge

2.1. DESICCATION RESISTANCE

Algae inhabiting hot deserts experience a variety of environmental challenges. In addition to aridity and temperature extremes, they are also subject to high light intensity and, in some cases, high salinity. Studies of soil microalgae and cyanobacteria from hot deserts have revealed that they have several mechanisms to deal with extreme conditions. Water availability, which includes precipitation, condensation, and water vapor, is the most important factor in limiting the growth of desert algae. In a thorough review of the desiccation tolerance of prokaryotic organisms, Potts (1994) gives particular attention to cyanobacteria.

2.1.1. Compatible Solutes

The ability to accumulate disaccharides, particularly trehalose, which function as compatible solutes under conditions of water stress, is documented in many bacteria including some cyanobacteria. The hydration of proteins by water molecules is important in maintaining their three-dimensional structure and consequently their function. At intermediate stages of water deficit, compatible

solutes act to protect the shell of water around proteins. Under the most extreme conditions, trehalose actually replaces the water shell and stabilizes protein structure. When desiccated cells are rehydrated, metabolic activity resumes quickly. The ability of trehalose to stabilize membranes has also received critical attention (Potts, 1994).

2.1.2. Extracellular Polysaccharide

The ability of cyanobacteria to produce and secrete extracellular polysaccharide (EPS) sheaths is also thought to contribute to desiccation tolerance. The EPS layers of cyanobacteria are complex, often with intricate ultrastructure. They are composed of compounds, typically polysaccharides, which tend to be hygroscopic and are often pigmented. In at least some cases, the EPS forms a gel. Several researchers have shown that the sheath of *Nostoc commune* UTEX 584 has distinct transition zones between gel and sol states. This finding implies that the structure of the sheath and hence its water retention properties can be regulated during the growth of the cells (Potts, 1994). The production of sheath pigments such as gloeocapsin, fuscorhodin, and fuscocochlorin has been reported to increase desiccation tolerance (Bewley, 1979).

2.1.3. Akinetes

Some cyanobacteria, including members of the genera *Nostoc*, *Anabaena*, and *Scytonema*, form thick-walled structures called akinetes. While akinetes are generally considered to be tolerant to freezing and desiccation, unambiguous evidence to support these claims does not yet exist. Neither has the mechanism leading to the formation of these structures been elucidated (Potts, 1994). In some cyanobacteria that do not form akinetes, the entire filament forms a dormant structure, which is contained within a thick sheath. When water is present, the filament germinates and resumes vegetative growth (Bhatnagar and Bhatnagar, 2005).

2.2. PROTECTION FROM ULTRAVIOLET LIGHT

While light is necessary for photosynthesis, too much light can be harmful to algae by causing an increase in mutation frequency or photooxidative bleaching. Both cyanobacteria and eukaryotic algae produce UV-absorbing pigments, which can protect them from the deleterious effects of light. Members of the cyanobacterial genera *Scytonema*, *Nostoc*, and *Calothrix* accumulate the pigment scytonemin in the external sheath that surrounds the vegetative filament (Garcia-Pichel and Castenholz, 1991). Many other cyanobacteria accumulate mycosporine-like amino acids (Potts, 1994). Many species of eukaryotic algae accumulate yellow, orange, or red carotenoid pigments, which have been shown to provide protection from UV light.

3. The Diversity

Three hot deserts exist in North America; these are the Chihuahuan, the Mojave, and the Sonoran deserts. The Chihuahuan desert is situated on a high plain between the Sierra Madre Oriental and Occidentale Mountains. The soils are derived mostly from limestone. The total precipitation is 200–300 mm annually and falls primarily during the summer. The Mojave desert lies in the rain shadow of the Sierra Nevada Mountains. Soil is composed of mixed sediments derived from limestone and igneous rock. Precipitation is 45–200 mm annually and falls primarily in the winter. Summer temperatures can be extremely high; air temperature can reach 54°C and ground temperatures can exceed 90°C. The Sonoran Desert extends from Sonora, Mexico, and north to east-central Arizona. Considerable variation exists within the Sonoran Desert with some areas experiencing less than 70 mm and others as much as 300 mm rain annually (Dick-Peddie, 1991; Rosentreter and Belnap, 2001).

3.1. MICROBIOTIC CRUSTS IN DESERT SOILS

In arid and semiarid areas where soil cover is sparse and patchy, open soil supports the development of microbiotic crusts. Microbiotic crusts are mixed communities of organisms which may include various combinations of bacteria, cyanobacteria, eukaryotic microalgae, lichens, fungi, mosses, and leafy liverworts. The relative proportion of these components is influenced by temperature, water availability, and soil type (Belnap *et al.*, 2001; Bhatnagar and Bhatnagar, 2005). Cyanobacteria can withstand the harshest conditions and generally dominate in poor sandy soils with neutral or slightly alkaline pH. They are less prevalent in acid soils. Lichen presence increases as the content of carbonate, gypsum, and silt increases. A detailed discussion of the structure and function of soil crusts is beyond the scope of this review. This topic has been explored in depth in a recent monograph (Belnap and Lange, 2001).

3.2. ALGAL DIVERSITY IN MICROBIOTIC CRUSTS

Given the magnitude of environmental challenges faced by microalgae inhabiting arid and semiarid soils, one might anticipate that algal communities would show limited diversity. But there is a growing body of evidence which argues against this assumption. Funded in part by a National Science Foundation grant, a group of researchers from John Carroll University, Brigham Young University, and the University of Connecticut have been examining the species composition of cyanobacteria, diatoms, eukaryotic non-diatom algae, lichens, and bryophytes in microbiotic crusts of arid and semiarid deserts in the USA and Mexico. Three

investigators (V.R. Flechtner, J.R. Johansen, and L.M. Lewis) have focused on characterizing the diversity of microalgae in microbiotic crusts from the Chihuahuan, Mojave, Sonoran, Colorado Plateau, and Great Basin Deserts. We have used a variety of techniques including direct microscopic observation of wetted soil samples, microscopic observation and life-cycle studies of individual taxa isolated on agar-solidified media, and ribosomal RNA sequence analysis. We have identified diverse algal communities including genera and species not previously reported in the scientific literature as existing in desert soils. The algal diversity uncovered encompasses both cyanobacteria and eukaryotic algae.

3.3. THE ALGAL FLORA OF HOT DESERT CRUSTS

3.3.1. *Studies Prior to 1970*

Early floristic studies of desert crusts focused more on the lichen and cyanobacterial components than on eukaryotic algae. Characterizations of the algal components of microbiotic crusts from these deserts carried out during the 1950s and 1960s (reviewed by Rosentreter and Belnap, 2001) provide a much more complete description of the cyanobacterial and diatom crust components than of the non-diatom eukaryotic microalgal components (Table 1).

The relative dearth of information on the non-diatom eukaryotic algal flora can be traced to several sources. First, most crusts have far fewer eukaryotic algae than cyanobacteria. Second, the area of expertise of the researchers studying the crusts prior to 1990 often did not include non-diatom eukaryotic microalgae. Third, characterization of eukaryotic non-diatom algae often requires clonal isolation of individual taxa, characterization of life-cycle history, and sometimes DNA sequence data. Such studies are labor intensive and can be expensive. However, when a polyphasic approach to algal identification is used, a rich non-diatom eukaryotic algal diversity can be identified even in crusts from hot deserts.

3.3.2. *An In-Depth Study of the Algal Flora Found in Microbiotic Crusts from Baja California, Mexico*

The most extensive characterization of desert crust algae published to date (Flechtner *et al.*, 1998) was done on soils from the Cataviña area of the Central Desert of Baja California, Mexico ($29^{\circ}47'N$, $114^{\circ}46'W$). This area resides in the southern portion of the Sonoran Desert and would be categorized as an arid desert. Mean annual precipitation ranges from 46 to 101.7 mm (Garćia, 1981; Blom and Clark, 1984). Soils are formed by the decomposition of Cretaceous granite and are coarse and sandy. Summer mean temperature is $25.8^{\circ}C$ and winter mean temperatures is $13.2^{\circ}C$; although the temperature occasionally reaches freezing, the ground does not freeze. The majority of the sites studied showed 80–100% crust cover; cyanobacterial crusts were the dominant form (Flechtner *et al.*, 1998).

Table 1. Algal taxa identified in crusts from the Chihuahuan, Mojave, and Sonoran Deserts.

	Chihuahuan	Mojave	Sonoran (north)
Cyanobacteria			
<i>Anabaena virabilis</i> (Kütz.)	+		
<i>Calothrix castellii</i> (Massal.) Born. & Flah.		+	
<i>Calothrix parietina</i> Thuret			+
<i>Lyngbya aestuarii</i> (Mert.)			+
<i>Microcoleus chthonoplastes</i> (Fl. & Dan.)		+	
Thuret & Gom.			
<i>Microcoleus paludosus</i> (Kütz.) Gom.		+	+
<i>Microcoleus sociatus</i> (W. & G.S. West) Gom.			+
<i>Microcoleus vaginatus</i> (Vaucher) Gom.	+	+	+
<i>Nostoc commune</i> Vaucher	+	+	+
<i>Nostoc microscopicum</i> (Carm.) Harv. & Hook			+
<i>Nostoc muscorum</i> Ag.		+	+
<i>Oscillatoria</i> sp.			+
<i>Phormidium</i> sp.	+		+
<i>Phormidium tenue</i> (Menegh.) Gom.		+	
<i>Plectonema nostocorum</i> Born.	+		+
<i>Schizothrix calcicola</i> (Ag.) Gom.	+	+	
<i>Schizothrix californica</i> Dr.	+		
<i>Schizothrix lamyi</i> Gom.	+		
<i>Scytonema hofmannii</i> Ag.	+	+	+
<i>Tolyphothrix tenuis</i> Kütz.			+
Bacillariophyceae			
<i>Navicula</i> sp.	+		+
<i>Nitzschia</i> sp.			+
<i>Pinnularia</i> sp			+
Euglenophyceae			
<i>Astasia</i> sp..	+		
Chlamydophyceae			
<i>Gonium</i> sp.	+		
<i>Chlorococcum infusionum</i> (Shrank) Meneghini		+	
<i>Chlorococcum</i> sp.	+		
Chlorophyceae			
<i>Chlorella vulgaris</i> Beijerinck	+		+
<i>Chlorella</i> sp.		+	
<i>Fritschella</i> sp.	+		
<i>Palmogloea protuberans</i> (Sm. & Sow.) Kütz.	+		+
<i>Protococcus viridis</i> Agardh. (Now <i>Diplosphaera chodatii</i> (Bialosuknia) R. & F. Chodat		+	
<i>Stichococcus subtilis</i> (Kütz.) Klerck			+
<i>Trochiscia hirta</i> (Reinsch.) Hansg.			+

The data presented in this table were compiled from Rosentreter and Belnap, 2001. Some of the taxa designations have been updated in recent years.

Previous studies (Grondin and Johansen, 1993; Wheeler *et al.*, 1993) have demonstrated that the distribution of microorganisms in soil displays considerable microheterogeneity. To optimize the probability of obtaining a representative

sample of the microalgal flora and to minimize the chance of missing rare taxa, composite samples of ten subsamples were collected from ten sites independently in this region. Initial identification of algae employed both direct observation of wetted soil samples and culture on agar-solidified algae as previously described (Flechtner *et al.*, 1998). Molecular analysis was employed to identify enigmatic species (Lewis and Flechtner, 2004). A richly diverse algal flora consisting of 15 species of cyanobacteria and 37 species of eukaryotic algae from 25 genera was identified (Table 2). Four of these isolates (*Cylindrocystis brebissonii* var *deserti*, *Elakatothrix obtusa*, *Fasciculochloris mexicana*, and *Scenedesmus bajacalifornicus*) had not been previously described in the scientific literature (Fig. 1). A comparison these data with the data for the northern Sonoran Desert presented in Table 1 reveals that the diversity of eukaryotic algae is much higher than suggested by earlier studies. It is interesting to note that the genera of eukaryotic algae such as

Table 2. Microalgal composition of microbiotic crusts from the southern Sonoran Desert in Baja California, Mexico.

Cyanobacteria

- Anabaena* sp.
Lyngbya digueti Gom.
Lyngbya pulealis Mont.
Microcoleus stenstrupii J.B.-Pet.
Microcoleus vaginatus (Vaucher) Gom.
Myxosarcina burmensis Skuja
Myxosarcina spectabilis Geitler
Nostoc commune Vaucher
Nostoc muscorum Ag.
Nostoc piscinale Kütz.
Nostoc punctiforme (Kütz.) Hariot
Plectonema tomasinianum var. *gracile* Hangs.
Schizothrix arenaria (Berk.) Gom.
Schizothrix calcicola (Ag.) Gom.
Scytonema ocellatum Lyngb.

Bacillariophyceae

- Hantzschia amphioxys* (Ehr.) Grunow
Hantzschia amphioxys f. *capitata* O. Müller
Luticola cohnii (Hilse) Mannq
Luticola mutica (Kütz.) Hariot
Nitzschia hantzschiana Rabh.
Nitzschia punctata var. *minor* Temp. & Perag.
Pinnularia borealis Ehr.
Pinnularia borealis var. *scalaris* (Ehr.) Rabh.
Staurosira construens (Ehr.) Williams & Round
Chlamydophyceae
Chlorococcum minutum Starr
Eustigmatophyceae
Vischeria helvetica (Vischer & Pascher) Hibberd

(Continued)

Table 2. Microalgal composition of microbiotic crusts from the southern Sonoran Desert in Baja California, Mexico—cont'd.

Chlorophyceae
<i>Apatococcus constipatus</i> (Printz) Printz
<i>Bracteacoccus aggregatus</i> Tereg
<i>Bracteacoccus cohaerens</i> Bischoff & Bold
<i>Bracteacoccus grandis</i> Bischoff & Bold
<i>Bracteacoccus minor</i> (Chodat) Petrová
<i>Bracteacoccus minutus</i> Schwarz
<i>Bracteacoccus pseudominor</i> Bischoff & Bold
<i>Chlorella ellipsoidea</i> Gerneck
<i>Chlorella vulgaris</i> Beijerinck
<i>Chlorosarcinopsis aggregata</i> Arce & Bold
<i>Chlorosarcinopsis arenicola</i> Groover & Bold
<i>Chlorosarcinopsis auxotrophica</i> Groover & Bold
<i>Chlorosarcinopsis bastropiensis</i> Groover & Bold
<i>Chlorosarcinopsis gelatinosa</i> Chant. & Bold
<i>Chlorosarcinopsis semipervirens</i> Groover & Bold
<i>Cylindrocystis brebissonii</i> var. <i>deserti</i> Flechtner & Johansen
<i>Dictyochloropsis splendida</i> Geitler
<i>Diplosphaera</i> sp.
<i>Elakatothrix obtusa</i> Flechtner and Johansen
<i>Elliptochloris subsphaerica</i> (Reis.) Ettl & Gärt.
<i>Fasciculochloris mexicana</i> Flechtner & Johansen
<i>Klebsormidium dissectum</i> (Gay) Ettl & Gärt.
<i>Klebsormidium flaccidum</i> (Kütz.) Sil., Matt.& Bl.
<i>Lobosphaera tirolensis</i> Reisigl
<i>Lobosphaeropsis lobophora</i> (Andr.) Ettl & Gärt.
<i>Muriella decolor</i> Vischer
<i>Muriella terrestris</i> J.B. Pet.
<i>Myrmecia astigmatica</i> Vinatzer
<i>Myrmecia biotorellae</i> (Tsch. & Plessl) = J.B. Pet.
<i>Myrmecia globosa</i> Printz
<i>Myrmecia incisa</i> Reisigl
<i>Myrmecia macronucleata</i> (Deason) Andr.
<i>S. bajacalifornicus</i> Flechtner & Lewis
<i>Spongiochloris minor</i> Chant. & Bold
<i>Stichococcus bacillaris</i> Nägeli

Chlorococcum, *Coccomyxa*, and *Klebsormidium*, which were cited by other authors (e.g., Belnap *et al.*, 2001; Bhatnagar and Bhatnagar, 2005) as being the main inhabitants of desert microbiotic, were infrequently isolated or not recovered at all from this site. The most dominant taxa at this site were members of the genera *Bracteacoccus*, *Chlorosarcinopsis*, and *Myrmecia* (Fig. 1).

Microscopic observation of cyanobacterial and eukaryotic taxa revealed that a number of these taxa manifest cellular characteristics previously shown to be associated with desiccation tolerance or UV-light protection. Members of the

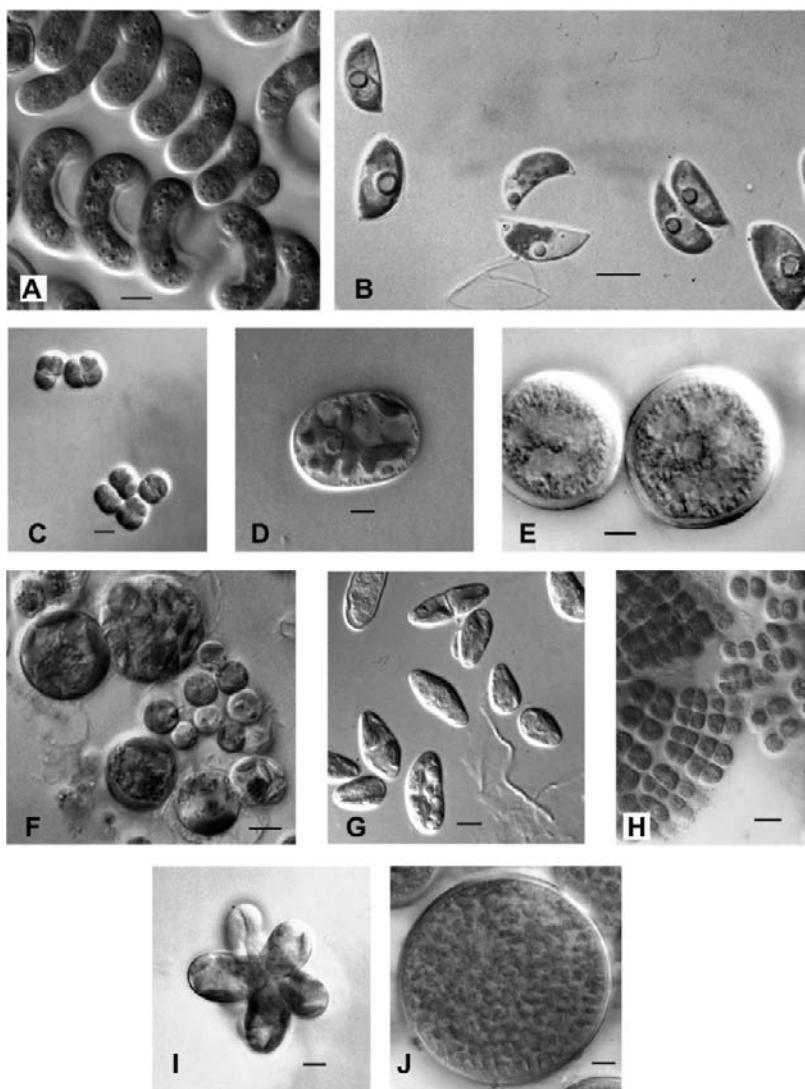


Figure 1. Microalgae isolated from North American desert soils. A. *Spirirestis rafaelensis*; B. *Scenedesmus deserticola*; C. *D. chodatii*; D. *C. brebissonii* var. *deserti*; E. *Spongiochloris* sp.; F. *Eustigmatos* sp.; G. *E. obtuse*; H. *F. mexicana*; I. *M. biotorellae*; J. *Bracteacoccus* sp. Scale bar = 10 μ .

cyanobacterial genera *Nostoc* and *Scytonema* elaborated an extracellular polysaccharide sheath, which in some cases became quite copious. Members of the genera *Scytonema*, *Myxosarcina*, and some members of the genus *Nostoc* produced a brown pigment previously shown to be responsible for UV-light resistance. Members of the genera *Anabaena*, *Scytonema*, and *Nostoc* produce

thick-walled akinetes and heterocysts. Among the eukaryotic algae, *S. bajacalifornicus*, *Spongiochlorsis minor* (Fig. 1), *Vischeria helvetica*, and some members of the genera *Bracteacoccus* (Fig. 1) and *Chlorosarcinopsis* produced orange or red carotenoid pigment previously shown to be associated with UV-light resistance. *E. obtusa*, *F. mexicana*, and *Chlorosarcinopsis gelatinosa* cells were embedded in extracellular matrices. But some of the most dominant taxa, including members of the cyanobacterial genus *Schizothrix* and the eukaryotic genus *Myrmecia* (Fig. 1), showed none of the characteristics known to be associated with either desiccation resistance or UV-light resistance. While members of the genus *Myrmecia* are often found in symbiotic association with fungi in lichen crusts, the crusts at the Baja site showed no evidence of lichen presence. It would be interesting to examine the physiological properties of these non-lichenized *Myrmecia* taxa to determine whether they accumulate trehalose or other compatible solutes under conditions of water stress.

3.4. ALGAL DIVERSITY: A COMMON FEATURE OF NORTH AMERICAN MICROBIOTIC CRUSTS

3.4.1. Scope of the Study

Over the course of 13 years, my collaborators and I have examined the cyanobacterial and eukaryotic algal flora from more than twenty geographically distinct sites in arid hot deserts (Chihuahuan, Mojave, and Sonoran), cold semiarid deserts (Colorado Plateau and Great Basin), and San Nicolas Island, one of the Channel Islands off the coast of California. We have been interested in determining whether taxa new to science exist within these sites, what level of algal diversity exists within sites, and the similarity of the algal flora in geographically distinct sites.

3.4.2. Taxa New to Science

It is clear that North American desert soils contain taxa new to science. *Spirirestis rafaelensis* (Fig. 1), a new genus of cyanobacteria, was isolated from crusts in an undisturbed region of the San Rafael Swell in the Colorado Plateau Desert (Flechtner *et al.*, 2002). This is the only location in which this taxon has been found. Four species new to science (*C. brebissonii* var *deserti*, *E. obtusa*, *F. mexicana*, and *S. bajacalifornicus*) were isolated from the study site in Baja California, Mexico (Flechtner *et al.*, 1998; Lewis and Flechtner, 2004). *S. bajacalifornicus* is morphologically similar to *Scenedesmus deserticola* (Fig. 1), which was found in crusts from multiple sites (San Nicolas Island, San Bernardino Co., CA, Otero Co., New Mexico, and Yuma Proving Grounds, Arizona) and *Senedesmus rotundus* which was found in crusts from Socorro Co., New Mexico. These enigmatic algae were originally misclassified because unlike most species of *Scenedesmus* in which daughter cells remain attached in a colony of four cells, the daughter cells of these taxa dissociate completely following cell division. Taxonomic placement was

resolved using nucleotide sequence data from the 18S and internal transcribed spacer of ribosomal RNA. Multiple isolates placed in the genus *Bracteacoccus* on the basis of morphology of vegetative cell structure and zoospore size and structure have been recovered from a number of sites in the Chihuahuan, Mojave, Sonoran, and Great Basin Deserts. Molecular analysis using 18S rRNA sequences revealed that six of these isolates recovered from five sites in the Great Basin Desert of Utah have sequences distinct from those of any previously described *Bracteacoccus* species (Lewis and Flechtner, 2002). The examples of *Scenedesmus* and *Bracteacoccus* just discussed illustrate the power of using a polyphasic approach to algal identification. Identification of algal taxa using microscopic examination of wet mount and clonal isolates has the advantage of being inexpensive and accessible to many researchers. But as pointed out by Lewis (Lewis and Flechtner, 2004), the problems of convergent evolution, morphological stasis, and phenotypic plasticity can confound accurate taxonomic placement. Coupling these classical methods to molecular analysis provides researchers with a powerful approach to answering a number of important questions about the diversity and relatedness of soil microalgae.

3.4.3. Studies of Crusts from Semiarid Sites

In a 1993 review, Johansen summarized the floristic data available for the Lower Columbia Basin, Great Basin, Colorado Plateau, and Sonoran Deserts (Johansen, 1993). While the lists of cyanobacterial and diatom taxa were fairly extensive (26 and 24 taxa, respectively), only 8 Chlorophyte and 3 Xanthophyte taxa were listed. Our studies reveal a much broader diversity in algal flora. In a study conducted over a 4-year period and which included multiple sites in Dugway Proving Ground (Great Basin Desert) located in Tooele County, Utah and the Wedge Overlook (Colorado Plateau) located in Emory County, Utah, 26 species of eukaryotic algae and 33 species of cyanobacteria were identified (Ng, 2002; Flechtner *et al.*, 2005; and unpublished data). Analysis of microbiotic crusts from seven sites on San Nicolas Island revealed the presence of over 40 different species of cyanobacteria and 23 species of eukaryotic non-diatom algae (Lewis and Flechtner, 2004 and unpublished data).

3.4.4. Ubiquitous Algal Taxa

Comparison of the microalgal flora from geographically distinct sites reveals that some taxa are found in most sites. Ubiquitous non-diatom eukaryotic taxa identified using microscopic techniques include *Diplosphaera chodatii* (Fig. 1), *Myrmecia biotorellae*, *Bracteacoccus* sp., *Chlorella* sp., *Pseudotetracystis* sp., and *Stichococcus* sp. It appears that some taxa are more prevalent in hot deserts (Chihuahuan, Mojave, and Sonoran) and others in cold, semiarid deserts (Great Basin and Colorado Plateau). Members of the class Chlorophyceae are encountered much more frequently than are members of the classes Chlamydophyceae and Xanthophyceae.

3.4.5. Geographically Distinct Patterns in Microbiotic Crust Algal Flora

Many of the sites contain a defining algal signature; they contain rare taxa or combinations of taxa not common in other sites. For example, *Spongiochloris* spp. were found only in crusts from the hot deserts. Eustigmatophytes were observed only in crusts from San Nicolas Island and Baja California, Mexico. *Xanthonema* spp. were found only in crusts from the Great Basin Desert. One of the most interesting similarities noted was in crusts from Baja California, Mexico and crusts from a xeric shrubland located near the southern tip of Lake Wales Ridge in Highlands County, Florida. Two of the species new to science identified in the Baja site (*E. obtusa* and *C. brebissonii* var *deserti*) were also present in the Florida site. A *Scenedesmus* isolate (originally misidentified as *Ettlia* sp.) was also isolated from this site (Hawkes and Flechtner, 2002). *Elakatothrix*, *Scenedesmus*, and *Cylindrocystis* were recovered relatively rarely from any site, and to have this particular combination of taxa appear in two widely separated sites is intriguing. The sites have little in common. The soils at the Baja site are derived from granite and are coarse and sandy-textured. Percent silt and clay range from 12.1% to 42.5%. The pH ranges from 6.3 to 7.5. The area receives 46–101.7 mm rainfall annually. The soils at the Florida site are excessively well-drained white sands (St. Lucie or Archbold soil types) with less than 2% silt and clay. They are much more acidic (pH 4.2–5.2). Annual precipitation is 1,331 mm, falling mainly in the summer. While the site is xeric, it is not considered a desert. The one feature the sites have in common is latitude; the Baja site is at 29°47'N 114°45'W and the Florida site is at 27°11'N and 81°21'W. Did the prevailing winds carry algal cells from the Baja site to the Florida site? Did morphologically similar taxa arise at these sites independently? Unfortunately, these questions cannot be answered because cultures of the Florida isolates are not available for molecular work. But as additional new algal taxa are isolated and identified from xeric soils, molecular approaches will make it possible to ask questions about the movement of taxa among closely adjacent and widely separated sites.

4. Summary

Microbiotic crusts are generally considered to provide important ecological benefits including stabilization of soil and nutrient enrichment due to both nitrogen fixation and production of organic carbon via photosynthesis. Several investigators have sought to remediate damaged soils by reestablishing microbiotic crust formation through the application of cyanobacterial amendments; thus far these studies have not produced encouraging results (Kubečková *et al.*, 2003; unpublished data). When one examines the literature prior to the mid-1990s one finds few extensive floristic studies of the microalgal components of desert soil crusts from North America. Our research group has undertaken extensive evaluation of microbiotic crusts from both hot arid and cold semiarid North American deserts.

We have used a variety of microscopic, culture, and molecular techniques to demonstrate the extensive diversity of both cyanobacteria and eukaryotic microalgae and have identified a number of taxa new to science. Some of these data have already been published and other manuscripts are in preparation. We are beginning to create a comprehensive database of the algae that are present in North American microbiotic crusts (Biotic Crust Project: <http://hydrodictyon.eeb.uconn.edu/bcp/>). Unfortunately, there is still an extremely limited body of work addressing the physiological and structural modifications that allow a wide spectrum of algae to survive in arid desert environments. Such information is not only interesting from an academic perspective, but might also be of practical use in guiding environmental biologists interested in reclaiming areas damaged by overgrazing or human impact. It is our hope that the cultures we are maintaining will provide a useful starting point for scientists interested in defining more clearly how microalgae cope with challenging desert environments.

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CHROOCOCCIDIOPSIS FROM DESERT TO MARS

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1. Introduction

In 1986 R.M. Powers (1986), a master space writer, stated: “We will go to Mars – that is taken for granted. Our children, or our children’s children, may not only go there, they may settle down and carry on as pioneers of the first really New World. The famous canals, the deserts, the immense canyons and looming volcanoes – and the dream of Mars – are all waiting for us on the Red Planet.” Powers hypothesized that the first Mars habitants should be bacteria, cyanobacteria, fungi.

At that time we knew very little about Mars surface compared to what we know today, thanks to the numerous and successful explorations by means of the Surveyor USA, 1996; Orbiter and Lander, 1998–1999; Internazionale Mars, 1996; Mars Pathfinder 1996, USA; Planet B, 1998–1999, Japan; Mars 01, USA, Orbiter and Lander; Mars 01, 2001, Russia; Mars 03, USA, Orbiter and Lander; Mars surveyor 05, 2005 (Sawyer, 2001). In 2003 the European Space Agency’s Mars Express orbiter and NASA’s rovers, Spirit and Opportunity, went to Mars: they are still acquiring data sets revealing new information about the history of Martian water and the possibility that the planet once harboured habitable conditions (Bibring et al., 2006).

Fundamental problems in investigating on extinct or extant life on Mars, concern the presence of water and finding out to what extent living organisms may survive at its very low temperatures and atmosphere conditions. The collected data transmitted to Earth from the above-mentioned missions and the fascinating images of Mars, allowed a detailed reconstruction of the Red Planet surface; conjunctures were made about water as not an unknown element, at least in the past, on the planet. Scientists have now to ascertain if the astronomic and meteorological data about the actual conditions of Mars are compatible with the concept of life that we have on Earth (McKay et al., 1996). Once established that on Mars the actual conditions of water, temperature and atmosphere might be compatible with extreme life forms, which earthly organisms could colonize the Red Planet? About that, many other questions arise, such as life ever existed on Mars in the past or if some of the pre-existing organisms are still present somewhere; if terrestrial organisms could adapt to Mars conditions (Friedmann, 1986; Friedmann and Ocampo-Friedmann, 1995; Beaty et al., 2005).

This review is dedicated to the memory of Roseli Ocampo-Friedmann. She with E. Imre Friedmann pioneered researches on Chroococcidiopsis and life in extreme environments.

In fact, only organisms which are capable of surviving at very low temperatures and water shortage on Earth, could represent hypothetical habitants of Mars. Biologists and NASA researchers conjectured about life outside our planet: in order to provide answers to this question, they focused their interest on terrestrial organisms, living in extreme environments, such as hot deserts and Antarctic regions, originating a new research field called “exobiology” (Friedmann, 1993).

2. The Life in Hot and Cold Deserts

Friedmann (1971) along with Ocampo-Friedmann (1985) started the study of life conditions in the hot and dry deserts of Asia and Antarctica, and then extended their researches to other continents. They found out that *Chroococcidiopsis* constantly appears in extreme dry environments and wherever it survives, it is often the only photosynthetic organism present.

Life in the desert is interesting from a biological point of view: there are numerous ways by which organisms can settle down in these environments. Bell (1993) reported that the lithobionts are mainly microbionts, which live inhabiting rocky environments. They are distinguished by location in: *epilithics* dwelling on the rock surface; *hypolithics* forming biofilms at the stone–soil interface; *endolithics* colonizing microscopic fissures (*chasmoeendoliths*) and structural cavities (*cryptoendoliths*) of rocks, and *euendoliths* actively boring into rocks. Cryptoendolithic microorganisms living inside rocks can provide a terrestrial model explaining what may have happened to life forms on Mars, when the planet became dry and cold. There are evidences of fossil traces of microbial rock colonization in Antarctica: similar structures might exist on Mars and may represent an easier target for life-detection systems, other than fossils of cellular structures. A paramount feature of the biology of Antarctic cryptoendoliths is their extraordinary slow growth, with exfoliation occurring when the microbial biomass reaches the carrying capacity of the cryptoendolithic habitat (Sun and Friedmann, 1999). Hypolithic communities containing a single *Chroococcidiopsis* morphospecies with heterotrophic associates were reported to colonize translucent stones along a gradient of aridity in the Atacama Desert in Chile (Warren-Rhodes et al., 2006). In the hyper-arid core of this desert, endolithic growth of *Chroococcidiopsis* and heterotrophic bacteria has been detected within halite rocks and representing the only life form present (Wierzchos et al., 2006). Among microorganisms able to withstand extreme conditions, the so called extremophiles (Rothschild and Mancinelli, 2001), *Chroococcidiopsis* become landmark for possible Mars inhabitants.

3. *Chroococcidiopsis* Cell Organization and Survival in Nature

Cryptoendoliths were first detected by Friedmann et al. (1967) in the Negev Desert. Later they were reported from the Middle East (Negev, Sinai), Central Asia (Gobi), North America (Sonora), South America (Chile), South Africa

(Natal) and McMurdo Dry Valleys (Antarctica). *Chroococcidiopsis* is a cyanobacterium that constantly appears in extremely dry, hot and cold places and wherever it survives, it is often the only photosynthetic organism present. This coccoid cyanobacterium was first reported from hot deserts together with *Gloeocapsa* (Friedmann, 1971). Rock-inhabiting communities in McMurdo Dry Valleys are wetted and metabolically active only for a total of 500–800 h per year (Ocampo-Friedmann et al., 1988); in the most arid areas of hot deserts, such as the Atacama Desert (Chile), instead, the number of metabolically active hours per year is considerably inferior to this (Warren-Rhodes et al., 2006). In these environments, biological and geological processes overlap, for this reason, the desert *Chroococcidiopsis* are thought to be extant representatives of “eoanhydrobiotes”, the old desiccation-tolerant cells (Billi and Potts, 2002), able to enter a dormancy state at the desiccation onset and resume metabolic activities when water becomes available, a phenomenon known as anhydribiosis (life without water).

Lithic *Chroococcidiopsis* has a complicated and variable life cycle with vegetative cells baeocytes (nanocytes) and resting cells. A single vegetative cell undergoes extensive binary fission in three planes to produce cell aggregates of about 10 μ diameter, within a fibrous envelope. Then, multiple fission occurs in almost all of the cells within an aggregate, followed by the release of small numerous baeocytes, non-motile cells of about 3 μ diameter and enveloped by fibrous materials. When released, the baeocytes enlarge and undergo binary fission to produce vegetative cells. These cells can modify their envelope and cytoplasm under the form of resting cells, able to survive in adverse life conditions.

4. Chroococcidiopsis in Culture

The cytology and life cycle of *Chroococcidiopsis* were studied in cultures obtained from strains CCMEE 34(N6909b) and CCMEE 29(N6904) isolated by Friedmann in 1969, from cryptoendolithic and hypolithic growth, in the Negev Desert (Israel). Cultures were stored in Tallahassee (Florida, USA) for a long period (over 66 months) on 1.5% agarized BG11 medium at 30°C and 20°C on a 16:8 h LD cycle under a photon flux of 25–35 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Samples of these cultures were studied at the University of Rome “Tor Vergata”. Here, the strain VRUC 176 was isolated from a sandstone sample collected in the Negev Desert and further investigated. Cultures of different age and period of desiccation allowed the investigations of the cytology of *Chroococcidiopsis* after long-term desiccation (Grilli Caiola et al., 1993); about the effects of desiccation on its envelope composition (Grilli Caiola et al., 1996a); the effects of nitrogen and phosphorus deprivation on its cellular organization (Billi and Grilli Caiola, 1996a); the physiological and ultrastructural effects of nitrogen limitation and starvation (Billi and Grilli Caiola, 1996b); of the subcellular distribution of iron superoxide dismutase (Fe-SOD) (Grilli Caiola et al., 1996b) and of calcium distribution as revealed by ESI and EELS techniques (Grilli Caiola M. and Canini

A., unpublished data). In addition, *Chroococcidiopsis* was tested to evaluate the resistance to ionizing-radiation (Billi et al., 2000a) and to simulated Martian UV flux (Cockell et al., 2005).

Previous studies supposed the existence of different *Chroococcidiopsis* species based on morphological, ecological and cultures studies (Dor et al., 1991). A method set up for the extraction and purification of genomic DNA from *Chroococcidiopsis* was first employed to identify the cell division gene *ftsZ* (Billi et al., 1998), then to investigate the genetic diversity among desert isolates of *Chroococcidiopsis* (Billi et al., 2001). Preliminary results based upon 16S rRNA gene sequencing, suggest that hot and cold isolates of *Chroococcidiopsis* are closely related and divergent from other Pleurocapsalean representatives (Billi et al., 2001). Due to the high sequence divergences present within the *Chroococcidiopsis* lineage (Fewer et al., 2002), including isolates from hot and cold deserts, it is still unclear whether some forms should be regarded as different species or genera.

Studies on old (Fig. 1) and young (Fig. 2) cultures of *Chroococcidiopsis* indicated that cell viability in aged cultures (40–66 months) was lower compared to that of young ones (2–5 months), being the first as low as 0.5–10% compared to 90–100% survival rate of the youngest ones (Fig. 3). Other significant differences

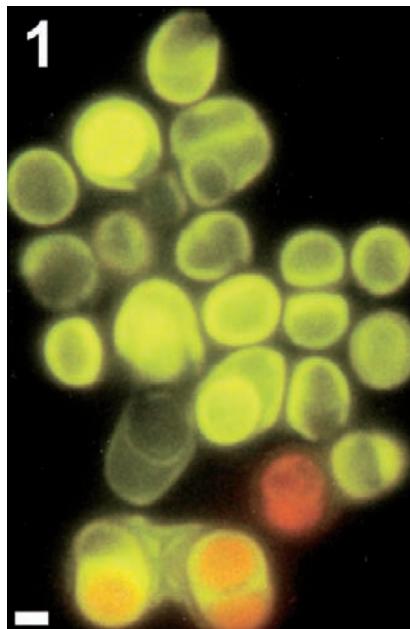


Figure 1. *Chroococcidiopsis* under light microscope. In 41-months-old culture of strain CCME 29(N6904), most of cells are unviable while few show red autofluorescence due to pigments. Bar = 1.5 µm.

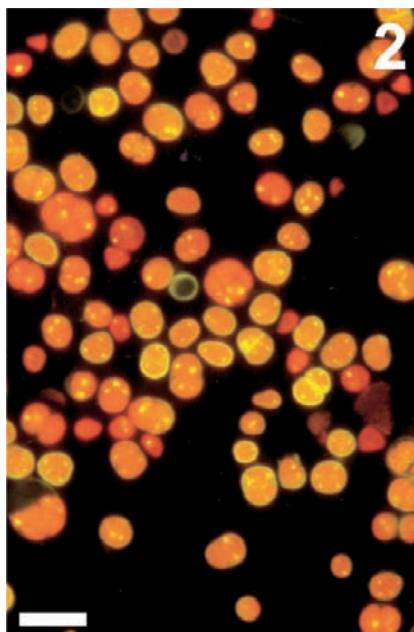


Figure 2. In 1-month-old culture of strain CCMEE 29(N6904), cells show a white fluorescence of the DAPI-stained nucleoids and red pigment autofluorescence. Bar = 10 μm .

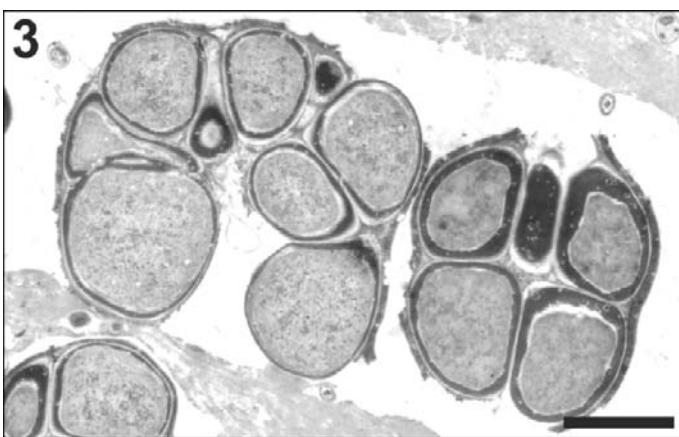


Figure 3. *Chroococcidiopsis* under transmission electron microscope. Mother cells with baeocytes in 1-month-old culture of strain VRUC 176. Bar = 1 μm .

concern the organization and composition of both the sheath and the ultrastructure of the cells adapted to survive to desiccation.

The sheath of aged cells was enriched with polysaccharides both in the baeocyte mother cells and the released baeocytes. The phycobiliproteins content

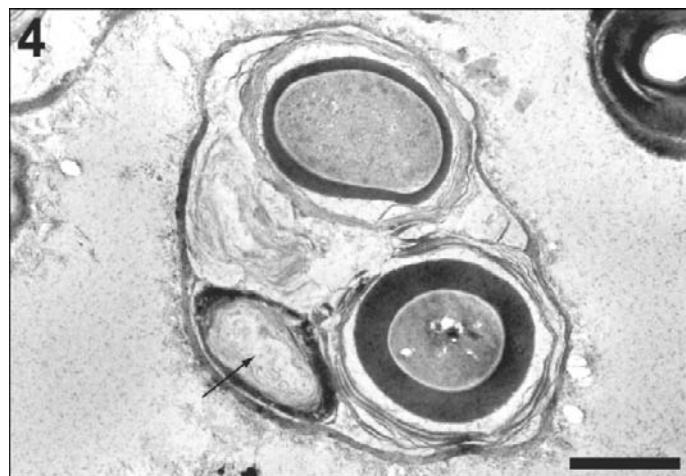


Figure 4. A cell aggregate with cells in different developmental stages and one dead cell (arrow) in 6-months-old culture of strain CCMEE 34(N6909b). Bar = 1 μ m.

decreased with aging. In most of the aged cultures, up to 66 months, cells occurred in various degenerating stages and among the dead cells, single living cells were scattered. These last cells (Fig. 4), showed an envelope organization similar to that peculiar of spores and akinetes, previously described in some *Nostoc* strains (Grilli Caiola and De Vecchi, 1980). The living cells showed a thick sheath, but also a cytoplasm with ribosomes and glycogen. Thus a selective process like a programmed cell death seems to occur in the old and desiccated cultures, although the occurrence within the same aggregate, of cells in different physiological states might influence their survival. Under these conditions, inside a single cell aggregate, single cells keep their usual structure pattern, while the others gradually deteriorate. The latter, generally contain carbohydrates in the envelope, fragmented thylakoids and carboxysomes, but no glycogen granules. Cells with multilayered and thick envelopes show intact cytoplasmic structures. The presence of pores (Fig. 5) crossed by membranes on the cell walls of the resting cells is peculiar; it is perhaps useful for intercellular or environmental exchanges. The presence of pores across the cell walls was previously reported in cyanobacteria (Grilli Caiola and De Vecchi, 1985). When old liquid cultures were transferred into fresh nutritional medium, aged cells created new aggregates by means of multiple divisions and the new aggregates released baeocytes, originating new young aggregates. When agarized cultures were allowed to dry, almost all the cells were organized in single cells with a thick envelope and cytoplasmic structures, similar to those observed in the aged cultures kept for many months in liquid culture (Fig. 6). So, the modification of the envelope and cytoplasm result to prove the main structural mechanism to survive to desiccation.

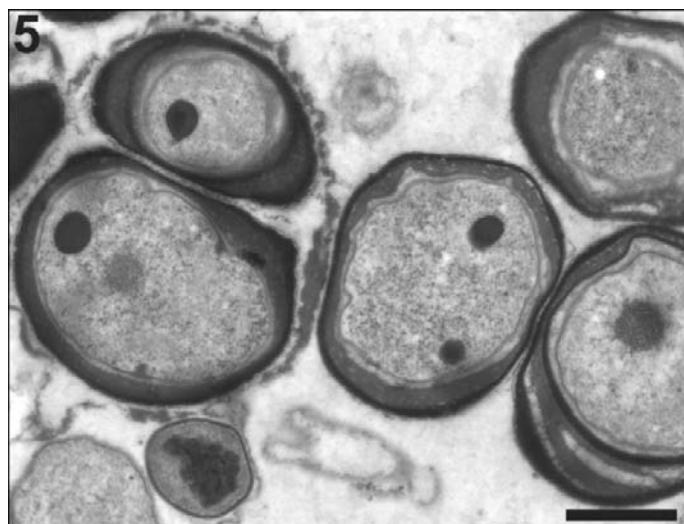


Figure 5. *Chroococcidiopsis* under transmission electron microscope. Resistant cells with thickened envelope from 1-year-old and desiccated culture of strain VRUC 176. Bar = 1 μm .

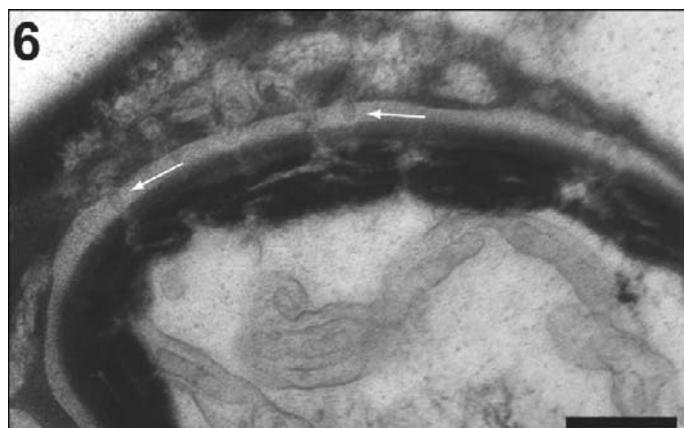


Figure 6. Envelope and cell wall of 1-year-old cell of strain VRUC 176. Pores crossed by membranes-like structures are visible (*arrows*) within the electron transparent wall. Bar = 0.5 μm .

Such a mechanism was confirmed also by experiments made with cultures deprived of nitrogen and phosphate. In cultures grown for 3 months in nitrogen-free medium only isolated viable forms were detected. In these cells a decreased content in chlorophyll and phycocyanin paralleled undetectable O_2 evolution and

depressed O₂ uptake. In addition, such isolated cells showed a multilayered envelope and cytoplasm with vesiculation of thylakoids, but no glycogen granules. N-limited and N-starved cells were able to recover their cellular organization and physiological activity upon N-repletion. These surviving cells had a spore-like feature comparable, from a functional point of view, to akinetes (Grilli Caiola and De Vecchi, 1980).

In cultures grown for one month in phosphate-free medium, cells were still dividing, though their pigment content was reduced compared to that of N-deprived cells. Viable cells were characterized by a complex envelope and vesiculation of thylakoid, undetectable oxygen evolution and oxygen uptake rates. In addition, spore-like forms occurred, suggesting that single cells might allow the survival under P- and N-depletion as well. After one month, P- and N-depleted cells were able to recover upon nutrient repletion.

In order to get information about the capacity of *Chroococcidiopsis* to survive to desiccation, the sheath composition was analyzed with an electron transmission microscopy (TEM) by means of cytochemical reactions, ESI and EELS techniques. The studied samples were obtained both from quartz flint collected in the Negev Desert (Israel) and wet- and laboratory-desiccated cultures.

The envelope of cells from rock as well from desiccated cultures, showed the presence of sporopollenin-like compounds, acid sulphate and beta-linked polysaccharides, positively charged glycoproteins, other than lipids and proteins. These compounds form a very elaborate structure in the envelope of the cells present in both stones and desiccated cultures. In addition, the thickness of the envelope increased with the age and desiccation, suggesting that the envelope represents a crucial mechanism in preventing water loss. However, such a mechanism might cause the cell death by reducing exchanges between the cells and the environment. Isolated living cells occurring in desiccated cultures, might be considered as resting forms allowing the survival of the cyanobacterium. Looking at a cell aggregate, it is difficult to establish which cells will die in order to assure the survival of the others. A similar process recalls the "programmed cell death", occurring in eukaryotic organisms, both animal and plant.

In compliance with the aims above reported, the cell sheath was analysed by ESI and EELS techniques in order to ascertain the presence of calcium and nitrogen in the envelope. By ESI it was possible to localize the different elements on the ultrathin sections of a cell previously identified by TEM. It was possible to detect on the same section different elements and thus to evaluate their amount in a target part of the cell. These techniques indicated that in cells from one-month-old cultures, the envelope contains calcium as well as nitrogen. However, EELS spectra indicated that the calcium amount was very high compared to that of nitrogen. In fact, nitrogen was present in such a low rate that was almost undetectable in the envelope, whereas it resulted more abundantly in the cell wall. The composition of the envelope was similar in both cell aggregates and single cells. Inside the cell, nitrogen was mainly present in the thylakoids, whereas the calcium amount was low. The EELS spectra acquired on the envelope of cells from one-year

old cultures revealed a very high peak of calcium, higher than that detected in the young ones, whereas nitrogen was present in a very low amount. In the old cells, when calcium and nitrogen were checked in cyanophycin storage granules, both elements resulted present.

The oxidative stress is also an important parameter to take into account, in order to highlight the survival strategies in *Chroococcidiopsis*. As term of comparison, the presence and distribution of iron Fe-SOD was measured by means of immunogold technique, using an antibody produced against Fe-SOD purified from *Anabaena cylindrica* (Grilli Caiola et al., 1993). In one-month-old cultures, the enzyme was localized mainly in the nucleoplasm and in the cell envelope. In one-year-old cultures, where mainly single cells occurred, Fe-SOD was localized in the peripheral cytoplasm and in the envelope, but it was absent in the nucleoplasmic areas (Fig. 7). This finding suggests that Fe-SOD could work as a barrier against oxygen radicals both in the cytoplasm and in the extracytoplasmic area. The presence of Fe-SOD reinforces the idea that *Chroococcidiopsis* is an ancient cyanobacterium, and that this enzyme originated very early on Earth, when oxygenic photosynthesis arose. The presence of Fe-SOD in periplasmic and apoplastic compartments was recently reported in other prokaryotes and eukaryotes. In the desiccation-tolerant cyanobacterium *Nostoc commune* the presence of a highly stable and active Fe-SOD in the extracellular matrix was reported after prolonged desiccation (Shirkey et al., 2000).

The great similarity between fossil and modern cyanobacteria indicates that cyanobacteria changed very little, if not at all, since their appearance on Earth billions of years ago. This accounts for their evolution low rate. The fossil taxa show slight or not evident morphological changes over hundreds of millions of years. This morphological evolutionary preservation is well documented for both coccoid and filamentous forms by numerous researchers (Kremer, 2006).

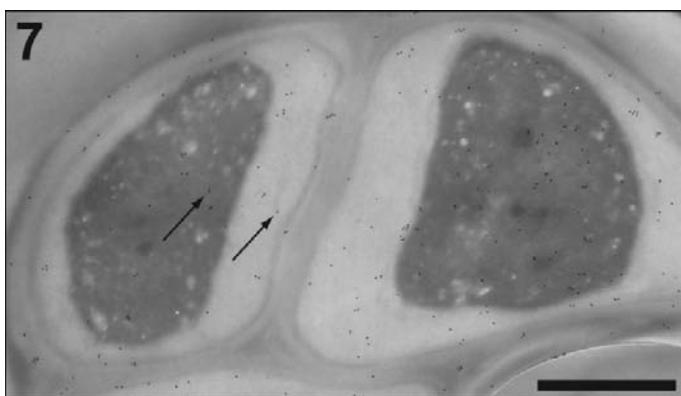


Figure 7. Gold particles indicating Fe-SOD localization in the periplasm and cell wall of *Chroococcidiopsis* cells from 1-year-desiccated culture of strain VRUC 176. Bar = 1 μ m.

Chroococcidiopsis is not only capable to survive to extreme desiccation, but it shows also a remarkable resistance to high doses of ultraviolet radiation (Martian UV-flux; Cockell et al., 2005) and ionizing radiation (up to 15 KGy; Billi et al., 2000a). In addition, since it is the only desiccation-tolerant cyanobacterium fit to genetic manipulation (Billi et al., 2001), the molecular analysis of its desiccation tolerance and radioresistance might reveal how to enhance its survival potential and obtain an organism better suited to extraterrestrial conditions.

5. Molecular Contributions to Decipher Drought Tolerance in *Chroococcidiopsis*

The capability to face the physiological constraints imposed by the complete removal of water, the storage in the dried state and subsequent rewetting, is a complex phenomenon, which involves every level of the cellular organization, through mechanisms not yet completely understood (Billi and Potts, 2000, 2002).

In order to survive desiccation, *Chroococcidiopsis* must avoid and/or repair several damages, spanning from those mediated by the oxidative stress to those due to the transition phase of the phospholipid bilayers (Potts, 2001). All these events generate injuries to proteins, lipids, nucleic acids and membranes and lead to the death of the majority of the organisms. It was speculated that the extraordinary radioresistance of hot and cold desert strains of *Chroococcidiopsis* is due to their ability to repair DNA damages (Billi and Potts, 2002). Indeed, the exposure to high doses of X-rays radiation (up to 15 KGy) induces the complete fragmentation of the genome of desert strains of *Chroococcidiopsis*, which is repaired within few hours (Billi et al., 2000a). This capability makes the resistance to ionizing radiation of *Chroococcidiopsis* comparable to that reported for the non-spore-forming bacterium *Deinococcus radiodurans* (Cox and Battista, 2005). At the moment, there are no data available on the effects of desiccation on the genome of *Chroococcidiopsis*. While, it was reported that after prolonged desiccation, the genome of the desiccation-tolerant cyanobacterium *Nostoc commune* is covalently modified, but protected from oxidative damage and degradation (Shirkey et al., 2003). In order to understand how *Chroococcidiopsis* reconstructs its genome from the remaining fragments, it must be pointed out that DNA repair pathways other than nucleotide excision repair should be employed. In fact, under circumstances of complete DNA fragmentation, even the search for the lost information via homologous would be necessarily useless, even in microorganisms containing multiple genome copies. In *D. radiodurans* the numerous genome copies represents only a passive contributor to ionizing resistance and biochemical mechanisms are employed to limit DNA degradation and restrict the fragment diffusion (Cox and Battista, 2005). For this bacterium a previously unknown mechanisms for fragments reassembly requiring at least two genome copies was reported (Zahradka et al., 2006). When approaching the DNA repair mechanisms in *Chroococcidiopsis*, it might be also relevant to undertake ultra-

structural to investigate whether the genome of this cyanobacterium is arranged in toroids, as reported for *D. radiodurans* (Cox and Battista, 2005).

However, in addition to DNA repair, molecules and enzymes with antioxidant activity are likely to play a central role in the desiccation and radiation tolerance of *Chroococcidiopsis*, taking into account that the oxidative stress is exacerbated by the photosynthetic process (Potts, 2001). The role of trehalose and sucrose in desiccation tolerance and cryoprotection has been widely documented (Crowe et al., 1997). These disaccharides prevent *in vivo* and *in vitro* the phase transition of cellular membranes and stabilize dried proteins. The expression of cyanobacterial sucrose-6-phosphate synthase in the desiccation-susceptible bacterium *Escherichia coli*, increased its survival after both freeze-drying and air-drying (Billi et al., 2000b). Trehalose was also reported to stabilize purified and dried plasmid DNA (Shirkey et al., 2003). Desert strains of *Chroococcidiopsis* accumulate trehalose and sucrose in response to osmotic stress, but no data are available about their presence and abundance upon desiccation. In deciphering the molecular basis of *Chroococcidiopsis* survival under extreme conditions, it might be important to investigate the role played by molecules known to contribute to the desiccation tolerance of anhydrobiotes, such as the Late Embryogenesis Abundant Proteins and Heat Shock Proteins. Finally, the possibility to genetically manipulate desert strains of *Chroococcidiopsis* provides the challenge to decipher the molecular basis of its desiccation tolerance (Billi et al., 2001).

Compared to UV radiation, drought and nutrient depletion resistance studies dealing with chilling and interplanetary space resistance of cyanobacteria are very few. Experiments carried out with the purpose to test the symbiotic system *Azolla-Anabaena azollae* resistance by means of exposing it to space weightlessness for 6 days, indicated that microgravity does not affect the main biological characteristics of both fern and cyanobacterium (Carrapico, 2001). Concerning the chilling effects on cyanobacteria, Nishida and Murata (1996) have revealed that changes in membrane fluidity could be involved in the initial event leading to the expression of genes for desaturases. But the extent of cyanobacterial resistance to chilling is still unknown. There is no information available about the behaviour of cyanobacterial cultured cells after storage at -196°C (Whitton, 1987). Laboratory experiments on *Hemicloris Antarctica* – a free living alga often present in Antarctic cryptoendolithic communities – at temperature oscillations in the range 5°C and -5°C or -10°C did not damage the cells. In addition, *H. antartica* appeared to be undamaged after slow or rapid cooling at -50°C (Meyer et al., 1988).

6. *Chroococcidiopsis* Towards Mars?

As we have forwarded in the introduction, Mars dream goes on. But the researches on the actual meteorological and atmosphere conditions on the Red Planet indicate that they are limiting life to terrestrial organisms exclusively. However, studies on *Chroococcidiopsis* offer a frame of information useful for

future researches about life on it as well as in the interplanetary spaces. In addition, they allow the planning of new missions to the Red Planet. Among the numerous terrestrial organisms, *Chroococcidiopsis* show some features worthy of attention such as its capacity to survive to long-term desiccation and deprivation conditions of basic nutritional elements, such as nitrogen and phosphorous, and then to recover when water and nutritional elements are available. It can survive to ionizing radiation by repairing DNA damages and it may be genetically modified in order to enhance its resistance to extraterrestrial conditions.

Chroococcidiopsis is able to live in conditions proximal to the limits of life on Earth. At the moment it is impossible to establish whether or not it will be able to survive to the limiting life conditions present on Mars. However, today we have accumulated a great deal of information on Mars and its possible past and future inhabitants. Opinions for and against the real chance of colonizing Mars will continue to be debated (Beaty et al., 2005). Other studies and missions will be needed to monitoring the conditions on it compatible with the green world, such as the soil composition, the water content, the absence of the gravity effects, and the details about its atmosphere. With these data, perhaps, also *Chroococcidiopsis* could become a pioneer paving the way for the advent and settling down of more evolved organisms on the Red Planet.

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CHLOROPHYTA ON LAND:

Independent Lineages of Green Eukaryotes from Arid Lands

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1. Introduction

Familiar examples of green algae (Chlorophyta) on land include those that participate in symbiotic associations with fungi, forming lichens (e.g., *Coccomyxa*, *Myrmecia*, *Stichococcus*, *Trebouxia*, Ahmadjian, 1958; Friedl, 1997), and taxa that grow richly on natural and man-made surfaces or on leaves of citrus and magnolia trees (e.g., *Prasiola*, *Trentepohlia*, *Cephaleuros*, Rindi and Guiry, 2004; Rindi et al., 2005). Besides these examples, green algae can occur in rock (endolithic), or at the surface (epidaphic), or just below the surface (endedaphic) of soil (Friedmann et al., 1967; Bell, 1993). Green algae are components of desert soil communities known as biological soil crusts or cryptogamic crusts (Evans and Johansen, 1999; Belnap and Lange, 2001). Crust communities are found on all continents on Earth, in arid and semi-arid habitats, where soil moisture is limiting and vascular plant cover is sparse (e.g., Johansen, 1993; Evans and Johansen, 1999; Green and Broady, 2001). Along with cyanobacteria, fungi, lichens, diatoms, and bryophytes, desert green algae form water-stable soil aggregates that have important ecological roles in nutrient cycling, water retention, and stabilization of soils (Evans and Johansen, 1999). The fragile nature of desert crust communities makes them highly susceptible to disturbance by trampling and fire, and has lead to numerous studies on the recovery of crusts after disturbance (Belnap and Eldridge, 2001; Nagy et al., 2005). Reviews of the ecology of crusts can be found in West (1990), Eldridge and Greene (1994), Evans and Johansen (1999), and Belnap and Lange (2001). This paper provides background information about the taxonomy of green algae from arid soil communities, and highlights recent studies that address the fine scale distribution, evolutionary relationships, diversification, and origins of Chlorophyta on land.

Desert soils are typically dominated by cyanobacteria and lichens, and green algae and other photosynthetic eukaryotes can be overlooked because they are often present in small numbers, and because their identification is challenging. Some crust communities appear to lack green algae altogether (Garcia-Pichel et al., 2001), including environments of pH above 5.5–6 where cyanobacteria are the major taxa (Metting, 1981; Garcia-Pichel, 2000). The biodiversity and ecological roles of green algae in these desert soil communities are not as well known as cyanobacteria and lichens (Evans and Johansen, 1999). However, green algae have

been reported from crust types on gypsum, limestone, and sandstone, across a wide range of pH, and endolithically. Genera such as *Bracteacoccus*, *Chlorella*, *Chlamydomonas*, *Chlorococcum*, *Desmococcus*, *Klebsormidium*, *Myrmecia*, have been reported in deserts of Africa, Antarctica, Asia, Australia, Israel, North America, and South America (Evans and Johansen, 1999; van Thielen and Garbary, 1999; Eldridge, 2001; Green and Broady, 2001; Ullmann and Büdel, 2001). Johansen (1993) demonstrated that green algae are more dominant than cyanobacteria in the sagebrush steppe of the Lower Columbia Basin in Washington. Additional examples that green algae can be the dominant photosynthetic eukaryote in certain arid soil communities, particularly in more acidic soils, are being discovered (see Part 2).

The earliest taxonomic surveys of desert algae were published beginning in the 1950s (e.g., Durrell, 1959; Cameron, 1960, 1964; Chantanachat and Bold, 1962; Shields and Drouet, 1962; Bischoff and Bold, 1963; Cameron and Blank, 1966; Friedmann et al., 1967). These authors determined different genera and species of green algae using light microscopy on the dominant life history stage available for examination, the vegetative cell, and relied on keys and descriptions that utilized vegetative morphology. These authors expanded our understanding of the range of habitats in which green algae can exist. For example, 19 different species of green algae were found to occur in desert soils or in rock in the Negev Desert, Israel (Friedmann et al., 1967). Metting (1981) compiled a list of over 100 taxa of terrestrial green algae from previous studies, and provided an extensive review of the biology, distribution, and taxonomy of soil algae. He stressed the need to apply principles of Bold (1970) that used vegetative cell features (size and shape of cells, plastid morphology, pyrenoid presence) in conjunction with zoospore features (presence of walls, length of flagella) to distinguish genera.

Johansen et al. (1993) and Flechtner and colleagues (Flechtner et al., 1998; Flechtner, 1999) provided even more detailed taxonomic surveys of green algae in xeric habitats. Flechtner (1999) discussed the occurrence of algae from desert habitats in the western USA and Mexico, and the problematic nature of identification of soil green algae. Most notably, the lack of regional keys, resulting in the practice of shoe-horning observed forms into existing descriptions for other geographical regions, and the lack of zoospores in some taxa. As discussed by Flechtner (1999), the use of light microscopy to examine green algae directly from desert soils leads to the scoring of a relatively small number of genera and species of green algae from deserts. Compared to other algae, unicellular green algae have very few easily diagnosed morphological traits. In addition, they can be highly reduced in size and complexity (some as small as 5 µm), and identification can require culturing and examination of alternate life history stages such as motile, asexual zoospores or gametes.

Through culturing and an examination of alternate life history stages, many more taxa can be identified. Applying these methods, Flechtner et al. (1998) found 37 taxa in 19 genera of green algae from Baja, Mexico, some of which were new species. They also concluded that a number of coccoid chlorophyte genera have

widespread distributions, including *Apatococcus*, *Bracteacoccus*, *Chlorococcum*, *Chlorosarcinopsis*, *Chlorella*, *Desmococcus*, *Klebsormidium*, *Myrmecia*, *Neochloris*, *Stichococcus*. While these floristic studies provided a better picture of algal diversity than studies that relied solely on microscopic examination of vegetative cells, the lack of regional keys still resulted in the practice of assigning observed forms into existing descriptions for other geographical regions.

Although culturing methods significantly increased the number of isolates of green algae found in samples from xeric soils, difficulties still remain in identifying particular green algae, such as *Chlorella*, which apparently do not produce motile cells (Flechtner, 1999). In addition, numerous examinations of the ultrastructure of the vegetative cells and zoospores of unicellular green algae that were published beginning in the 1980s demonstrated that common desert genera (e.g., *Characium*, *Chlamydomonas*, *Chlorococcum*, *Chlorosarcina*, *Neochloris*) contained species that were members of different families, orders, or classes (e.g., Melkonian, 1978; Deason and Floyd, 1987; Watanabe and Floyd, 1989). The subsequent taxonomic separation of some morphologically defined genera into two or three genera in different orders and even classes of Chlorophyta indicated that the vegetative morphological characteristics originally used to typify some genera were the result of convergent evolution. In the 1990s, analysis of nucleotide sequence data helped to confirm that vegetative morphology generally did not reflect phylogenetic relationships, but that relationships predicted by the ultrastructural traits of motile cells were taxonomically useful (e.g., Lewis et al., 1992; Friedl and Zeltner, 1994). At present, there is an emphasis on natural or monophyletic genera, based on phylogenetic relationships, rather than grouping species into genera based on morphology alone. Most phylogenetic studies rely on sequence data from the 18S rDNA gene or other genes that provide an adequate number of characters for analysis. Molecular studies demonstrate that green algae of xeric habitats are diverse and have distinct evolutionary origins (see Parts 3 and 4).

2. Fine-Scale Distribution of Green Algae in Arid Soils

This section discusses four recent studies that reveal important clues into the distribution and diversity of green algae in xeric soils. Hu et al. (2003) used light and scanning electron microscopy to examine the vertical micro-distribution of photosynthetic eukaryotes and cyanobacteria in soils from the Tengger Desert in central China. In this extreme habitat, soil temperatures range from 74°C to -25°C, and although this desert receives rain, evapo-transpiration rates are very high, leading to xeric soil conditions. The soil community examined was lichen-poor; instead, it was dominated by cyanobacteria and green algae. Hu et al. (2003) characterized the algae from one “uncrust” and five “crusted” sites. Soil pH ranged from 7.76–8.33 in the five “crusted” sites. Their detailed microscopic investigation showed that most cyanobacteria and green algae occur in the upper

100 µm of the soil, with the most resistant cyanobacterial taxa located 30–50 µm below the surface of the soil, below a thin layer of inorganic material. The green algae occupied a layer 50–100 µm below the surface. Importantly, this study demonstrated a case in which green algae are dominant in soils with neutral or alkaline pH. Green algae made up from 8.6% to 76.7% of the algal biovolume in the five “crusted” sites, with *Desmococcus olivaceus* as the dominant green eukaryote. Other green algae reported include *Chlorococcum humicola*, *Chlorella vulgaris*, *Chlamydomonas* sp., and *Palmelloccoccus miniatus*.

The next three studies that will be summarized examined algae on more sandy and acidic soils, which are expected to support fewer cyanobacteria (Metting, 1981; Garcia-Pichel, 2000). Hawkes and Flechtner (2002) conducted a detailed taxonomic survey of soil algae in the Lake Wales Region of central Florida, a relictual Pleistocene coastline. The vascular plant vegetation is adapted for dry conditions, and includes the “Florida rosemary” (*Ceratiola ericoides*), scrub oaks (e.g., *Quercus inopina*, *Q. chapmanii*), and sand pines (*Pinus clausa*). Although receiving regular rainfall, this region is characterized by deep, sandy soils that easily lose water. Most crusts were distributed between the vascular plants, and were less than six millimeters thick. Using light microscopy and culturing, in addition to cyanobacteria, 16 taxa of green algae were identified. Some taxa, including *Cylindrocystis brebissonii* v. *deserti*, *Elakatothrix obtusa*, *Klebsormidium flaccidium*, *Klebsormidium montanum*, and three unknowns, occurred in high abundance. The authors noted that due to a lack of sufficient keys, it became necessary to lump multiple (probably unrelated) morphological forms into one taxon (“*Chlorella* sp.”), and that this would result in an underestimate of diversity. Similar to the results of Grondin and Johansen (1993), Hawkes and Flechtner (2002) found heterogeneity of species at small spatial scales. In addition, the authors noted that similar genera (e.g., *Cylindrocystis brebisonii*, *Elakatothrix obtusa*) were found between geographically separate locations (Florida and Baja California, Mexico), perhaps due to similar habitat requirements, present day dispersal, historical reasons, or glaciation. However, Hawkes and Flechtner (2002) pointed out that because of the difficulties associated with identification of unicellular green algae, it will be important to survey the similar-looking algae with molecular data.

The algae of a sandy terminal moraine in central Brandenberg, Germany, were characterized by Hoppert et al. (2004). At this location, the soil is depleted of organic nutrients and characterized by rapid permeability and low water-holding capacity. In full sunlight, soil temperature in summer reaches 50°C. Through light and electron microscopic investigation and culturing, Hoppert et al. (2004) detected no cyanobacteria, but found that the upper-most 1.0–1.5 mm of soil was dominated by the green algae *Zygonium ericetorum* and *Ulothrix* sp. Similarly, Smith et al. (2004) surveyed sites in the Cape Cod National Seashore, USA. They used light microscopy, DGGE analysis, and sequencing of 16s plastid rDNA to identify the dominant green algae, and to verify the near absence of cyanobacteria. In the upper 3–5 mm of the substrate, the dominant

taxa were filamentous *Klebsormidium* and *Geminella*, which formed sheaths. Smith et al. (2004) also characterized three plastid 16S rDNA haplotypes from the Cape Cod algae as matching a cultured, freshwater-derived *Klebsormidium* from the SAG culture collection (Sammlung von Algenkulturen der Universität Göttingen) with 98% to 92% sequence similarity. The authors point out that *Klebsormidium* has been found from a large number of extreme environments, including highly acidic streams, snow, and deserts.

3. Molecular Phylogenetics and the Number of Transitions to Land

Since Flechtner et al. (1998) and Flechtner (1999), phylogenetic analysis of data from the nuclear small subunit rRNA gene (18S rDNA) has been instrumental in uncovering a wide variety of green algae in desert soils, and in determining their evolutionary affinities (Lewis and Flechtner, 2002, 2004; Smith et al., 2004; Lewis and Lewis, 2005). The need for molecular data stems from the nonmonophyly of common crust green algae (such as *Chlorella*, *Chlorococcum*, *Chlamydomonas*) determined using ultrastructural data from motile cells and nucleotide sequence data analysis (Huss and Sogin, 1990; Lewis et al., 1992; Wilcox et al., 1992; Friedl and Zeltner, 1994; Friedl, 1995; Nakayama et al., 1996; Watanabe and Floyd, 1996; Buchheim et al., 1997). Using 18S rDNA data, Lewis and Flechtner (2002, 2004) and Lewis and Lewis (2005) determined that desert green algae fall within three of the five traditional classes of green algae, Chlorophyceae, Trebouxiophyceae, Charophyceae s.l., the classes that contain primarily fresh water members.

Phylogenetic analyses of sequences from desert algae indicate that the members of one subset of the isolates are related to known genera of terrestrial green algae. Chlorophyceae examples include the familiar *Chlorococcum*, *Bracteacoccus*, but also perhaps surprisingly, *Scenedesmus*. Although *Scenedesmus* is typically associated with freshwater habitats, examples of soil-derived representatives were reported previously (Hilton and Trainor, 1963; Verses and Trainor, 1966). Several lineages of desert Trebouxiophyceae have been identified, including typical lichen photobionts as well as free-living soil algae, *Myrmecia*, *Chlorella* (*sensu* Huss et al., 1999), and other lineages of *Chlorella*-like taxa which are yet formally taxonomically unnamed. Charophyte examples include *Cylindrocystis* and *Klebsormidium*, genera that were mentioned in Part 2. Whether these North American isolates are the same taxonomic entity as those found from disparate habitats will be discussed further in Part 4. A separate subset of the sequences from the desert algae do not group closely with known genera and has low 18S sequence similarity to those in public sequence databases, resulting in new and “deeply branched” lineages on the overall green algal phylogenetic tree. Such a pattern could be explained if the closest relatives have not yet been sequenced, or if the desert isolates are indeed very different than the aquatic taxa that have been surveyed. Further sequencing of green algae from a wide range of habitats will clarify our estimation of the phylogenetic placement of these new desert lineages.

With the molecular phylogenetic data in hand, it is possible to estimate the number of separate transitions to land in Chlorophyta. Figure 1 is a summary of phylogenetic relationships for classes of green algae and the embryophytes, and is based on studies that used molecular and/or ultrastructural information (see Lewis and McCourt, 2004). Embryophyte green plants are the typical “land plants” such as bryophytes, ferns, gymnosperms, and angiosperms. Embryophytes are primarily terrestrial but some have become secondarily aquatic, whereas the

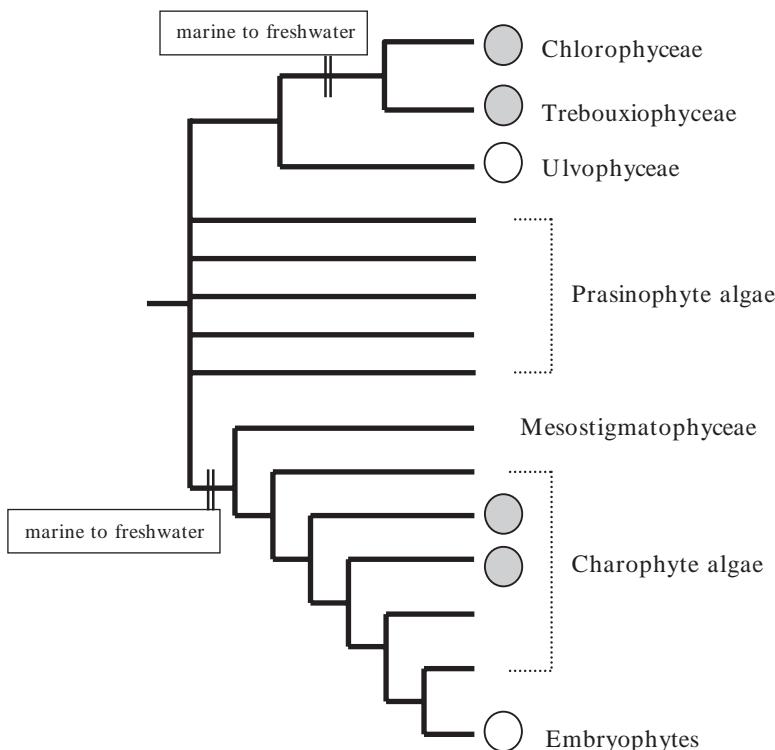


Figure 1. Phylogenetic relationships among the main groups of Chlorophyta, including green algae and embryophyte plants (adapted from Lewis and McCourt, 2004). Classes of green algae are indicated, along with two former classes that are currently recognized as nonmonophyletic groups, the prasinophyte and charophyte algae (dotted brackets). All green plants evolved from a marine, aquatic ancestor that likely resembled a swimming, unicellular prasinophyte alga, although the earliest diverging prasinophyte lineage has not been unequivocally determined. Cross-bars mark the transitions from marine to freshwater habitats in two distinct lineages. Circles indicate the lineages possessing at least some terrestrial members (or in the case of embryophyte plants, primarily terrestrial members). Filled circles indicate the algal lineages with one or more separate cases of desert representatives. Conservatively, 14–17 independent transitions from aquatic freshwater to desert habitat were estimated, with at least nine in Chlorophyceae, and four in Trebouxiophyceae (Lewis and Lewis, 2005).

remainder of Chlorophyta is primarily aquatic in marine or fresh water. The groups indicated by the open circles in Fig. 1 are those that have at least some terrestrial representatives. Ulvophyceae examples include *Trentepohlia* and *Cephaleuros*, loosely filamentous algae that grow on rock surfaces and are known to grow on the leaves of citrus and *Magnolia*. Conservatively, 14–17 independent transitions from aquatic freshwater to desert habitat were estimated using Bayesian methods and parsimony reconstructions on phylogenetic trees of 18S rDNA data (Lewis and Lewis, 2005), at least nine in Chlorophyceae, and four in Trebouxiophyceae. The groups indicated by closed circles possess desert algae. Note that the algae cultured and sequenced from arid habitats have their evolutionary origins in freshwater lineages. To date, no desert representatives of the class Ulvophyceae have been reported, even though algae such as the common intertidal marine green algae *Ulva* (sea lettuce) can desiccate and recover following tidal cycles, and aerial algae such as *Trentepohlia* are known.

Overall, 18S rDNA sequence data have helped improve our understanding of xeric green algae, by providing much more accurate diagnosis of the lineages that occur in deserts, and by allowing an estimation of the number of transitions to terrestrial habitats. Another important benefit of sequence data is that the published sequences are comparable across studies, and they provide a more direct way in which future researchers can test taxonomic similarities. Even those scientists who are not experts in green algal taxonomy can obtain data that are applicable to future comparison. That said, however, new information indicates that 18S data may be too evolutionarily conserved to allow us to obtain accurate species lists. As will be discussed below, we will need to target sequence data from a more variable region of the genome if the species are to be accurately compared across different geographical regions or habitats.

4. Diversification of Desert Lineages

In this section, the application of ITS rDNA (Internal Transcribed Spacer of rDNA) data to questions of species identification will be discussed. As highlighted by Hawkes and Flechtner (2002), Smith et al. (2004), or even earlier by Flechtner (1999), there are many examples of morphologically similar taxa from distinct habitats that might represent distinct species or other ranks. 18S rDNA molecular data have provided important insights into the evolution and diversity of xeric green algae, but it is becoming increasingly clear that data from this gene provide only coarse-grained phylogenetic resolution. If we want to determine if the morphologically similar green algae isolated from different habitats or geographic locations are the same, then we will need to collect information with more resolving power. Lewis and Flechtner (2004) isolated six morphologically similar isolates from deserts in western North America. These were very small cells, on the order of 20 µm in diameter, and resembled unicellular forms of the freshwater species *Scenedesmus obliquus* using light microscopy. At the level of 18S rDNA

sequence similarity, the six desert isolates shared a > 99.9–99.6% similarity to the *Scenedesmus obliquus* isolates from freshwater habitats in Sweden. That is, they had between 1–7 differences out of 1,758 aligned base pairs, and for most purposes would be considered “identical” to the aquatic isolates. However, using phylogenetic analysis of ITS rDNA sequence data, it was determined that the six desert isolates had between 97.9–95.94% sequence similarity to the aquatic isolates (14–27 of 665 aligned base pairs). The desert isolates formed two distinct and well-supported clades, separate from each other and from the aquatic *Scenedesmus* isolates. Examination of the desert strains using scanning electron microscopy revealed morphological distinctions as well. These data tell us that algae found in different habitats, such as freshwater lakes and desert soils, can have “identical” 18S rDNA haplotypes, but that variation at the ITS rDNA level provides clues that they are indeed distinctly diverged lineages. It will be interesting to learn what an analysis of ITS rDNA data would say about the relationship among strains of *Klebsormidium*, *Cylindrocystis*, or any other genera that have been isolated from very different habitats. Are these algae phenotypically plastic, being able to grow in many different environments, or do we see distinct lineages with habitat specificity? Work from the desert *Scenedesmus* would support the interpretation that these isolates are indeed specialized, but the other cases mentioned similarly need to be studied.

5. Summary

The goal of this review was to provide background information on the distribution and taxonomic identity of green algae from xeric habitats, and to demonstrate that the identification of green algae from xeric habitats has improved through methods that incorporate culturing and examination of motile stages, and through the addition of nucleotide sequence data. Ever finer taxonomic resolution can be obtained using 18S rDNA sequences, and by molecular characterization using ITS rDNA sequences. Molecular characterizations tell us that the green algae of xeric soils are more diverse than previously known, and have allowed estimations of the number of independent transitions from aquatic to desert habitats. In addition, molecular data have helped to clarify the precise identity of taxa that possess similar morphology. As more desert soil communities are studied using 18S and ITS rDNA data, we should begin to resolve the important questions related to species distribution. If we wish to know whether or not xeric green algae have cosmopolitan distributions or are endemic to certain regions, or to determine the abiotic or biotic factors influencing the distribution of green algae in xeric soils, then a more fine-scale approach will be needed. It is clear that similar genera have been assigned to different geographic locations or soil types. However, given the convergent evolution of vegetative morphology in unicellular green algae, preparing lists of genera and species based on vegetative morphology alone will not yield sufficient resolution to make valid comparisons.

The application of 18s rDNA sequence data alone, or in conjunction with ITS rDNA sequence data, will be necessary to more precisely identify and associate particular green algae species or strains with certain habitats. In the future, such detailed phylogenetic information collected from green algae across many different habitats will allow us to reveal the rapidity with which such major habitat switches can be made.

Although Bacteria and Archaea are well known from extreme habitats, there is an increasing number of diverse green algae that have been determined from extreme habitats such as highly acidic waters (Gerloff-Elias et al., 2005; Pollio et al., 2005), high metal content (Soldo et al., 2005), high salinity (Liska et al., 2004), and cold temperatures (Hoham et al., 2002). The biology of many strains of extremophilic green algae has received attention in the past (e.g., Trainor, 1962; Liska et al., 2004). What are the specific physiological traits that allow green algae to survive through extended periods of drought and temperature extremes, for example? Besides these questions, it will also be important to connect phylogenetic and physiological studies to address questions such as: Given that so many distinct lineages of xeric green algae have been identified, do adaptations to a specific habitat involve the same or different mechanisms across the desert green algae? Such questions can be answered only in a phylogenetic context.

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The biodata of **Ulf Karsten** and **Rhena Schumann** are printed adjacent to their contributions in this book.

Biodata of **Anika Mostaert** co-author (with **Ulf Karsten** and **Rhena Schumann**) of the chapter "*Aeroterrestrial Algae Growing on Man-made Surfaces – What are the Secrets of their Ecological Success?*"

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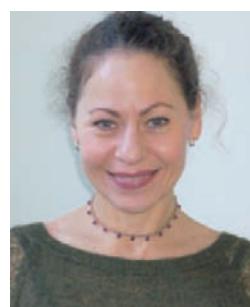
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AEROTERRESTRIAL ALGAE GROWING ON MAN-MADE SURFACES: *What are the Secrets of their Ecological Success?*

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1. Distribution and Habitat

Aeroterrestrial phototrophic microorganisms typically form conspicuous biofilms in all climatic zones at the interface between any type of solid substratum and the atmosphere. In temperate regions such as North-Western Europe, eukaryotic green microalgae (Chlorophyta) are the most abundant aeroterrestrial organisms (see also Rindi, this volume), whereas cyanobacteria dominate warm-temperate to tropical regions (Ortega-Calvo et al., 1995; Tomaselli et al., 2000). Aeroterrestrial green microalgae grow epiphytically and epilithically on natural surfaces such as tree bark, soil and rock, and are known to be the photobionts of lichens (Ettl and Gärtner, 1995). These organisms also occur in urban areas on anthropogenic surfaces such as roof tiles, concrete, building facades and other artificial surfaces where they cause aesthetically unacceptable discolouration known as patinas and incrustations (Gaylarde and Morton, 1999; Tomaselli et al., 2000).

Colonization by aeroterrestrial green microalgae, as well as by other microorganisms may accelerate weathering of substrata (Gorbushina and Krumbein, 2005, and references therein). Once established, algal biofilms attract heterotrophic bacteria, fungi and protozoa thereby forming complex microbial biocoenoses (Gaylarde and Morton, 1999). Algal primary production and dead algal cells provide organic molecules that serve as a source of carbon and nitrogen for heterotrophic organisms. In turn, bacterial and fungal remineralization of these organic compounds supports new growth of phototrophs, for example recycling of nutrients. Bacteria and fungi are well known to be powerful deteriogens of concrete, mortar, marble, stone and metal by the production of organic and inorganic acids (May et al., 1993; Brehm et al., 2005). Whether aeroterrestrial green microalgae actively corrode materials directly remains under debate (Ortega-Calvo et al., 1995). Nonetheless, cryptoendolithic growth of the green microalga *Stichococcus bacillaris* has been implicated in biocorrosion of granite from monuments (Ortega-Calvo et al., 1995), and indirect effects of aeroterrestrial green microalgae to the process of biodeterioration are undeniable, for example those mediated by the production of extracellular polymeric substances

(EPS). Once deposited on a substratum, EPS act as a coating that can change the physico-chemical surface properties, for example, by decreasing hydrophobicity and increasing water binding capacity as well as surface roughness. In addition, the phototrophic biofilm can undergo large changes in volume during dry and wet intervals, or during freezing and thawing, both processes contributing mechanically to weathering (Gaylarde and Morton, 1999).

Algae are a ubiquitous component of all aquatic and terrestrial habitats. The colonization of man-made surfaces is established through the settlement of spores or probably more importantly of cells from the atmosphere (Fig. 1) where they occur together with dust particles, that is the so-called aeroplankton (Tormo et al., 2001).

Rain water in the city of Rostock (Germany) can contain up to 1,000 green microalgal cells per milliliter (Schumann et al., 2004). In addition, other aeroplanktonic microorganisms such as bacteria and fungi are transported through wind in high cell densities, up to 100,000 spores per cubic meter air (Nay, 2003). Successful establishment of the deposited spores or cells is dependent on local environmental conditions and the ability to attach rapidly and strongly to a surface. Their ability to adhere to a wide range of natural and artificial surfaces is achieved by attachment via a two-stage process, initial and permanent, mediated by EPS. It is generally accepted that the EPS of algae are predominantly polysaccharide-protein complexes, namely glycoproteins, which may strengthen over time through a curing process (Fletcher and Callow, 1992).

While studies continue to concentrate on chemically characterizing algal EPS, mainly of diatoms (Stal, 2003, and references therein), recent studies

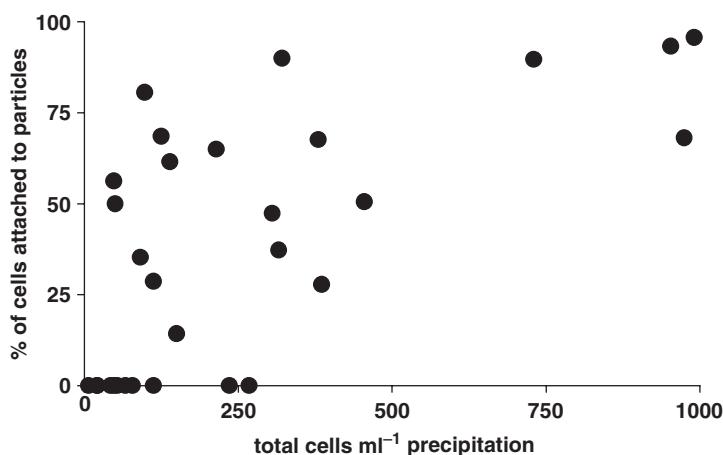


Figure 1. Fraction of phototrophic cells attached to dust particles compared to total cells observed in rain water between May 2003 and January 2004 in Rostock, Germany (Spearman Rank Order Correlation significant: $r_s = 0.65$, $P < 0.001$, $n = 30$).

(Dugdale et al., 2005; Mostaert et al., 2006) have begun to investigate the nanomechanical properties of algal adhesives using the atomic force microscope (AFM) (Binnig et al., 1986). This instrument allows measurement of the mechanical properties of single molecules such as DNA, modular proteins and polysaccharides, and is able to directly probe mechanical properties of natural adhesives of living organisms *in situ*. The underlying mechanisms of the strength of a natural adhesive is thought to be revealed by the mechanical properties of molecules, rather than the biochemical identification of the specific molecules present (Smith et al., 1999).

While the chemical composition of terrestrial algal adhesives remains essentially unknown, the work of Mostaert et al. (2006) has been able to provide new insights into the underlying mechanical design for adhesive strength and attachment at the molecular level. The authors showed that the adhesive of a terrestrial green alga from coastal lagoons exhibits high mechanical strength and toughness due to the presence of 'sacrificial bonds' and 'hidden length' within the adhesive molecules. A similar AFM force data 'signature' was shown from the adhesive pads secreted by the centric diatom *Toxarium undulatum* (Dugdale et al., 2005), and these authors attributed their data to large, single modular proteins arranged into parallel polymers, called adhesive nanofibers, that function as a cohesive unit. The authors based their mechanism on previously reported AFM measurements of isolated biopolymers and their signature force data.

Mostaert et al. (2006) proposed an alternative mechanism for adhesive strength based on small protein molecules cross-linked within a quaternary amyloid structure. Amyloid structures are proteinaceous β -sheets fibrillar structures usually associated with neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. This proposed mechanism was based not only on mechanical signatures (Fukuma et al., 2006), but was also strengthened by histological evidence from the amyloid-specific dye Congo red. Further data for four other species of aerotrestrial green algae from Germany and Ireland (unpublished data) supported the proposal of amyloid structures for mechanical strength in the adhesives of these algae. Additional evidence has been provided by confocal microscopy and Raman spectroscopy of the adhesives of these terrestrial algae *in situ*, and Fourier transform infrared spectroscopy of isolated adhesive proteins (unpublished data). Emerging evidence exists for the occurrence of non-pathological amyloid occurring in nature (Fowler et al., 2006), and the generic nature of the amyloid fold could provide a widespread mechanism for mechanical strength in other natural adhesives.

The life of phototrophs in aerotrestrial biofilms is subjected to specific obstacles at least for a substantial portion of cells. The most important factor for algal growth is light availability, which is reduced dramatically after passing the outermost cell layers. If one single benthic diatom absorbs 30–80% of incident light (Iturriaga et al., 1988) the smaller green algae may absorb somewhat less. If an absorption of 20% is assumed, photon fluence density is reduced by >2/3 after passing the fifth cell layer. Thus, the vertically arranged cell layers are light acclimated

differently, and vary in photosynthetic activity. Moreover, different species show well defined spacial positions inside a microbial community according to their tolerance levels and competitive strength (Oren et al., 1995). Light seems to be of special importance for endolithic algal growth, but the regulation of settling depth and zonation inside rocks is not fully understood yet (Matthes et al., 2001). Unlike filamentous cyanobacteria and benthic diatoms, most aeroterrestrial green algae are immotile and, therefore, unable to migrate to the most suitable layer inside the biofilm. The mucoid matrix of biofilms is, furthermore, a diffusion barrier for nutrients and gases, like oxygen or carbon dioxide. While carbon dioxide should be easily provided by microheterotrophs associated with the phototrophs (Kühl et al., 1996), it is unknown so far, if bacteria and fungi remineralize inorganic nutrients sufficiently. However, nutrient limitation is at least partly counteracted by the accumulation of hydrolytic enzymes aiding remineralization in the mucoid matrix (Thompson and Sinsabaugh, 2000). In aquatic communities, remineralization is further improved by micrograzers, for example protists or rotifers (Arndt et al., 1992). The presence of such organisms in terrestrial phototrophic biofilms is not known. However, it is reasonable to assume that the activity of potential micrograzers is restricted due to regular desiccation, which rapidly induces inactivation and encystment (Bamforth, 2004). On the other hand, lower transport rates or the binding of substances to the polymeric matrix of a biofilm reduces or even prevents toxication of phototrophs by anthropogenically applied herbicides and heavy metals. This seems of great ecological advantage for biofilm forming organisms allowing them to colonize environmentally harsh habitats such as, for example, mine tailings reservoirs (Garcia-Meza et al., 2005).

2. Taxonomy and Biodiversity

Most aeroterrestrial green algal taxa exhibit only a few morphological characters that can be used for reliable light microscopic identification (Rindi and Guiry, 2004). This is particularly true for natural samples where motile stages are mostly absent. While it is unknown if the high degree of morphological similarity is related to adaptation to the terrestrial habitat, it seems that single cells arranged in consortia, and thick cell walls may be attributed to growth and survival of these organisms in mostly dry conditions.

Recent studies demonstrate a much higher genetic biodiversity than expected from pure morphological characters indicating a high incidence of evolutionary convergence among aeroterrestrial green algae (Karsten et al., 2005; Friedl, unpublished results). So far, phylogenetic analysis based on 18S rRNA genes document that most aeroterrestrial green algae belong to the class Trebouxiophyceae, a sister group of the Chlorophyceae (Karsten et al., 2005). In many cases the DNA sequences did not fit to known taxa from the database, and thus point to new, undescribed genera, or even families within the Trebouxiophyceae. Ongoing phylogenetic studies are therefore crucial in order to better understand the evolution, biodiversity and taxonomy

of these organisms. While at least 150 unicellular species of aerotrestrial Chlorophyta as well as their motile stages are morphologically described (Ettl and Gärtner, 1995), neither their adaptation to environmental conditions, nor their phylogenetic relationships are well understood.

3. Environmental Conditions

Compared to freshwater and marine environments, aerotrestrial algae of terrestrial habitats are exposed to harsher environmental conditions, such as desiccation and high insolation (PAR and UV). Extensive exposure of aerotrestrial algae to the atmosphere results in dehydration, which strongly affects photosynthesis and growth. In North-Western Europe, for example, water availability frequently fluctuates from fluid droplets after rain or snow, to extended periods of dryness. The occurrence of condensation water on a surface depends on their heat storage capacity and on the diurnal air temperature regime. Weather changes can lead to sudden air temperature drops to less than 15 K, which is similar to the night–day air temperature difference. However, on sun exposed surfaces such as roof tiles, diurnal temperatures can reach 50 K in summer. Water availability is therefore the key ecological prerequisite for long-term survival of aerotrestrial algae, because only fully hydrated cells will be physiologically functioning.

Solar radiation is essential for life on Earth. Through photosynthesis, aerotrestrial green microalgae convert light energy into chemically bound energy, which is used for growth and cell division. Changes in irradiance and light quality can promote photosynthesis, but can also inhibit many biological processes if radiation becomes excessive (Barber and Andersson, 1992), or if short wavelength radiation with high energy content, such as ultraviolet B radiation (UVB; 280–315 nm), is absorbed by biomolecules such as nucleic acids and proteins (Vass, 1997). Consequent photodamage or conformational changes to important cellular components in algal metabolism may result in reduced photosynthetic and general metabolic activity leading to a decrease or inhibition in growth and cell division (Franklin and Forster, 1997). Although the biological consequences of changes due to high doses of UV-radiation in aquatic and terrestrial ecosystems are not fully understood, many phototrophic organisms are strongly affected (Franklin and Forster, 1997; Day, 2001), and changes in biodiversity and decreases in productivity are likely.

As with higher plants, aerotrestrial green algae require inorganic nitrogen and phosphorus for their metabolic activities, particularly to support growth and cell division. In most aquatic ecosystems the introduction of high concentrations of nitrogen and phosphorus is due to eutrophication, and therefore excessive growth of phytoplankton is concomitant with increased agricultural activities. The nutrient supply of aerotrestrial algae, however, has not been so well understood. Studies have begun to demonstrate that the atmosphere carries high concentrations of nitrate, ammonium and phosphate (Wright et al., 2001; unpublished data). Aerosols

Table 1. Concentration ranges of Dissolved Inorganic Nitrogen (DIN as the sum of Ammonium, Nitrite and Nitrate) and Soluble Reactive Phosphorus (SRP, $\mu\text{mol L}^{-1}$) in rain water sampled between 1996 and 2003 ($n = 703$ and 757, respectively) in Zingst, northeastern Germany, and on Plaster ($n = 16$) exposed to the atmosphere for 1 year in Zingst as well as on Roof Tiles ($\mu\text{mol m}^{-2}$) sampled throughout Germany ($n = 16$).

Rain water	DIN 7–643		SRP 0–19	
	New	Exposed	New	Exposed
Plaster	15–551	146–357	0–1040	3–36
Roof tiles	77–1960	42–5440	1–90	12–189

from agricultural fertilizer, dust particles from farmland, and nitrogen emissions from animal production and traffic represent the main anthropogenic sources, from which nutrients are easily transported into the atmosphere. The deposition of airborne nutrients is mainly mediated by rain and snow (Table 1).

Therefore, this water can be characterized as a fertilizer supplying essential nutrients to aeroterrestrial algae. In addition, heterotrophic bacteria and fungi, which benefit from dead or lysed algal cells, as well as from any deposition, will mineralize EPS to release inorganic phosphorus and nitrogen, which then supports new algal growth. Thus, a continuous cycling of the essential bioelements can be maintained indefinitely.

4. Adaptive Mechanisms

Although aeroterrestrial microalgae are interesting and important from ecological and applied points of view, their ecophysiology has not been investigated in detail. The colonization of terrestrial habitats involves exposure to harsh environmental conditions, such as desiccation and high insolation (PAR and UV).

The degree of water loss to algae in aeroterrestrial habitats is related to external factors such as temperature and air humidity, which exhibit diurnal and seasonal fluctuations. Internal factors also affect the ability of algal cells to retain cellular water. These factors include the algal surface to volume ratio, or morphological features such as thick cell walls and mucilage layers. For example, small organisms are more susceptible to dehydration, and thicker cell walls may produce a higher resistance against water loss. From a physiological standpoint, a consistent, high cellular water content is an essential feature for viability, because all metabolic activities would be optimally functioning. While morphological structures may avoid or delay water loss, the biosynthesis and accumulation of high concentrations of organic compounds such as sugar alcohols are also thought to contribute to desiccation tolerance (see also Oren, 2007 this volume). Sugar alcohols exert multiple functions in metabolism, that is besides their roles as organic

osmolytes and compatible solutes, they can also act as antioxidants, heat protectants (stabilization of proteins), and rapidly available respiratory substrates (energy supply for a maintenance metabolism under stress and for repair processes). The major physiological goal is to keep homeostasis as long as possible and to minimize or repair any damage of the algal cells as fast as possible. Aeroterrestrial green algal taxa such as *Prasiola*, *Stichococcus* and *Trentepohlia* have been found to synthesize and accumulate high concentrations of a range of sugar alcohols such as glycerol, erythritol, ribitol, arabitol, mannitol, sorbitol and volemitol (Feige and Kremer, 1980) (Fig. 2).

Despite these adaptations, if desiccation stress persists for extended periods of time, algal cells will inevitably lose water. Under such conditions, spores (i.e. resting stages) are formed to cope with the environmentally unfavourable period. Spores are characterized by thick, impregnated cell walls, which guarantee long-term survival in dry habitats. If the spores encounter fluid water, they usually germinate within a short time initiating growth of a new algal population.

In situ photosynthesis of an aeroterrestrial algal biofilm colonizing a building facade was investigated over six months, with an emphasis on changing water availability and air humidity (Häubner et al., 2006). The fluorimetric measurements of

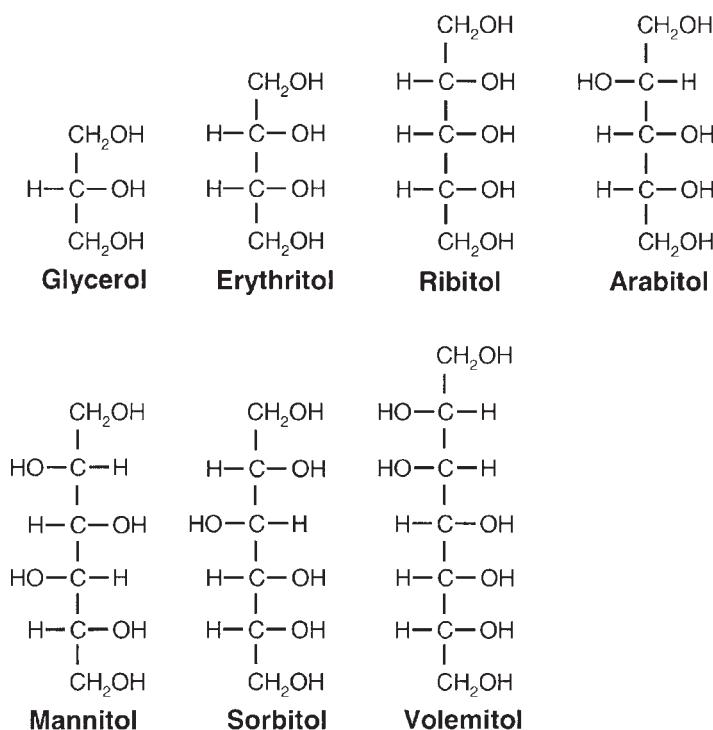


Figure 2. Typical sugar alcohols found in aeroterrestrial green algae.

the quantum efficiency (F_v/F_m) indicated diurnal activity patterns. On most sampling dates, the photosynthetic performance of the green microalgae was particularly high in the morning. This activity profile of the algal community correlated with the presence of condensation water onto the facade. With increasing solar radiation during the day, the water film evaporated, leading to desiccation of the organisms and inhibition of photosynthesis. It was observed that some degree of photosynthesis was possible as long as liquid water was present, and that recovery of photosynthesis in dried cells occurred within minutes of artificial moistening. Nevertheless, the ecological consequence of diurnally and seasonally changes, that is regularly inhibited photosynthesis, is a reduced primary production.

A laboratory study under controlled conditions with three aeroterrestrial green microalgal strains (*Stichococcus* spp., '*Chlorella luteoviridis*') showed that photosynthesis and growth required either fluid water or 100% air humidity (Häubner et al., 2006). This study indicated that by only taking up water vapour from the surrounding air, important physiological processes could be supported. Furthermore, decreasing air humidities were accompanied with increasing inhibition of photosynthesis and growth. Similar results have been described for the aeroterrestrial green microalga *Apatococcus lobatus* (Bertsch, 1966), as well as for green algal lichens from Utah (USA) (Lange et al., 1997) and cryptoendolithical lichens from hot and cold deserts (Palmer and Friedmann, 1990). *A. lobatus* was able to take up CO₂ for photosynthesis down to 68% relative air humidity (Bertsch, 1966). Green algal lichens differ physiologically from cyanobacterial lichens in that they are capable of photosynthesizing in the presence of water vapour, whereas cyanobacterial lichens are dependent on liquid water (Lange et al., 1989). In addition, both lichen types exhibited different speeds of restitution of the photosynthetic apparatus after sudden hydration of dry thalli by liquid water. Green-algal lichens recovered significantly faster, and hence resemble in this respect aeroterrestrial green microalgae. The underlying mechanisms remain unclear.

Only few data are available on the effect of temperature on growth of aeroterrestrial algae. Two *Stichococcus* species and *C. luteoviridis* have been reported to grow between 1° and 30°C with optimum rates at 20–23°C indicating eurythermal characteristics (Häubner et al., 2006). The observed growth optimum temperatures are similar to the optimal CO₂ uptake rates at 18–20°C determined for *A. lobatus* (Bertsch, 1966). Since isolates grew near the freezing point, they should be able to grow and photosynthesize well in winter, as long as water is available. This assumption is supported by the regular inspection of various buildings in North Germany and Ireland during winter (unpublished results).

Aeroterrestrial green algae well photosynthesize under high photon flux densities. Although in *Stichococcus* spp. optimum growth at 450 µmol photons m⁻² sec⁻¹ was observed, it reasonably grew up to 920 µmol photons m⁻² sec⁻¹. The cell-specific contents of chlorophyll *a* and organic carbon remained constant over a broad range of photon fluence densities tested. High amounts of luteine and β-carotene were accumulated as light protection pigments, which are directly involved in fluorescence quenching in certain green algal taxa (Niyogi et al., 1997;

Casper-Lindley and Björkman, 1998). Physiological adaptation mechanisms include the xanthophyll cycle and an efficient non-photochemical quenching (unpublished data). The antioxidant function of xanthophylls may play a special role in counteracting long periods of high insolation to avoid any photodamage (e.g. Baroli et al., 2003). Both processes are closely connected in all higher plants (Gilmore and Yamamoto, 1991; Demmig-Adams and Adams, 1996), but in green algae, this relationship is not so pronounced resulting in an effective measure to adapt to high photon fluence densities (Masojídek et al., 1999). Whether in aerotrestrial green algae these photophysiological abilities are particularly well developed remains to be studied.

Various strategies have evolved in phototrophic organisms to counteract the damaging effects of ultraviolet radiation, such as the formation of antioxidants or efficient DNA repair mechanisms (Franklin and Forster, 1997; see also the chapter of Pattanaik et al., this volume). Another protective strategy is the biosynthesis and accumulation of UV-absorbing sunscreens, such as mycosporine-like amino acids (MAAs) in various marine and terrestrial organisms (Dunlap and Shick, 1998; Kogej et al., 2006; Volkmann and Gorbushina, 2006). MAAs are water soluble, low molecular weight molecules, with maximum absorption bands between 310 and 360 nm (Cockell and Knowland, 1999). So far, about 20 different MAAs have been identified in aquatic organisms from a wide taxonomic range (Cockell and Knowland, 1999).

In a recent study, a specific UV-absorbing MMA, previously only known from the aerotrestrial green macroalgal genus *Prasiola* (Trebouxiophyceae) (Hoyer et al., 2001), was identified in other closely related aerotrestrial green microalgae colonizing building facades (Karsten et al., 2005). This particular UV-absorbing compound was first described in the green macroalga *Prasiola crispa* ssp. *antarctica*, which lives almost terrestrial near penguin colonies (Hoyer et al., 2001). These authors reported high concentrations of a UV-sunscreen with an absorption maximum at 324 nm, which was characterized as a putative MAA due to its chromatographic properties. Gröninger and Häder (2002) confirmed the occurrence of 324 nm-MAA in *Prasiola stipitata* from the supralittoral zone of the rocky island Helgoland (North Sea). In addition, although not further specified, Reisser and Houben (2001) reported the occurrence of UV-absorbing compounds in various other species of terrestrial green microalgae.

The 324 nm-MAA has only been identified in members of the Trebouxiophyceae, and appears to be absent in members of the Ulvophyceae and Chlorophyceae (Karsten et al., 2005). When mapped on a 18S rRNA green algal phylogeny, the distribution of this sunscreen compound showed a high chemosystematic value for the Trebouxiophyceae (Karsten et al., 2005). In addition, preliminary experiments with various aerotrestrial green algal strains show an accumulation of this MAA with increased UV-exposure, thus supporting its function as a putative UV-sunscreen. However, in contrast to numerous ecophysiological studies on the effects of UV-stress on terrestrial cyanobacteria, fungi and lichens (Garcia-Pichel and Castenholz, 1991, 1993; Brenowitz and Castenholz, 1997; Büdel et al., 1997;

Bjerke et al., 2005; Volkmann and Gorbushina, 2006), aeroterrestrial green algae have been largely unstudied.

5. Conclusion

Phylogenetic analysis based upon 18S rRNA gene sequences of various morphologically similar green microalgal isolates from roof tiles and building facades have indicated a much higher biodiversity than expected. New lineages among the class Trebouxiophyceae have been made known. The critical environmental factor ensuring ecological success of these organisms in the terrestrial habitat appears to be water availability. Aeroterrestrial algae have developed various protective strategies to minimize water loss such as thick cell walls, extracellular mucoid layers, desiccation tolerance of photosynthesis and growth, as well as the biosynthesis and accumulation of metabolically multi-functional sugar alcohols. Their strong attachment to surfaces is believed to be due to the presence of β -sheet-rich proteinaceous fibrillar structures in their adhesives that provide mechanical strength. Recent effects of the Earth's global warming due to enhanced CO₂ emissions, seems to be milder winters in Europe, that is fewer days with freezing temperatures, which may favour the growth and survival of aeroterrestrial microalgae in urban environments. However, to better understand the ecological success of the aeroterrestrial microalgal flora more basic research is urgently needed, particularly with regards to taxonomy and ecophysiology.

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THE SYSTEMATICS OF SUBAERIAL ALGAE

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1. Introduction

Over the past millions of years the land on our planet has been the testing ground for many experiments or, more dramatically, the battleground for many invasions. A myriad of ancestral plant forms came from the sea and lakes to exploit the terrestrial environment. Those life forms were algae, simple photoautotrophic organisms that eventually prepared the land for the terrestrial flora and fauna that were to follow. They successfully conquered the land in terms of making it a useable new habitat for themselves and developed new forms and processes to adapt. Those plant “invaders” or “conquerors” are represented today by algae living among us populating soils and other terrestrial habitats.

Most of the photosynthetic organisms that occur nowadays in aquatic habitats belong to this heterogeneous category generally called algae. These organisms are phylogenetically unrelated, or only distantly related, and differ enormously in terms of gross morphology, ultrastructure, biochemical traits and many other important features. Several lineages of algae successfully colonized terrestrial environments. Although from the ecological point of view the most important conquest of land was that of the green algae of the streptophytan lineage (those that gave rise to land plants), several other groups did succeed in becoming terrestrial. Representatives of these lineages are presently commonly found in terrestrial environments and unlike land plants, have maintained a very similar morphology to that of their aquatic relatives. The Charophyta and the Chlorophyta sensu stricto are the two groups of eukaryotic algae that along with the prokaryotic Cyanobacteria (blue-green algae) have been most successful in colonizing terrestrial environments.

Subaerial algae are terrestrial algae that live on stable exposed surfaces above the soil (Nienow, 1996), they are perhaps the most obvious, and yet most overlooked, group of algae. They produce rich growths (in the form of green, red, brown or black streaks) on a wide range of surfaces, including natural rocks, urban walls, woodwork, metal, bark and leaves of trees and hair of animals. Subaerial algae are particularly abundant and widespread in areas with humid climates sometimes causing significant economic problems. This problem is serious in tropical areas (such as Singapore), where legislation forces owners of

houses to keep their homes algae free! In countries with ancient and rich cultural heritage (i.e. Spain, Italy and Greece), the effects of algal deterioration on monuments has been studied in detail and great efforts have been made to limit their development. Cyanobacteria are well-known agents of biodeterioration of monuments in tropical regions (Gaylarde and Gaylarde, 2000), but remain underexplored and relatively unknown in terms of modern systematic assessments (Crispin and Gaylarde, 2004). Often overlooked in these habitats, the total diversity of these taxa is typically underestimated (Ferris et al., 1996). In this report, we summarize the current state of knowledge of the diversity and systematics of the subaerial algae, with special attention to some important types of habitats (in particular the tropical rainforest), for which the subaerial algal flora is poorly known and in urgent need of further investigation.

2. Floristics

Subaerial algal studies are available mostly from Europe with very limited coverage from other continents, although in the last few decades Antarctica has been studied in great detail (e.g. Broady, 1996). In terms of habitats, natural rock surfaces are the most extensively studied from Central Europe (e.g. Frémy, 1925; Jaag, 1945; Golubic, 1967), South America (e.g. Büdel, 1999), North America (e.g. Johansen et al., 1983), Africa (e.g. Frémy, 1930; Büdel, 1999) and Asia (e.g. Fritsch, 1907a, b). Algae growing on concrete and other artificial surfaces have gained great attention in the last decades, due to their biodeterioration effect for both temperate (e.g. Ortega-Calvo et al., 1991; Gaylarde and Gaylarde, 2000; Rindi et al., 2003; Rindi and Guiry, 2003, 2004) and tropical regions (e.g. Wee and Lee, 1980). Epiphytic algae occur on a variety of plant substrata, with tree bark inhabiting forms the best known and studied in Europe (e.g. Schlichting, 1975), Africa (e.g. Printz, 1921), Asia (e.g. Fritsch, 1907a, b; Islam, 1960; Handa and Nakano, 1988; Nakano et al., 1991), South America (e.g. Möbius, 1888; Lagerheim, 1890; Schmidle, 1901) and North America (e.g. Cox and Hightower, 1972; Wylie and Schlichting, 1973; Dillard, 1989; John, 2003). Little information exists about algae growing on animals; although sloths (Weber Van Bosse, 1887; Thompson, 1972), spiders (Cribb, 1964), lizards (Gradstein and Equihua, 1995), polar bears (Lewin and Robinson, 1979), prosimians (Sanderson, 1963) and birds (Schlichting et al., 1978) have all been found to support subaerial algae.

3. Biodiversity and Systematics

The vast majority of the photosynthetic microorganisms occurring in terrestrial habitats belong to four groups: the Cyanobacteria (Komárek, 2003a), the Chlorophyta and the Charophyta (sensu Lewis and McCourt, 2004) and the Heterokontophyta (sensu Andersen, 2004). From a numerical point of view,

the prokaryotic Cyanobacteria and the eukaryotic Chlorophyta account for the largest numbers of species currently described. The Chlorophyta consist of four main lineages. Three of these, Chlorophyceae, Trebouxiophyceae and Ulvophyceae, form well-supported monophyletic groups and are usually separated at the class level (Lewis and McCourt, 2004; see Adl, 2005, for recent classification). The fourth lineage, Prasinophytes, is basal and it has been shown to be non-monophyletic. Most species of subaerial Chlorophyta are currently ascribed to the class Trebouxiophyceae, which includes several widespread genera (e.g. *Chlorella*, *Myrmecia*, *Prasiola*, *Stichococcus* and *Trebouxia*). The Chlorophyceae are mostly freshwater algae, but the class includes also some common subaerial genera (i.e. *Bracteacoccus* and *Chlorococcum*). The Ulvophyceae is largely formed by the marine green algae (Sluiman, 1989); however, it also includes an important lineage of subaerial algae, the order Trentepohliales (Lopez-Bautista and Chapman, 2003). Recently, a second ulvophycean subaerial lineage, represented by the genus *Spongiochrysis* (order Cladophorales), has been discovered (Rindi et al., 2006). The Charophyta are members of the other major lineage of green plants (the Streptophytes) and the phylum includes the closest relatives to the algae that gave rise to the land plants. Charophyta occur mostly in freshwater habitats but the phylum also includes some subaerial taxa, including one of the most widespread genera, *Klebsormidium*. The Heterokontophyta are a large group of eukaryotic algae separated into several classes and varying greatly in gross morphology. Subaerial forms are found in two different classes, the Bacillariophyceae, or diatoms, and the Xanthophyceae or yellow-green algae. To date, the main taxonomic treatments concerning algae of terrestrial habitats are still entirely based on traditional morphological concepts (Ettl and Gärtner, 1995). Early phycologists described several subaerial algae that produce populations recognizable with the unaided eye (e.g. Agardh, 1824; Kützing, 1849; Rabenhorst, 1868). Most subaerial algae, however, are small in size and occur in nature in only small amounts; they can therefore be observed and examined accurately only when growing in unialgal cultures (John, 1988). Thus, the number of described taxa increased considerably in the second half of the last century, with the widespread development of culturing techniques. This has particularly been the case with unicellular green algae, for which many taxa have been described entirely on the basis of cultured material (studies by Ettl, Hindák, Komárek and co-workers from Europe, and by Brown, Bold, Deason, Trainor and co-workers in the USA).

3.1. SYSTEMATICS OF SUBAERIAL CYANOBACTERIA

Although the cyanobacteria are some of the most widely distributed, ubiquitous organisms on the planet they are also historically understudied. Among the most prevalent and abundant algal lineages with subaerial members, cyanobacteria serve an important role in community succession (Gerrath et al., 2000). Growing

on such diverse habitats as rocks, soils (Jaag, 1945), tree bark (Desikachary, 1959) and dripping walls (Johansen et al., 2004), the taxonomy and biodiversity of these taxa is still mostly unknown (Komárek, 2003b). Among the oldest recorded fossils, the cyanobacteria were significantly involved in the formation of life on the planet as some of the earliest colonizers (Schopf, 1996). Unfortunately, they are also among the most poorly characterized and catalogued organisms, in part due to the prevailing belief among classic monographers (i.e. Geitler, 1932; Desikachary, 1959) that many species are cosmopolitan. However, recent studies have noted that these organisms may not be as cosmopolitan as previously assumed (e.g. Komárek, 1999) and endemic taxa are currently being described from newly explored habitats, especially subaerial ecosystems (e.g. Flechtner et al., 2002, Casamatta et al., 2005, Casamatta et al., 2006, Kastovska et al., in press). However, cyanobacterial diversity is still underreported due to difficulties in employing morphological characters to identify species and a lack of specialists exploring novel habitats. Thus, the subaerial habitats are a nearly unexplored treasure of cyanobacterial biodiversity.

The taxonomy of cyanobacteria has gone through major rearrangements in the last two decades, culminating in the recent revisions of Komárek and Anagnostidis (Anagnostidis and Komárek, 1985, 1990; Komárek and Anagnostidis, 1989, 1999, 2005). Their approach is based on a combination of morphological, ultrastructural, biochemical and (in part) genetic data and has proposed a substantial rearrangement in comparison with the traditional (i.e. Geitler, 1932) and the simplified Drouetian system (Drouet, 1981). Many new genera have been erected and the circumscription of many traditional genera (including some widespread in subaerial habitats, such as *Gloeocapsa*, *Lyngbya* and *Phormidium*) have been substantially rearranged. In the last 15 years, an increasing number of investigations have studied the phylogeny of the cyanobacteria, mainly using 16S rDNA gene sequences (Wilmotte, 1994; Otsuka et al., 1999; Wilmotte and Herdman, 2001; Suda et al., 2002; Casamatta et al., 2005; Rajaniemi et al., 2005). This molecular approach, coupled with careful ecological and morphological assessments, has enabled researchers to begin cataloguing the great biodiversity undoubtedly present in this lineage.

Preliminary observations on subaerial cyanobacterial taxa have revealed several intriguing facets. First, there are many more species of cyanobacteria (particularly the filamentous Oscillatoriales and coccoid Chroococcales lineages) than are evidenced by employing traditional taxonomic keys, and consequently there are numerous new species to be described. Second, in a number of broadly defined genera, such as *Leptolyngbya*, *Microcoleus* and *Nostoc*, the 16S rRNA and 16-23S internal transcribed spacer (ITS) sequence data demonstrate that more genera must be recognized if monophyly is to be achieved. Third, ITS regions vary widely between strains, and have been informative for making systematic decisions at both the genus and species levels. Fourth, in *Leptolyngbya* the ITS regions are not always congruent with phylogenies based on 16S rRNA sequences, and we suspect that at least some of the absence of congruence is due

to multiple operons within genomes. Fifth, when distinctive habitats of geographically/climatically isolated regions are studied closely, they always have endemic species of cyanobacteria. For example the Nostocacean lineage, composed of members that may produce nitrogen-fixing specialized akinete cells, is among the most common components of subaerial algal communities, and two new endemic genera in this group have recently been described: *Rexia* (Casamatta et al., 2006) and *Mojavia* (Kostakova et al., in review). Thus, as these habitats are explored we anticipate that more new taxa, as well as emendations of some existing genera of cyanobacteria are anticipated from the subaerial habitat (Casamatta et al., 2005; Kastovska et al., in press).

3.2. SYSTEMATICS OF SUBAERIAL CHLOROPHYTA

The taxonomy of the green algae is also in a phase of major rearrangements. The existence in the Chlorophyta of four main groups, three of which represent monophyletic lineages (Chlorophyceae, Trebouxiophyceae and Ulvophyceae), is now widely accepted. Arrangement at all other taxonomic levels, however, is still unclear. The flood of molecular data collected in the last two decades has given rise to dramatic modifications at every level of classification, and chlorophytan taxa widespread in terrestrial habitats have been among the most affected. A number of traditional orders (Chlorellales, Chlorococcales, Chlorosarcinales) originally circumscribed using vegetative morphology, are now known to contain phylogenetically unrelated taxa. Reclassification into different groups has taken place even at lower taxonomic levels, especially the genus level. Several common genera of subaerial chlorophytes, such as *Chlorella*, *Chlorococcum*, *Neochloris* and *Trebouxia*, have been shown to include species that in fact belong to different classes (Huss et al., 1999; Friedl and O'Kelly, 2002; Krienitz et al., 2003; Lewis and McCourt, 2004). Splitting of groups described on traditional morphological basis and erection of new genera is a general trend in the current microchlorophyte taxonomy (e.g. Krienitz et al., 2004; Buchheim et al., 2005). In the Charophyta, molecular studies have led to the recognition of six main groups (Mesostigmatophyceae, Chlorokybales, Klebsormidiales, Zygnematales, Coleochaetales and Charales), but the arrangement at lower taxonomic levels has been comparatively less affected.

Some algal groups, which are currently studied in detail in our laboratories, are selected to illustrate recent developments in the systematics of subaerial green algae.

3.2.1. *Trentepohliales*

The order Trentepohliales is a subaerial order of the class Ulvophyceae (Chapman, 1984; Lopez-Bautista et al., 2002) widely distributed in humid climates, and particularly abundant and diverse in tropical regions. They are usually found growing on leaves, tree bark, stem and fruits as well as rocks and

human constructions (buildings, fences, etc.). Conflicting hypotheses have been presented on the systematic position of this order. Phragmoplast-type cytokinesis suggested an affinity with the Charophycean lineage (Streptophytes) while the components of the flagellar apparatus suggested an affinity with the Ulvophyceae in the Chlorophycean lineage (Chlorophytes). Recent phylogenetic analyses (Lopez-Bautista et al., 2002; Lopez-Bautista and Chapman, 2003) using the nuclear-encoded 18S rDNA sequences from taxa representing both the Chlorophytes and Streptophytes unequivocally indicated that the subaerial Trentepohliales are closely related to marine ulvophycean algae in the Chlorophyta lineage. The subaerial trentepohlialean flora appears to be the sister group to the siphonous and hemisiphonous marine ulvophycean algae. As presently circumscribed, the order contains one family and six genera, which are entirely separated on morphological grounds: *Trentepohlia*, *Physolinum*, *Printzina*, *Phycopeltis*, *Cephaleuros* and *Stomatochroon*. Approximately 70 species are currently included in this order but the taxonomic position of many is uncertain. Despite their relatively simple morphology (i.e. branched uniseriated filaments) trentepohlialean taxa are a taxonomically difficult group. A highly plastic morphology is correlated to environmental factors making the circumscription of species and genera not only difficult but also confusing. More recent studies (Lopez-Bautista et al., 2006a) on the systematic of trentepohlialean taxa using 18S rDNA indicate that the morphological criteria traditionally used for the circumscription of genera and species are not correlated with phylogenetic patterns. Representatives of the genus *Cephaleuros* formed a well-supported monophyletic clade, while *Trentepohlia* resulted polyphyletic and strains of *Trentepohlia*, *Phycopeltis*, *Printzina* and *Physolinum* are assorted in several different lineages. Furthermore, nuclear DNA content analyses in Trentepohliales (Lopez-Bautista et al., 2006b) are correlated with phylogenetic advancement in the Trentepohliales with basal taxa having the smallest genomes while more derived taxa have substantially larger genomes. At the species level, *Cephaleuros virescens* is a textbook example of a common taxon considered to have a worldwide distribution in tropical and subtropical regions. However, in our phylogenetic analyses *C. virescens* seems to represent a complex of morphologically similar entities, with the real *C. virescens* (type) probably restricted to tropical South and Central America (Lopez-Bautista et al., 2006a).

3.2.2. *Prasiolales*

The order Prasiolales is an order of marine, freshwater and terrestrial green algae widespread in temperate regions. Although terrestrial taxa do not represent the majority of the order, they include some subaerial species that are very common and widespread in temperate regions with humid climate (*Prasiola crispa*, *P. calophyllla*, *P. furfuracea* and *Rosenvingiella radicans*). The phylogenetic position of this group has long been uncertain and molecular evidence supporting their inclusion in the Trebouxiophyceae has become available only quite recently (Friedl and O'Kelly, 2002; Karsten et al., 2005). As presently circumscribed, the order

contains four accepted genera: *Prasiococcus*, *Prasiola*, *Prasiolopsis* and *Rosenvingiella*. *Prasiola* and *Rosenvingiella* are the two largest genera and their relationships have long been uncertain; for a long time it has been believed that species of *Rosenvingiella* were developmental forms of *Prasiola*. Utilizing a combination of field studies, culture experiments, and chloroplast-encoded *rbcL* sequences the relationships at the species and genus level have been recently clarified (Rindi et al., 2004). The same study model was applied to elucidate the taxonomic position of *R. radicans*, one of the most common subaerial algae in northwestern Europe. The taxonomic identity of this species has been uncertain for more than two centuries; this has traditionally been one of the most problematic aspects of the taxonomy of this group. In the past this alga has been mostly considered a filamentous form of *P. crispa*; in the last few decades, it has been frequently regarded as a terrestrial form of the marine species *Rosenvingiella polyrhiza*. Culture studies and phylogenetic analyses of the *rbcL* data consistently indicated that this alga is in fact an independent species, and the new name *Rosenvingiella radicans* was proposed for it. The combination of culture studies and molecular data showed that in this group at least three different species have an identical morphology (*R. radicans*, the filamentous form of *P. crispa* and an alga of uncertain identity indicated as *Prasiola* sp. Manchester; Rindi et al., 2004).

3.2.3. *Spongiochrysis Hawaiensis*

During recent fieldwork in Hawaii several samples were collected from subaerial habitats. The microscopic examination of a bright golden-yellow coating occurring on the bark of many trees on several beaches along the windward coast of O'ahu revealed that they were produced by a unicellular green alga with a very unusual pattern of cell division. Production of new cells took place by a budding-like mechanism, which has been reported so far only for two genera of Trebouxiophyceae, *Marvania* (Hindák, 1976) and *Stichococcus* (a single species, *Stichococcus ampulliformis*; Handa et al., 2003). Complete 18S rDNA sequences for two populations of this alga resulted into a surprising discovery (Rindi et al., 2006). Phylogenetic analyses positioned this intriguing alga as a member of the Cladophorales/Siphonocladales clade of the Ulvophyceae. This was a discovery of enormous interest (Rindi et al., 2006) as it showed that a subaerial habit has developed in an algal group that was formerly believed to be almost entirely aquatic (the Cladophorales) and that a second subaerial lineage (beside the Trentepohliales) exists in the class Ulvophyceae. Once again, it also showed that identical morphologies and identical mechanisms of reproduction have developed independently in completely separated green algal lineages, and studies based only on morphological methods are insufficient to understand the diversity of many subaerial microchlorophytes.

3.2.4. *Klebsormidium*

Klebsormidium is one of the most common genera of terrestrial Charophyta. It consists of about 20 species of green algae occurring on soil, subaerial surfaces

and semi-aquatic habitats all around the world. The systematics of this genus has been entirely based on traditional morphology (Ettl and Gärtner, 1995; Lokhorst, 1996; John, 2002). Although molecular data are available, these have been obtained as part of investigations aimed at high-level phylogenetic relationships; the species-level relationships in *Klebsormidium* have not been examined with molecular tools. We have obtained and isolated into unicellular cultures numerous strains of this genus from subaerial habitats in a wide range of locations from Europe. Although the morphology of the field material was virtually identical and attributable to *Klebsormidium flaccidum*, preliminary experiments in culture have shown a very large range of morphological variation (Rindi, unpublished data). Whereas most strains produce only tridimensional filaments that remain completely submerged, some populations produce also a superficial layer of parallel filaments that cover completely the surface of the medium (as reported typical for the *K. flaccidum* by Lokhorst, 1996). In other strains, after a few weeks in culture the filaments get fragmented into many short fragments, giving the cultures the appearance of a green “soup.” Overall, our preliminary experiments indicated that even in this group a great deal of genetic diversity is hidden behind a very similar morphology. It is evident that *Klebsormidium* is one of the genera for which comparative studies based on morphology and molecular data are most needed.

4. Tropical Rainforests

Previous investigations by earlier naturalists in tropical rainforests (Fritsch, 1922; Frémy, 1930; Gardner, 1932) were not specifically focused on the subaerial habitats and did not attempt to provide an inventory of algal diversity in such habitats. Until recently, no studies have focused specifically on these habitats and the information available is based entirely on a classical morphological approach. No investigations have thus far attempted to characterize the subaerial algal flora of these forests and to examine in detail their biodiversity using state-of-the-art methods. This is unfortunate from a practical point of view since tropical rainforests can be considered highly specialized chemical factories, with many molecules proven to be highly effective drugs or very valuable compounds (Del Campo et al., 2000, Kotake-Nara et al., 2001, Hyunsuk et al., 2005, Wu et al., 2005). Gaps in the biology and diversity of subaerial algae are even more substantial for microalgae from tropical rainforests. To present a synthesis of the biodiversity status for subaerial algae of tropical forests is difficult or even impossible (Andersen, 1992).

Rainforests are highly humid and wet, thus particularly suitable for subaerial algae. It has been largely demonstrated that rainforests, especially tropical, are among the most diverse ecosystems on the planet, and are repositories of a large number of endemic taxa (Therezien, 1985; Williams et al., 2003, Burnham and Johnson, 2004; Funk and Berry, 2005); the necessity of their conservation is

largely accepted and justified. Several taxa of Trentepohliales that have been described in the last decade were originally discovered in tropical forests (Thompson and Wujek, 1992, 1997; Neustupa, 2003, 2005); and *S. hawaiiensis*, the second known terrestrial lineage in the Ulvophyceae, was recently discovered in an environment that is essentially rainforest-like (Rindi et al., 2006). Rainforests have also been shown to be centres of diversity for many animal, plants and algae (i.e. Trentepohliales), and it is perfectly reasonable to expect that they also host a much higher diversity of subaerial algae than currently appreciated. Unfortunately, tropical rainforests are among the most endangered ecosystems in the world and are rapidly disappearing due to deforestation, climatic changes and other human activities (Bulte and Van Kooten, 2000, Williams et al., 2003, Fearnside and Laurance, 2004, Laurance et al., 2004). The risk that many tropical algal lineages will become extinct before they are discovered is largely acknowledged.

5. Morphological Convergence

It is now generally accepted that gross morphology and reproductive features do not reflect phylogenetic patterns in most groups of green algae, both in the chlorophytan lineage (Krienitz et al., 2003; Fawley et al., 2004; Henley et al., 2004; Krienitz et al., 2004; O'Kelly et al., 2004) and in the charophytan lineage (McCourt et al., 2000; Gontcharov et al., 2003). Furthermore, in several different lineages of subaerial algae, the structure of the thallus has converged towards a relatively limited number of morphological types. Consequently, a great deal of genetic diversity is often hidden behind identical or very similar morphologies. Although this is a phenomenon common to many plant and animal groups, for terrestrial green algae this makes their taxonomy particularly problematic, due to the limited number of characters useful for a reliable morphological identification. With almost no exception, subaerial green algae have a simple thallus and a reduced size. Three main types of thallus morphology (Fig. 1) are found: (i) unicellular; (ii) sarcinoid (regular packets formed by a small number of cells); (iii) unisexual filamentous. In terms of number of species, the unicellular morphology is clearly the most widespread (Fig. 1A); this is typical of many common genera shown to be polyphyletic (*Bracteacoccus*, *Chlorella*, *Chlorococcum*, *Muriella*, *Myrmecia*, *Stichococcus*, *Tetracystis* and *Trebouxia*). The sarcinoid morphology (Fig. 1B) occurs in a more limited number of genera (e.g. *Apatococcus*, *Chlorokybus*, *Chlorosarcina*, *Desmococcus* and *Prasiococcus*) but it is characteristic of some of the most successful taxa, that is *Desmococcus olivaceus* is often reported as the most common green alga in the world (Laundon, 1985; Ettl and Gärtner, 1995). Unisexual filaments are found in a relatively limited number of species (Fig. 1C), mainly belonging to two different groups: *Rosenvingiella* and *Prasiola* in the Trebouxiophyceae (Rindi et al., 2004) and *Klebsormidium* in the Charophyta (Karol et al., 2001; Turmel et al., 2002). *Klebsormidium*, however, is

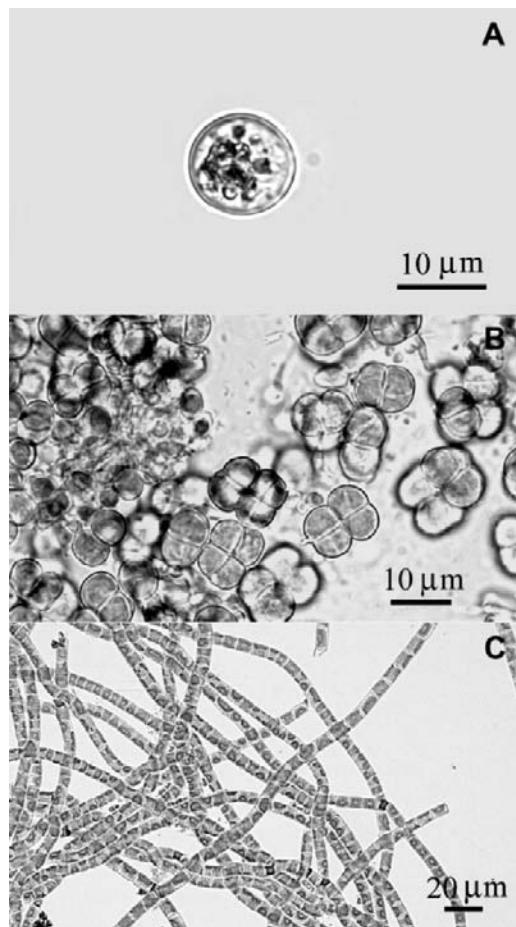


Figure 1. (A) *Spongiochrysis hawaiensis*; (B) *Desmococcus olivaceus*; (C) *Klebsormidium flaccidum*.

one of the most successful and widespread genera on a global scale and occurs in a wide range of habitats.

Although the causes for this morphological convergence are not well understood, it is clear that a simplification of the thallus from aquatic to subaerial habitats has generally been favoured. Other adaptations include, but are not restricted to, the capacity to utilize water in the form of vapour (Lange et al., 1990, Ong et al., 1992), the production of mucilaginous envelopes retaining moisture (Nienow, 1996), the production of resistance stages such as akinetes, the production of pigments acting as a protection from solarization (Whitton and Potts, 1982, Siefermann-Harms, 1987), the production of anti-freezing compounds (Raymond and Fritsen, 2000) and the production of mycosporine-like

amino acids as protection from UV radiation (Lange et al., 1990; Karsten et al., 2005). Since most terrestrial algae cannot survive in extremely dry conditions, it is therefore reasonable to assume that the possibility of a wide dispersal, allowing colonization of habitats with favourable conditions, plays a fundamental role in the survival of these organisms. Most studies in which algae have been isolated from air samples have shown a preponderance of small unicellular forms (Brown et al., 1964; Rosas et al., 1987; Kristiansen, 1996). It is considered that a spheroid unicell up to 12 µm in diametre, as found, for example in species of *Chlorella* and *Stichococcus*, is the ideal airborne alga (Roy-Ocotla and Carrera, 1993).

6. Conclusions

Although economically and ecologically important, subaerial algae have been largely understudied compared to marine and freshwater algae, and many basic aspects of their biology are still poorly understood. For historical reasons, most studies on diversity, taxonomy and ecology of subaerial algae have been carried out in Europe. Very few investigations, almost entirely limited to Europe, have examined comprehensively the subaerial algal flora of a certain geographical area. Studies on the taxonomy of subaerial algae are available for several groups. However, the large majority of these morphology-based investigations were conducted between the early 1800 and 1960. Work carried out in recent decades has been much more limited and has usually focused on a few, specific groups often involving biodeterioration. Amazingly, the few studies of subaerial algae in North America have yielded a wealth of putatively new taxa to science. Despite this body of information, in general the knowledge of subaerial algae is fragmentary, and even an estimate of their biodiversity is impossible at the present time. In the last 20–25 years algal systematics has changed dramatically due the development of PCR and DNA sequencing techniques. The molecular data that are currently available for subaerial algae have been produced as part of more general studies, focused primarily on algal phylogeny at the class or order level. However, the taxonomic coverage of the molecular data currently available is still uneven; whereas large datasets exist for several algal groups, for other species or genera, even those that are very common, nothing or very little is available. For example to date no sequences have been deposited in GenBank for *D. olivaceus* and *Apatococcus lobatus*, which are the two most common subaerial green algae reported in the world (Laundon, 1985). Given these reasons, it is clear that for many subaerial algae a correct taxonomic circumscription and an understanding of their diversity would be achieved only through a combined approach, in which morphological datasets based on both field collections and cultured materials are combined with extensive datasets of molecular information. As the diversity of many groups of terrestrial algae is still very poorly understood, the availability of new collections, inventories of their biodiversity and molecular characterization is particularly urgently needed.

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DIVERSITY, DISTRIBUTION AND ECOLOGY OF GREEN ALGAE AND CYANOBACTERIA IN URBAN HABITATS

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1. Introduction

Eukaryotic algae and cyanobacteria occur virtually in every terrestrial habitat on our planet. Organisms belonging to these groups are present even in some of the most extreme terrestrial environments, such as rocks in hot and cold deserts (Friedmann and Ocampo-Friedmann, 1984), Antarctic soils (Broady, 1996) and highly acidic post-mining sites (Lukešová, 2001). As early as the beginning of the nineteenth century, it was realized that microalgae occur also on walls, masonry and other man-made substrata (e.g. Dillwyn, 1809; Agardh, 1824); however, very little attention has been devoted to this type of algal communities until recently. Cities are artificial environments in which artificial substrata (such as concrete, asphalt, glass and metal) provide the largest part of the surfaces available for the colonization of microorganisms. The surfaces of many urban buildings are exposed to full sunlight; organisms growing on such surfaces are therefore frequently subjected to extremely high light irradiance, high levels of UV radiation and extreme dehydration (Crispim and Gaylarde, 2004; Karsten et al., 2005). The temperature of walls and roofs is subjected to a high range of variation and, in tropical regions, can reach 60–70°C (Tripathi et al., 1990). Most urban habitats are also affected by large amounts of pollutants, such as gases (SO_2 , CO , NO_x , hydrocarbons, ozone), aerosols, dusts and heavy metals (Seaward, 1979; John, 1988). Due to such a negative combination of factors for organisms of aquatic origin, for microalgae and cyanobacteria cities can be certainly considered extreme environments. Reports on algae and cyanobacteria from urban habitats have gradually appeared in the last few decades. Most studies on this subject concern European, Asiatic and South American cities; at present, there is almost no information published for other continents. In general, the knowledge of the diversity and ecology of these communities is still rudimentary, because most studies have focused much more on the biodeterioration operated by these organisms on artificial surfaces than on their biology. In this chapter, the information currently available on cyanobacteria and green algae of urban environments is summarized. General aspects of the diversity and distribution of these organisms in urban habitats are discussed, and the composition and ecology of the most common algal assemblages in these environments are described in detail.

2. Diversity of Algae and Cyanobacteria in Urban Habitats

Algae and cyanobacteria occurring in cities are not exclusive to urban environments; they are mostly cosmopolitan species, which are also frequently recorded from soil, rocks and other natural substrata (Caneva et al., 1992a, b; Ortega-Calvo et al., 1993a, b). To date, at least 98 species of green algae (Chlorophyta and Streptophyta) and 101 species of cyanobacteria have been reported from cities all around the world (Rindi, unpublished data). The species most frequently recorded include the green *Apatococcus lobatus* (Chodat) Boye Petersen, *Chlorella vulgaris* Beijerinck, *Desmococcus olivaceus* (Peerson ex Acharius) Laundon, *Klebsormidium flaccidum* (Kützing) P.C. Silva, Mattox & Blackwell, *Rosenvingiella radicans* (Kützing) Rindi, McIvor & Guiry and *Stichococcus bacillaris* Nägeli, and the blue-green *Calothrix parietina* Nägeli ex Bornet & Flahault, *Leptolyngbya tenuis* (Gomont) Anagnostidis & Komárek, *Microcoleus vaginatus* (Vaucher) Gomont, *Phormidium autumnale* (C. Agardh) Trevisan ex Gomont and *Tolypothrix byssoides* (Berkeley ex Bornet & Flahault) Kirchner. For several reasons, however, it is currently impossible to provide an exhaustive and taxonomically reliable catalogue of the species occurring in urban environments. In some studies, although the presence of algae on artificial surfaces is mentioned, the characteristics of the areas sampled are not described in detail (Boye Petersen, 1928; Brook, 1968; Schlichting, 1975; Hoffmann, 1986; Gómez-Alarcón et al., 1995; Tripathi et al., 1997); it is therefore unclear if the sampling sites must be considered urban or not. In other studies, records of terrestrial algae are reported collectively from a number of sites, both urban and rural; in some cases it is not clearly specified which records refer to urban sites and which to rural sites (Joshi and Mukundan, 1997; Gaylarde and Gaylarde, 1999, 2000; Rifón-Lastra and Noguerol-Seoane, 2001; Gaylarde et al., 2004). For most terrestrial microalgae and cyanobacteria, identification is difficult and requires observations conducted on both fresh field samples and material grown in culture (John, 1988; Broady, 1996). For this reason, in many studies referred to cities, identification only at the genus level (or above) has been the option chosen (Tiano et al., 1995; Flores et al., 1997; Gaylarde and Gaylarde, 2000, 2005; Lamenti et al., 2000; Tomaselli et al., 2000; Crispim et al., 2003, 2004; Gaylarde et al., 2004). It should be also remarked that several green algae found in cities are taxonomically problematic organisms, for which the arrangement at the species and genus level is still unclear or has been clarified only recently (e.g. John, 2002; Rindi et al., 2004). It is expectable that some entities (in particular, species of *Klebsormidium* and *Stichococcus*), as currently circumscribed, will prove to be a complex of morphologically similar species, rather than independent taxa; if this is the case, the taxonomic identity of many records will require a reassessment.

At present, relatively detailed lists of green algae and cyanobacteria with identifications at species level are available for Singapore (Wee and Lee, 1980; Lee and Wee, 1982), Athens, Greece (Anagnostidis et al., 1983), Rome, Italy (Ricci et al., 1989; Caneva et al., 1992b; Bellinzoni et al., 2003), Varanasi, India (Tripathi et al., 1990), Salamanca, Sevilla and Toledo, Spain (Ortega-Calvo et al., 1991,

1993c), Catania and Ragusa, Italy (Poli Marchese et al., 1995; Giaccone and Di Martino, 1999), Bratislava, Slovakia (Kapusta and Kovacik, 2000; Uher and Kovacik, 2004), Kyiv and Olvia, Ukraine (Darienko and Hoffmann, 2003), Galway City, Ireland (Rindi and Guiry, 2003) and Murcia, Spain (Uher et al., 2004a, 2005). It should be stressed, however, that most reports are referred to only one or few sites and do not provide a general account for the urban area considered. Since problems of biodeterioration have been the focus of most investigations, the sites studied are usually churches, historical palaces or other monuments. To date, the report of Rindi and Guiry (2003) for Galway City is the only study that has attempted to provide a detailed account of the subaerial algal flora of an individual urban area.

3. Distribution and Ecology: General Aspects

The distribution of algae and cyanobacteria in urban environments is characterized by great variability at several spatial scales, such as hundreds to thousands of kilometres (Rindi and Guiry, 2004; Gaylarde and Gaylarde, 2005), between sites located tens to hundreds of metres in the same city (Rindi and Guiry, 2002, 2003) and at microscales, such as millimetre to centimetre (Schlichting, 1975; Seaward, 1979; John, 1988). The distribution of many species and assemblages is very irregular and depends on a complex interaction of factors. Seaward (1979) summarized the properties and factors operating on the cryptogamic flora of urban habitats as follows:

1. Urban climate: precipitation (quantity, duration, chemistry including pH); humidity; temperature; light intensity; wind (direction, velocity); gaseous chemistry, including pollutants;
2. Surface effects: inclination; aspect; chemistry; texture (porosity, water-holding capacity; age; maintenance; heat absorption (e.g. colour); continuity (e.g. mortarwork);
3. Proximity of other surfaces: overshadowing; wind turbulence; continuity (e.g. effect of propagation);
4. Internal climate of buildings (e.g. temperature);
5. Internal properties of wall: water regime; temperature; chemistry;
6. Microenvironmental influence of wall in still air (=c. 8–10 cm);
7. Soil deposition.

Considering that such a high number of factors can affect the distribution of algae and cyanobacteria in urban habitats, it can be stated that the knowledge of their distributional patterns and the processes that affect them is still rudimentary. To date, all studies on this topic are entirely descriptive; the information available is based on observational evidence and an experimental assessment of the effect of one or more of the factors has never been attempted. A quantitative assessment of distributional patterns based on statistical analyses has been done

in very few investigations and only for few species (Rindi et al., 1999; Rindi and Guiry, 2004; Menéndez et al., 2006). Collection of samples of algae and cyanobacteria from sites where microbial growths were visually detectable (and subsequent examination by either light microscopy or SEM) is the approach most frequently used. When information on the distribution of microbial communities is provided, the importance of local conditions of high humidity is usually stressed. It is often mentioned that growth of algae and cyanobacteria occurs on surfaces where drainage of rainwater takes place (Wee and Lee, 1980; Danin and Caneva, 1990; Caneva et al., 1992a) or in microhabitats with conditions of high moisture, in particular on cornices, in holes, in crevices or beneath crusts, where water is retained and evaporation is slow (Anagnostidis et al., 1983; Ortega-Calvo et al., 1993a; Caneva et al., 1995). In other reports, a great importance has been attached to the type of substratum, in particular its chemical or physical properties. A strict substratum-specificity has been reported for species of Trentepohliales in Galway City (Rindi and Guiry, 2002). Schlichting (1975) found *Chlorella* abundant on bricks of Irish buildings, whereas the cyanobacteria *Chroococcus* and *Schizothrix* were confined to the mortar between them; differential growth between limestone and sandstone surfaces was also noted. Greater growth of algal biofilms on concrete than on limestone has been reported for buildings in South America (Crispim et al., 2003). Experimental growth in laboratory of some microalgae isolated from marble statues has shown a preference for certain types of stone substrata over others (Tiano et al., 1995).

Very limited information is available about the temporal dynamics of algal assemblages in urban habitats. In Galway City, the composition of algal assemblages at sites dominated by Prasiolales (Rindi et al., 1999) and Trentepohliales (Rindi and Guiry, 2002) did not show any variation over a study period of 1 year. Ortega-Calvo et al. (1991) noted the persistence of some algal species in different seasons at some sites on the cathedral of Seville.

Physiological studies conducted *in situ* on green and cyanobacteria in urban habitats are also very scanty (Tripathi et al., 1990; Ong et al., 1992; Häubner et al., 2006; for further details on this topic, see chapter “Aeroterrestrial Algae Growing on Man-Made Surfaces: What are the Secrets of Their Ecological Success?” by Karsten et al.). The papers of Rosas et al. (1987) and Roy-Ocotla and Carrera (1993) for Mexico City are the only studies so far that have considered dispersal of microalgae in urban habitats.

4. The Most Common Species and Types of Algal Assemblages in Urban Environments: Composition, Distribution and Ecology

4.1. CYANOBACTERIAL ASSEMBLAGES

Cyanobacteria are among the most widespread photosynthetic organisms and occur in all urban areas for which investigations of the microbial flora have been

carried out. They are particularly abundant in tropical regions, for which the dominance of the blue-green element is a typical feature of the subaerial vegetation (Nienow, 1996). Cyanobacteria are common on surfaces of both modern and ancient buildings (Tripathi et al., 1990; Crispim and Gaylarde, 2004); due to their capacity to resist very harsh conditions, they are among the most important pioneer microorganisms (Grant, 1982; Hoffmann, 1989; Whitton, 1992). Studies of cyanobacteria in urban environments have mostly concerned populations growing on monuments, for which these organisms are a frequent cause of biodeterioration (Ortega-Calvo et al., 1993a; Kovacik, 2000; Crispim and Gaylarde, 2004). Cyanobacterial assemblages produce grey or black discolorations on exposed surfaces; when their growth is profuse, they often appear as black vertical stripes, called "Tintenstriche," that is ink stripes. This type of assemblage has been studied with great detail from natural rocks (e.g. Jaag, 1945; Golubic, 1967) but little is known about its composition in urban environments. In cities, ink stripes occur at sites where bad drainage of rainfall takes place, such as leaking pipes, corners and bases of windows (Fig. 1A and B; Tripathi et al., 1990; Caneva et al., 1995; Rindi and Guiry, 2003; Crispim et al., 2004). Cyanobacteria growing in crevices or pits of urban buildings have not been examined in detail; the cyanobacterial flora occurring in the pits of roman monuments, in particular the Trajan's column, is the best documented (Danin and Caneva, 1990; Caneva et al., 1992b, 1994). Only few studies on urban buildings have reported the presence of endolithic cyanobacteria (Anagnostidis et al., 1983; Saiz-Jimenez et al., 1990).

Taxonomically, cyanobacteria of urban habitats consist of coccoid forms of the order Chroococcales (*Chroococcidiopsis*, *Chroococcus*, *Gloeocapsa*, *Myxosarcina*, *Synechocystis*) and filamentous forms of the orders Oscillatoriales (*Leptolyngbya*, *Lyngbya*, *Microcoleus*, *Oscillatoria*, *Phormidium*) and Nostocales (*Calothrix*, *Nostoc*, *Scytonema* and *Tolyphothrix*). A great variation in the composition of cyanobacterial assemblages has been reported in different studies. Assemblages dominated quantitatively by Chroococcales have been reported from walls in Geneva, Switzerland (Turian, 1981, 1985), in urban areas of southern Brazil (Gaylarde and Gaylarde, 1999, 2000), in Galway City, western Ireland (Rindi and Guiry, 2003) and from the region of Murcia, Spain, where *Chroococcidiopsis kashaii* Friedmann is the most common microorganism on artificial surfaces (Uher et al., 2004b, 2005). *Chroococcus lithophilus* Ercegovic, *Myxosarcina spectabilis* Geitler and *Synechocystis pevakii* Ercegovic are the most common species on the walls along the river Tiber in Rome, Italy (Bellinzoni et al., 2003); species of *Myxosarcina*, *Gloeocapsa* and *Synechococcus* have also been reported from several Roman monuments (Danin and Caneva, 1990; Caneva et al., 1992b, 1994, 1995). Conversely, communities dominated by Oscillatoriales have been described from cathedrals in Spain (mainly *Microcoleus* and *Phormidium*: Ortega-Calvo et al., 1991, 1993c) and architectural paints in India (mainly *Lyngbya* and *Plectonema*: Joshi and Mukundan, 1997). Assemblages formed by a mixture of coccoid and filamentous forms are also common and have

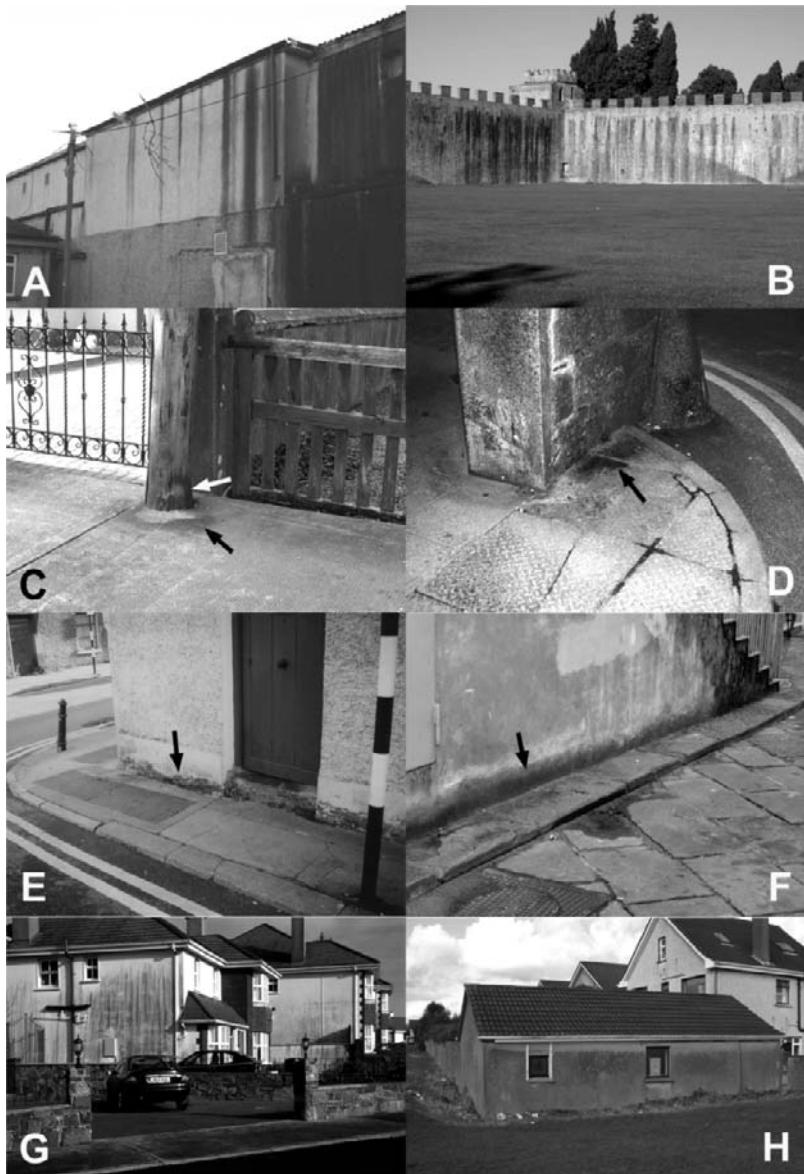


Figure 1. (A) Cyanobacterial stripes produced under broken, leaking rain pipes. Area of NUI Galway campus, Galway, Ireland. (B) Cyanobacterial stripes produced under wall openings. Ancient city walls, Square of the Miracles, Pisa, Italy. (C) Base of electricity pole with coating of *Desmococcus olivaceus* (white arrow) and patch of *Prasiola calophylla* (black arrow) on the surrounding ground. Upper Canal Road, Galway. (D) Patch of *Rosenvingiella radicans* (arrow) at the base of corner. Gaol Road, Galway. (E) *Rosenvingiella radicans* growing at the base of a wall (arrow). St. Augustine Street, Galway. (F) *Klebsormidium* producing dark green belt at the base of a wall. Back of Theatre Verdi, Pisa. (G) *Trentepohlia iolithus* forming streaks on urban houses. Knocknacarra, Galway; Prof Michael Guiry. (H) Building densely covered by *Trentepohlia iolithus*; note the absence of the alga from the top part of the wall, sheltered by the protruding roof. Laurel Park, Galway.

been reported for walls in Singapore (Wee and Lee, 1980), the Parthenon, Athens (Anagnostidis et al., 1983), buildings in Varanasi, India (Tripathi et al., 1990), statues in the Boboli Gardens, Florence, Italy (Lamenti et al., 2000) and churches in Porto Alegre, Brazil (Crispim et al., 2004). In general, the knowledge of urban blue-green assemblages is still limited and insufficient to allow generalizations about patterns of community structure from a geographical point of view.

So far, the investigation of Tripathi et al. (1990) is the only physiological study that has examined *in situ* urban populations of cyanobacteria; however, physiological traits promoting their growth in other terrestrial environments are likely to play also a major role in the colonization of these organisms in urban habitats, especially in tropical regions. Survival in urban habitats requires a great tolerance to very harsh conditions, such as very high/low temperatures, prolonged dry periods and extreme light intensity and UV radiation. A high capacity to withstand the removal of cellular water, maintenance of protein stability and structural integrity in dehydrated conditions, elaboration of extracellular glycans and production of heat-shock proteins and lipids (Lüttge, 1997; Potts, 1999; Tanaka and Nakamoto, 1999; Mikami and Murata, 2003) are certainly attributes of great importance for the colonization of urban walls. Compounds with function of UV sunscreens include mycosporine-like amino acids (Garcia-Pichel et al. 1993) and extracellular coloured pigments, such as scytonemin (Turian, 1985; Garcia-Pichel et al., 1992). Their abundance and intensity varies with the degree of exposure; colonies of *Gloeocapsa* growing on walls are often densely pigmented on the side facing light and colourless in the part adhering to the substratum (Turian, 1979; Rindi and Guiry, 2003).

Cyanobacteria may also affect some characteristics of the surfaces colonized, for example temperature; in Israel, blackish surfaces colonized mostly by cyanobacteria were found up to 8°C warmer than non-colonized areas (Garty, 1990). It is believed that cyanobacterial biofilms on exposed stone surfaces may accelerate the accumulation of atmospheric pollutants (Crispim and Gaylarde, 2004). Very little, however, is known about the effect of urban pollutants on the physiology of cyanobacteria, although there is evidence that some taxa are capable of adsorbing and using pollutants. Ortega-Calvo et al. (1995) reported that the growth of a biofilm of *Gloeothecace* in controlled conditions was optimal when sulphated crusts removed from the cathedral of Seville were used as substratum.

4.2. DESMOCOCCUS AND APATOCOCCUS

The morphological habit of sarcinoid colony (i.e. consisting of regular packets of cells) is common in terrestrial green algae. It has long been known that algae with this morphology are the main component of a widespread subaerial assemblage, which has usually the appearance of a powdery, hydrorepellent green coating. This community is well known for urban habitats and it has been indicated with various names, such as *Pleurococcetum vulgaris* (Barkman, 1958), proto-pleurococcoid

assemblage (John, 1988) and *Pleurococcetum* (Nienow, 1996). For the most common species, attribution to several different genera (*Protococcus*, *Pleurococcus*, *Desmococcus*, *Apatococcus*) has been proposed in different studies. A detailed taxonomic assessment of this assemblage is very difficult, especially for more dated studies (John, 1988). It is likely, however, that most frequently the main species involved is *Desmococcus olivaceus*. This is considered “the commonest green alga in the world” (John, 1988) and records of it are available for virtually all regions where subaerial algae have been studied. *Apatococcus lobatus* is also a cosmopolitan sarcinoid alga occurring on many different surfaces, including concrete, bricks and other man-made substrata (Ettl and Gärtner, 1995; Gärtner and Stoyneva, 2003); in some cases, this is probably the main component of the community. These two species may also be found mixed (Rindi and Guiry, 2003). Other green algae belonging to common terrestrial genera, such as *Chlorella*, *Chlorococcum*, *Coccomyxa*, *Stichococcus* and *Trebouxia*, are present in this assemblage, but usually in much lower amounts (John, 1988; Gärtner, 1994; Rindi and Guiry, 2003).

This community has been reported for several habitats and substrata. Tree bark is the substratum on which it is best developed and observable (Barkman, 1958; John, 1988; Gilbert, 1991). This assemblage can also be found on different types of woodwork; in Galway City, *Desmococcus olivaceus* forms bright green coatings at the base of electricity and telephone poles (Fig. 1C; Rindi and Guiry, 2003). Although less frequently, this type of community has also been reported from dry-stone walls (Darlington, 1981), walls of buildings (Rindi and Guiry, 2004) and ironwork (John, 1988). Barkman (1958) provided a detailed account of its ecology on tree bark in Europe and remarked that it is the most tolerant sub-aerial algal community. He stressed its capacity to develop when the environmental conditions are adverse to other algal and lichen communities, such as unsuitable tree species, polluted air, very high nitrogen concentrations, deep shade and situations that would be too dry for other algal assemblages (Barkman, 1958). In particular, the *Pleurococcetum* is considered highly toxotolerant and capable to thrive in urban areas where most lichens have disappeared due to high atmospheric pollution; it has been reported as the only epiphytic association present on trees in heavily polluted urban areas (Barkman, 1958; Segal, 1969; Seaward, 1979; John, 1988). In large Dutch cities in the 1950s, its development became so widespread to be considered a “green pest” (Barkman, 1958). Recently, a decline of *Desmococcus olivaceus* has been reported for several cities and linked to an improvement of the air quality, which has favoured the return of more pollution-sensitive lichen communities (Bates et al., 1990). However, reports of an increase of this alga with decreasing levels of pollution are also available (Pisut and Lisicka, 2000; Bates et al., 2001). Unfortunately, the distribution of *Desmococcus* is characterized by a great small-scale variability, which makes it extremely difficult to separate the effect of pollution from factors operating on small spatial scales. Direction of prevailing winds, patterns of dispersal and availability of suitable substrata are likely to be more important than air pollution. In Britain, if other local factors are discounted, the *Pleurococcus* assemblage has

been reported to develop mainly on surfaces facing North (Darlington, 1981); in Galway, *Desmococcus* may cover completely the base of some electricity poles and be more or less absent on identical poles located a few tens of metres apart in the same road. Further studies, based on appropriate sampling designs, are necessary to establish rigorously the effects of the factors affecting the distribution of this assemblage.

4.3. GREEN ALGAE OCCURRING AT THE BASES OF WALLS

The bases of urban buildings have been particularly neglected with regard to algae and cyanobacteria. The foundation of many cities, especially in Europe, dates back to millennia or many centuries. In many European cities, the central parts (which are usually the oldest) still follow a medieval design, with narrow streets and tall buildings. For this reason, the bases of walls are often sheltered from direct sunlight and are characterized by more humid and shaded conditions than the upper parts; they often represent a favourable habitat for the growth of green algae and cyanobacteria. At suitable sites, green patches formed by algae are visible in every European city with relatively ancient origins (Fig. 1D and E). They are usually most evident in spots located around the discharge mouth of rain pipes and at other sites characterized by high humidity; in favourable conditions, they may extend to produce green belts several metres long (Fig. 1F). It is therefore surprising that the first detailed studies on this habitat have been published only very recently (Rindi et al., 1999; Menéndez and Rico, 2001; Rindi and Guiry, 2003, 2004; Menéndez et al., 2006). To date, no published information is available yet for other continents. It is possible that the different structure of urban environments in other continents, with larger streets and therefore more exposed conditions, does not favour similar algal growths; however, this is most probably due to the fact that no attention at all has been devoted to this habitat elsewhere.

Most of the information available for the algae of the bases of walls has been provided by the study of Rindi and Guiry (2004), in which extensive collections from ten European cities were examined. These authors showed that in Europe this is the typical habitat of two different algal assemblages, both dominated by filamentous green algae: (i) the prasiolalean assemblage, associated with moist regions of Atlantic Europe (in particular Ireland and North-western Spain), in which species of *Prasiolales* are the most abundant; (ii) the *Klebsormidium* assemblage, present in the other parts of the continent, in which species of *Klebsormidium* are dominant.

The *Prasiolales* are green algae with great ecological amplitude, distributed primarily in cold temperate and polar regions. Although the group is mainly marine, several species are common in freshwater and terrestrial habitats. The occurrence of *Prasiolales* in terrestrial habitats of Atlantic Europe has long been known, but their abundance in urban environments was almost completely

ignored until the study of Rindi et al. (1999) for Galway City. Shortly afterwards, Menéndez and Rico (2001) showed similar growths of these algae in Oviedo and León, North-western Spain. In all these cities, the green patches observable at the bases of walls, corners and protrusions of buildings consist primarily of *Rosenvingiella radicans*. This is a common filamentous alga, occurring in a wide range of terrestrial habitats as well as in supralittoral and upper littoral habitats; it is an exceptionally euryhaline organism, able to grow equally well in culture media for marine and freshwater algae (Rindi et al., 2004). Its taxonomic identity was recently clarified by Rindi et al. (2004); see this study for details about the synonymies and the complicated nomenclatural history of this alga. *Prasiola calophylla* (Carmichael ex Greville) Kützing is frequently mixed with *Rosenvingiella radicans*; this species may also produce pure populations, which occur frequently on ground around electricity or telephone poles (Fig. 1C; Rindi et al., 1999; Rindi and Guiry, 2003). *Prasiola crispa* is not as abundant as the two previous species, and produces large populations only at a few sites; in Galway, however, small plants can be found mixed in the mats of *Rosenvingiella radicans*. Other species of green algae are found only rarely and in small amounts in the prasiolalean assemblage; *Phormidium autumnale* is the only cyanobacterium common in it (Rindi and Guiry, 2003, 2004).

On large spatial scales (100s to 1000s of kilometres), a cool and humid climate seems to be a key requirement for a vigorous development of the prasiolalean assemblage. The association of the Prasiolales with cold temperate and polar regions is striking; no records of terrestrial species are available for tropical and subtropical regions. The two European regions for which their abundance in urban habitats has been documented (Ireland and North-western Spain) are directly affected by the North Atlantic Drift of the Gulf Stream. This produces a temperate climate with limited seasonal variation, summer temperatures relatively low if compared with most of Europe (rarely higher than 20–22°C) and high levels of atmospheric humidity and rainfall throughout the year. On small scales (centimetres to tens of metres), the development of the prasiolalean assemblage is probably influenced by conditions of high moisture. Intervening space, type of habitat and orientation of the surface available interact to affect local conditions of humidity. Walls facing north or north-west (which, in the northern hemisphere receive less direct sunlight than surfaces with other orientations) and with short intervening space (as is typically the case in narrow streets) are the most favourable places. Large availability of nutrients, especially in the form of organic nitrogenous compounds, may also favour a luxuriant growth of Prasiolales. The abundance of these algae in dirty places, affected by bird guano or persistent mammalian urination, is well documented. Observational evidence suggests that organic nitrogen supplied as canine or human urine may be responsible for the abundance of Prasiolales at some sites in Galway City. Despite regulations forbidding it, to let dogs roaming freely is a widespread bad habit in Galway. Some of the largest patches of *Rosenvingiella*

radicans can be observed in habitats that dogs habitually use as signal posts, such as corners and bases of poles and trees (Fig. 1E; Rindi et al., 1999). Similar observations were made by Gilbert (1991), who defined the base of urban trees as the “canine zone” and reported that the filamentous form of *Prasiola crispa* (probably *Rosenvingiella radicans*) is the dominant species in it. For the old town of Oviedo, an important input of organic nitrogen may be supplied by the rain collected from the roofs, which brings to the ground the residuals dropped by the large populations of pigeons, sparrows and other birds (Menéndez et al., 2006). It must be stressed, however, that further studies are needed to assess in detail the effect of this factor, as no experimental evidence of its importance has so far been provided.

Klebsormidium is a very common genus of subaerial and soil algae. *Klebsormidium flaccidum* is the species most frequently recorded in urban environments (Ortega-Calvo et al., 1991, 1993a, c; Darienko and Hoffmann, 2003; Rindi and Guiry, 2003; Uher et al., 2005); however, the capacity of *Klebsormidium* to form large populations at the bases of walls was not noted before the study of Rindi and Guiry (2004). In this community, *Klebsormidium* is largely dominant. Other species, such as the green *Desmococcus olivaceus* and *Stichococcus bacillaris* and the cyanobacterium *Phormidium autumnale*, can be occasionally observed, but always in very small amounts. Although the observations available for the *Klebsormidium* assemblage are not as detailed as those for the prasiolalean assemblage, it is likely that on small spatial scales the development of this community is also linked to local conditions of moisture. Well-developed patches of *Klebsormidium* occur in the same type of habitats and are, in particular, a common sight around the mouth of rain pipes. On large spatial scales, the scarce data presently available suggest that in Europe the *Klebsormidium* assemblage replaces the prasiolalean assemblage in all the parts of the continent in which the climate is not as damp as in the humid Atlantic regions. This is not surprising, as species of *Klebsormidium* are known to be very tolerant to different types of stressing conditions, in particular extreme dehydration. The metabolic changes taking place in *Klebsormidium* following dehydration stress have been described in detail (Fritsch and Haines, 1923; Morison and Sheath, 1985); these algae produce thick-walled akinetes filled with abundant lipid and starch granules, able to withstand long dry periods and reproduce new populations when environmental conditions become favourable (Morison and Sheath, 1985). *Klebsormidium flaccidum* occurs in cities of southern Spain, in which summer temperatures can reach 45°C (Ortega-Calvo et al., 1991); differently from the Prasiolales, *Klebsormidium* has also been reported for tropical regions (Lee and Wee, 1982; Joshi and Mukundan, 1997). A high tolerance to several forms of chemical pollution, which has been reported for populations occurring in other habitats (Lukešová, 2001; Verb and Vis, 2001; Stapper and Franzen-Reuter, 2004), might also contribute to the success of *Klebsormidium* in urban environments.

4.4. TRENTEOHliaLES ON URBAN BUILDINGS

The order Trentepohliales includes subaerial green algae widespread in regions with humid climates, where they occur on a great range of substrata (Ettl and Gärtner, 1995; López-Bautista et al., 2002). These algae have their centre of diversity and abundance in the tropics, but they are also largely distributed in temperate regions (López-Bautista et al., 2002). Although not generally widespread in cities, species of Trentepohliales produce large populations on urban buildings in regions with humid climates. When well developed, these populations have a characteristic appearance. Large amounts of carotenoid pigments, considered a protection against high irradiance (Siefermann-Harms, 1987), occur in the cells of the Trentepohliales and give them a bright red, orange or yellow colour. Populations growing on urban walls produce conspicuous red or orange streaks, which are considered a major aesthetic nuisance. Most of the information available on Trentepohliales in urban habitats concerns Singapore (Wee and Lee, 1980; Lee and Wee, 1982; Ho et al., 1983; Ong et al., 1992) and Galway City (Rindi and Guiry, 2002, 2003).

In Singapore, *Trentepohlia odorata* (Wiggers) Wittrock is the most common alga on artificial substrata. This species is widespread on exposed building surfaces, on which it is often the primary colonizer (Wee and Lee, 1980; Lee and Wee, 1982); Ong et al. (1992) studied in detail its physiology. Its photosynthetic rates vary greatly with the time of the day, with high values in the early morning and lower values at mid-day and in the evening (Ong et al., 1992). *Trentepohlia odorata* is capable of utilizing water vapour. The early hours of the morning, characterized by high atmospheric humidity and low irradiance, are the most favourable; in the central hours of the day, with decreased humidity and high irradiation, rates and efficiency of photosynthesis are reduced. Interestingly, *Trentepohlia odorata* seems to have developed an internal rhythm adapted to this situation; such depression of the photosynthesis occurs also on rainy days, when favourable conditions of humidity and light intensity persist later in the day (Ong et al., 1992). Very few details are available about small-scale patterns of distribution of *Trentepohlia odorata*. Remarks and pictures by Sing (2002), however, suggest that surfaces cooled by air-conditioning systems, on which condensation occurs, are favourite places for the development of this species and other algal growths. Remarkably, *Trentepohlia odorata* has not been recorded in large-scale investigations of algae occurring on buildings in India and other tropical regions (Joshi and Mukundan, 1997; Gaylarde and Morton, 1999). The possible reason is that these investigations specifically concerned painted surfaces; further studies, however, are required.

Among the five species of Trentepohliales occurring in the urban area of Galway (Rindi and Guiry, 2002, 2003), *Trentepohlia iolithus* is largely the most common. Due to the displeasing discolorations produced on many buildings, this species is locally considered a pest and called popularly “the red fungus.” Concrete walls, either painted or not, are the typical habitat of *Trentepohlia*

iolithus. Great differences in the abundance of this alga are observable between different walls; availability of propagules, patterns of dispersal and age of the surface colonized concur to determine its abundance. Wind and rain are the main agents of dispersal for the Trentepohliales. These algae possess a unique type of zoosporangium, borne at the top of a supporting cell with a characteristic retorted pedicel (López-Bautista et al., 2002). Alternation of dry and wet periods causes the breakage of the connection between zoosporangium and pedicel. The zoosporangium is then carried by the wind and settles when it hits a wet surface (presumably the same may happen with detached vegetative fragments). Release of zoospores will then take place; the zoospores will swim in the film of water present on the wet wall and, after settlement, will germinate and produce new plants. Incident rain plays clearly an important role in bringing down the zoosporangia and other propagules carried by the wind; this is indicated by the fact that *Trentepohlia iolithus* is usually absent in the uppermost parts of walls, which are sheltered from the rain by the protruding roofs. Recent populations typically produce vertical stripes (Fig. 1G); this suggests that spores, vegetative fragments or other propagules released by the newly established population are carried down by the rainwater flowing along the wall and settle in lower parts, extending the population. Not unexpectedly, the largest populations of *Trentepohlia iolithus* usually occur on old, unpainted walls with a wide intervening space; a wide intervening space will offer more exposure to wind and rain, and therefore a higher probability that the wall will be hit by propagules of the alga. The time of exposure is also important; the probability to be colonized will be obviously higher for old walls, which have been exposed for a longer time. In cases of particularly vigorous growth, populations of this alga may eventually assume the habit of a uniform layer of red paint (Fig. 1H). *Trentepohlia iolithus* in western Ireland represents an excellent example of alga directly associated with the human presence; despite intensive and careful search, it has never been found on rocks, soil or other natural substrata (Rindi and Guiry, personal observations). Although information for other regions is very limited, this species is also probably common in other cities of humid Atlantic parts of Europe. Rindi et al. (2003) recorded it from buildings in several towns of Normandy, northern France. It is also likely that reports of *Trentepohlia monilia* De Wildeman from artificial substrata in Galicia, northern Spain (Noguerol-Seoane and Rifón-Lastra, 1997; Rifón-Lastra and Noguerol-Seoane, 2001) are a misidentification of this species.

Three more species of Trentepohliales occur on artificial substrata in the urban area of Galway (*Printzina lagenifera*, *Trentepohlia aurea* and *Trentepohlia umbrina*); they may produce locally abundant populations, but they are not widespread as *Trentepohlia iolithus* and do not represent a similar practical nuisance. Records of Trentepohliales in urban areas are also available for Porto Alegre, Brazil (Crispim et al., 2004) and other urban areas in South America (Gaylarde and Gaylarde, 2000), Siracusa, Catania and Ragusa, Italy (Tomaselli et al., 1982; Poli Marchese et al., 1995; Giaccone and Di Martino, 1999), but with no details on their distribution and ecology and often without a species-level identification.

5. Conclusions

Rather than representing a definitive summary, this chapter highlights how limited the knowledge of green algae and cyanobacteria in urban habitats is. Basic biological and taxonomic aspects are among the aspects most neglected; accurate identification at the species level, ideally based on a combination of morphological and molecular data, is greatly desirable for many taxa. Detailed studies on assemblages composition and comprehensive floristic investigations for individual urban areas are highly needed. Physiology and photosynthesis in situ are of great importance to understand distributional patterns and potential effects of biodeterioration, and it is surprising how limited attention these aspects have received so far. Knowledge of dispersal patterns is another basic aspect on which almost nothing is known, and for which new and detailed information is urgently required. New information on any of these topics will be very valuable and my hope is that this chapter will encourage many phycologists to pay more attention to urban algae and cyanobacteria. There is no doubt that these organisms are worthy of much more attention and offer the possibility for a bulk of exciting and creative research in many different areas.

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DIVERSITY OF ORGANIC OSMOTIC COMPOUNDS AND OSMOTIC ADAPTATION IN CYANOBACTERIA AND ALGAE

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1. Introduction

The world ocean, where most aquatic oxygenic phototrophs, cyanobacteria as well as algae, are found, contains around 35 g/l of salt. As a consequence, marine phytoplankton has to be adapted to life in a saline environment. Many representatives of the cyanobacteria as well as of the algae have developed adaptations to life at far higher salt concentrations as well, and some can even be found in salt-saturated environments, such as the Dead Sea, the Great Salt Lake, Utah, and saltern crystallizer ponds in which the total salt concentration exceeds 300 g/l (Oren, 2002). Species of the unicellular green alga *Dunaliella* are among the most prominent inhabitants of such hypersaline environments.

All organisms living in saline solutions, including marine phytoplankton species, need to adjust their cytoplasm so that the cell contents are in osmotic equilibrium with the outside medium. Moreover, in environments of changing salinity (shallow marine lagoons, brine pools, any salt lakes, etc.), they will have to adapt osmotically to fluctuations in the external salt concentration. In species that do not possess a rigid cell membrane, such osmotic adjustment can be achieved by rapid shrinkage or enlargement of the cells by movement of water through the membrane. Such a behavior had been documented more than a hundred years ago by Teodoresco (1905) in his description of the genus *Dunaliella* (translation by the author):

These zoospores are devoid of a cellulose cell wall; instead there is a cell envelope that possesses a certain flexibility and a certain elasticity, which allows the body to take quite different forms, in accordance with the [salt] concentration of the water. ... Thus, when we place a drop of salt water that contains zoospores [=motile vegetative cells] on a microscope slide, one detects in the microscope that these present themselves in the above-described form. However, when we let the drop evaporate a little, one observes that the body starts to elongate and to lose its shape. ...; when we then add to the preparation a drop of fresh water, the zoospores suddenly round up.

None of the known oxygenic phototrophs appear to accumulate salt at concentrations equal to the external medium to achieve osmotic balance. In fact, the

strategy of salt accumulation as a mechanism of osmotolerance is used by only very few organisms, notably the halophilic Archaea of the family Halobacteriaceae, and a few groups of heterotrophic bacteria: the aerobic *Salinibacter* and the anaerobic fermentative Halanaerobiales (Oren, 2000a, 2002). In these groups KCl is used to counteract the osmotic pressure exerted by the salts outside, in which generally NaCl dominates. In such organisms all intracellular enzymatic

Table 1. Representative halophilic and halotolerant algae and cyanobacteria and the organic osmotic solutes they accumulate in response to the salt concentration of their environment.

Taxonomic group	Genera ¹	Principal compounds accumulated
Cyanobacteria	Freshwater species <i>Synechococcus</i> ; <i>Synechocystis</i> , <i>Microcoleus</i> <i>Aphanothecace</i> (<i>Halothecace</i>)	Sucrose, Trehalose Glucosylglycerol Glycine betaine Glutamate betaine
Rhodophyceae	<i>Hypnea</i> , <i>Iridophycus</i> , <i>Lomentaria</i> , <i>Porphyra</i> , <i>Rhodymenia</i> <i>Corallina</i> , <i>Porphyra</i> <i>Centroceras</i> , <i>Griffithsia</i> <i>Grateloupia</i>	Floridoside, Isofloridoside Floridoside Digenaside Floridoside, N-methyl-L-methionine sulfoxide, Isethionic acid
Chrysophyceae	<i>Monochrysis</i> <i>Poteriochromonas</i>	Cyclohexanetetrol Isofloridoside
Eustigmatophyceae	<i>Monallantus</i>	Mannitol, Proline
Phaeophyceae	<i>Ascophyllum</i> , <i>Colpomenia</i> , <i>Dictyota</i> , <i>Ecklonia</i> , <i>Fucus</i> , <i>Pylaiella</i> , <i>Scytosiphon</i> , <i>Pelvetia</i> , <i>Hormosira</i>	Mannitol; sometimes in combination with other polyols
Prasinophyceae	<i>Platymonas</i> , <i>Pyramimonas</i> <i>Asteromonas</i>	Mannitol Glycerol
Bacillariophyceae	<i>Cyclotella</i> , <i>Navicula</i> <i>Cylindrotheca</i>	Proline Mannose
Chlorophyceae	<i>Dunaliella</i> , <i>Chlamydomonas</i> <i>Chlorella</i> <i>Cladophora</i> <i>Klebsormidium</i> , <i>Stichococcus</i> <i>Ulva</i>	Glycerol Proline, Sucrose Glycine betaine Sorbitol, Proline Dimethylsulfoniopropionate, proline, sucrose

¹Not all species of the genera listed necessarily accumulate the solutes indicated in the last column. Adapted in part from Ben-Amotz and Avron, 1983 and references therein.

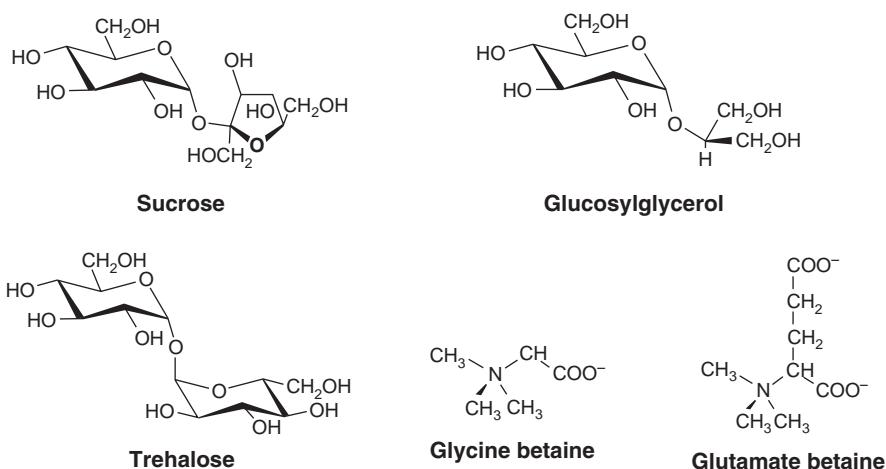


Figure 1. Organic compatible solutes found in halophilic and halotolerant cyanobacteria.

systems need to be active in the presence of high ionic concentrations, and this requires special adaptations of the cellular proteins. All other halophilic and halotolerant microorganisms (and some macroorganisms as well) accumulate organic solutes to osmotically equilibrate the cells with their medium. Such solutes are generally termed “compatible solutes” to indicate that their presence, even at high concentrations, does not inhibit enzymatic activity. The use of organic osmotic solutes therefore does not necessitate major adaptations of the intracellular enzymatic machinery.

Within the world of the salt-tolerant and salt-requiring oxygenic phototrophs, prokaryotic as well as eukaryotic, a tremendous variety of organic compatible solutes are found. Table 1 presents a representative selection of taxonomic groups and the osmotic solutes reported to accumulate and to be regulated according to the salinity of the medium. The chemical formulas of these solutes are given in Figs. 1 and 2. This chapter provides a short review of these organic osmotic solutes found in phototrophs that live at salinities varying from the relatively moderate salt concentrations in the marine environment up to the highest values in NaCl-saturated lakes. More in-depth reviews can be found in papers by Hellebust (1976), Brown (1978), Ben-Amotz and Avron (1983), Kirst (1994), and Bisson and Kirst (1995).

2. Compatible Solutes in the Cyanobacteria

Cyanobacteria are found from freshwater environments to hypersaline systems. The great morphological and physiological diversity within the group is reflected in the large variety of organic compatible solutes that have been

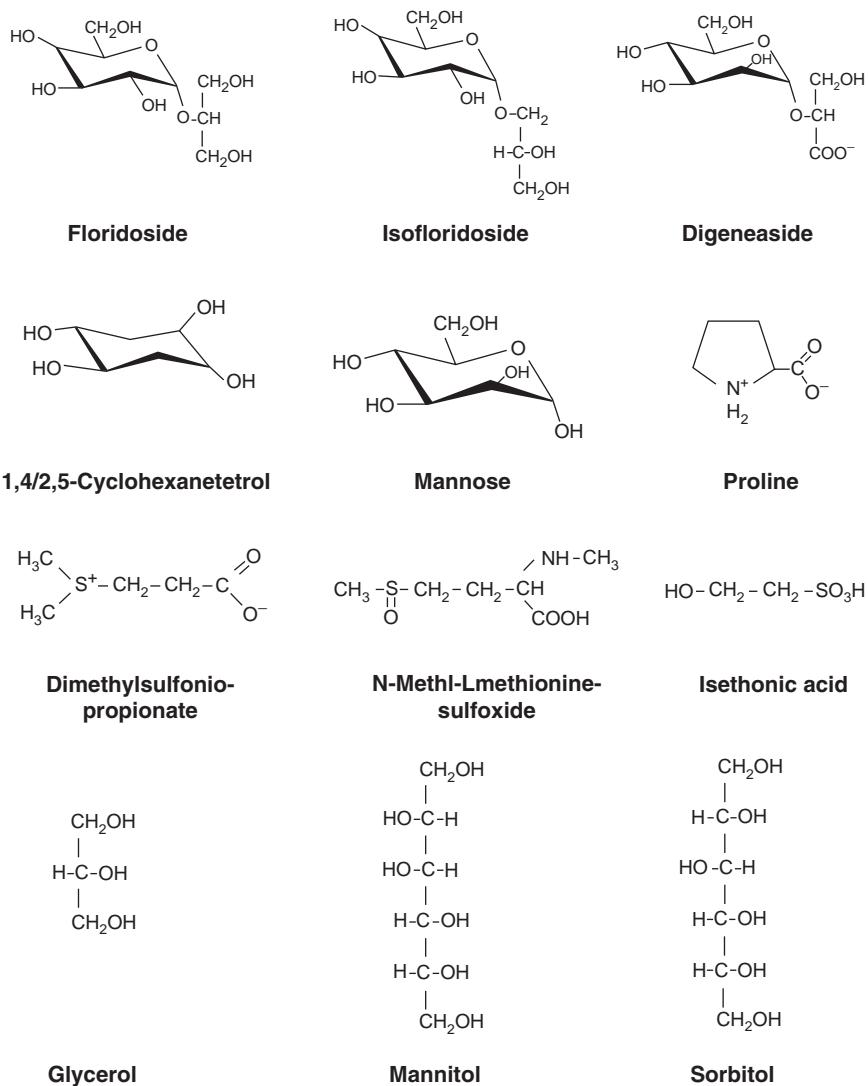


Figure 2. Some of the organic compatible solutes found in halophilic and halotolerant algae.

found to accumulate in different cyanobacteria as a reaction to increased salinity.

Figure 1 shows the main osmotic solutes of this group. They belong to different chemical classes: disaccharides (sucrose, trehalose), heterosides (glucosylglycerol), and methylated amino acids (glycine betaine and glutamate betaine) (Mackay et al., 1984; Reed et al., 1986; Oren, 2000b). There is a clear correlation between the salt tolerance of a cyanobacterium and the types of

compatible solutes it accumulates when subjected to salt stress. Freshwater species, not adapted to life at high salt, tend to produce disaccharides. The most halophilic representatives use glycine betaine to balance their cytoplasm with the outside salinity. Species that grow at intermediate salinities, including many marine types, generally produce glucosylglycerol (Oren, 2000b).

The metabolism of glucosylglycerol (O - α -D-glucopyranosyl-(1 \rightarrow 2)-glycerol) in halophilic and halotolerant cyanobacteria has been investigated in-depth. NMR studies in a halophilic *Synechococcus* showed a rapid increase in the concentration of the heteroside following salt upshock (Borowitzka et al., 1980; Mackay and Norton, 1987).

Microcoleus chthonoplastes, a filamentous species found worldwide in benthic microbial mats at salt concentrations from seawater up to 200 g/l and higher, is another well-known glucosylglycerol producer (Oren et al., 1994; Karsten, 1996), as is *Spirulina platensis* (Warr et al., 1985). The biochemistry and the molecular biology of glucosylglycerol production have been elucidated in *Synechocystis* PCC 6803. Glucosylglycerol is produced from ADP-glucose and glycerol-3-phosphate, with glucosylglycerol phosphate as an intermediate. Presence of salt and/or hypertonic conditions activates the glucosylglycerol forming enzyme system. Following salt downshock, *Synechocystis* releases its excess of the solute to the medium (Fulda et al., 1990; Erdmann et al., 1992; Hagemann and Erdmann, 1994; Hagemann et al., 1999).

Glycine betaine (trimethylglycine) is accumulated by the most halophilic among the cyanobacteria, including *Aphanothecce* (*Halothecce*), *Halospirulina* (*Spirulina subsalsa*), *Dactylococcopsis*, representatives of the *Lynbya* – *Phormidium* – *Plectonema* group, and others (Mohammad et al., 1983; Mackay et al., 1984; Reed et al., 1986; Gabbay-Azaria et al., 1988; Oren et al., 1994). Salt upshock in *Aphanothecce halophytica* led to enhanced synthesis of glycine betaine (Ishitani et al., 1993). Glutamate betaine is not commonly found, but its presence has been reported in a halophilic *Calothrix* strain (Mackay et al., 1984).

Another organic osmotic solute occasionally reported to occur in cyanobacteria is dimethylsulfoniopropionate (DMSP). It was found in *Microcoleus chthonoplastes* and in *Synechocystis*, *Lyngbya*, *Nostoc*, and *Anabaena* isolates. However, concentrations are low, and it is yet unclear to what extent these organisms rely on DMSP to establish osmotic equilibrium. Information about DMSP as an osmotic solute in algae is given in Section 3.1.

Finally, it has been reported that in a *Halothecce* species that abounds in a hypersaline gypsum crust in a saltern evaporation pond, two soluble UV-absorbing mycosporine-like amino acids accumulate at concentrations up to 0.1 M and possibly higher, concentrations sufficiently high to contribute to the cells' osmotic equilibrium (Oren, 1997). The first compound, which has its absorption maximum at 331 nm, was identified as mycosporine-2-glycine (Kedar et al., 2002); the second compound, which absorbs maximally at 362 nm, has recently been identified as 2-(E)-3-(E)-2,3-dihydroxyprop-1-enylimino-mycosporine-alanine (Volkmann et al., 2006). The possible role of these mycosporine-like amino acids was further

suggested by the fact that following salt downshock these compounds are excreted to the medium (Oren, 1997; Oren, 2000b).

3. Compatible Solutes in Marine Micro- and Macroalgae

In the diverse world of algae, the variety of organic osmotic solutes is even greater than in the cyanobacteria (Ben-Amotz and Avron, 1983; Fig. 2). Overall we can recognize a number of classes: heterosides (floridoside, isofloridoside, digeneaside), polyhydric alcohols (glycerol, mannitol, sorbitol, cyclohexanetetrol), simple sugars (mannose), amino acids (proline), and amino acid derivatives (DMSP, N-methyl-L-methionine sulfoxide). Some of these types of solutes (simple sugars, heterosides, methylated amino acid derivatives) are also found in the prokaryotic oxygenic phototrophs. The list of solutes presented in Fig. 2 and in Table 1 is by no means exhaustive: additional solutes have been detected in different species, and some of these are mentioned below.

The presence of certain osmotic solutes is correlated with the taxonomic position of the organism. The heterosides floridoside ($2-O-\alpha$ -D-galactopyranosylglycerol), L-isofloridoside, and D-isofloridoside are widespread in the Rhodophyceae and the Chrysophyceae (Kremer and Vogl, 1975; Kirst, 1980). Floridoside is formed by condensation of UDP-galactose and α -glycerolphosphate to give galactosyl-glycerolphosphate, which is then dephosphorylated. Intracellular concentrations increase with salinity (Kauss, 1967a, 1967b; Kirst and Bisson, 1979; Reed et al., 1980; Wiencke and Läuchli, 1981; Reed, 1985; Karsten et al., 1993; Karsten, 1999; Simon-Colin et al., 2004). The heterosides can be accumulated in high concentrations: up to 0.89 M floridoside + isofloridoside have been measured in *Porphyra purpurea* (Ben-Amotz and Avron, 1983). Following salt downshock, *Ochromonas* (*Poterochromonas*) *malhamensis* converts excess isofloridoside to the osmotically inactive polymer leucosin (beta(1 \rightarrow 3)-glucan) (Kauss, 1967b).

The chemically related digeneaside (α -D-mannopyranosyl-(1 \rightarrow 2)-glycerate) is a chemotaxonomic marker for the red algal order *Ceramiales* (Kremer and Vogl, 1975; Kirst and Bisson, 1979; Kirst, 1980; Reed, 1990). However, it is accumulated in concentrations too low to contribute significantly to the osmotic potential of the cells. Thus, a concentration of 0.024 M was reported in *Centroceras clavulatum* (Ben-Amotz and Avron, 1983). ^{13}C - and $^1\text{H-NMR}$ studies in the euryhaline *Caloglossa leprieurii* also showed low digeneaside concentrations, and these changed little with medium salinity. This organism accumulates also D-mannitol (Karsten et al., 1992b, 1994). In other organisms, however, the digeneaside concentration was reported to increase with medium salt concentrations (Kirst and Bisson, 1979). Although the distribution of different heterosides within red algal orders had been regarded as chemotaxononomically useful, recently it has been demonstrated that some genera of the *Ceramiales*, e.g., *Laurencia* and *Osmundea*, produce and accumulate floridoside and not digeneaside (Barrow et al., 1995).

Other biochemical exceptions in terms of major carbohydrates also are recorded in this order, with taxa such as *Bostrychia*, *Stictosiphonia*, and *Caloglossa* characterized by polyols such as sorbitol, dulcitol, and mannitol, compounds which are otherwise very unusual in the red algae (Karsten et al., 1992a, 1992b). Most interesting is the fact that all known low molecular weight carbohydrates found in the Ceramiales, i.e., heterosides and polyols, are evident in the Bangiophyceae as well (Karsten et al., 1999, 2003). Since the bangiophycean orders are considered to be more ancestral relative to the Florideophyceae, the ability to synthesize all of the same compounds found in the ‘advanced’ Florideophyceae indicate the maintenance of ancestral physiology in advanced taxa.

Another new low molecular weight carbohydrate was recently found in two *Hypoglossum* species (Ceramiales) in addition to digeneaside. Its chemical structure was elucidated as digalactosylglycerol. Digalactosylglycerol synthesis was strongly accumulated under hypersaline conditions, strongly supporting an osmotic function (Karsten et al., 2005).

Simple sugars are seldom found as osmotic solutes in algae. The report of the accumulation of mannose in the diatom *Cylindrotheca fusiformis* (Paul, 1979) appears to be a rare exception.

Polyols as osmotic solutes are common in the Phaeophyceae, the Prasinophyceae, and the Chlorophyceae (Reed et al., 1980, 1985; Ben-Amotz and Avron, 1983). Glycerol is the osmotic solute of choice for the most halotolerant among the algae. Its distribution and properties are described in Section 3.2. Other polyols include mannitol, sorbitol, and 1,4/2,5-cyclohexanetetrol (“cyclitol”). These polyols can accumulate in high concentrations: 0.22 M mannitol was found in *Platymonas subcordiformis*, more than 1.5 M sorbitol was detected in *Klebsormidium marinum*, and 0.74 M cyclohexanetetrol was measured in *Monochrysis luteri* (Ben-Amotz and Avron, 1983).

The amino acid proline is found as osmotic solute, often in combination with other compounds, in many diatoms (Bacillariophyceae) and green algae. Proline is the main compatible solute in diatoms over the whole extent of their salinity tolerance, from oligohaline to highly hypersaline waters (Liu and Hellebust, 1975; Ben-Amotz and Avron, 1983; Fujii et al., 1995). Increased water stress induced the synthesis of proline in *Cyclotella meneghiniana* (Schobert, 1974). *Stichococcus bacillaris*, a typical aeroterrestrial species from soil, tree bark, or building facades which shows a large salt tolerance, was found to contain 0.28 M proline and 0.52 M sorbitol when grown in 0.7 M salt (Brown and Hellebust, 1978).

In addition to the above-discussed, widely distributed organic solutes, a number of additional compounds have been identified in different groups of algae to contribute to the establishment of osmotic balance. One of these is isethionic acid, first found in *Ceramium flaccidum* (Barrow et al., 1993). In *Gratelouphia doryphora* (Rhodophyceae), isethionic acid is found together with another novel osmotic solute: N-methylmethionine sulfoxide. The concentrations of both compounds were found to increase with salinity (Simon-Colin et al., 2002, 2004). 4-Hydroxyprolinebetaine was first reported in the red alga *Ahnfeltia*

paradoxa (Hori et al., 1979), and was detected in some other species as well (Blunden et al., 1982, 1992).

The list of compounds discussed below is by no means exhaustive, and it is to be expected that systematic surveys of different algal groups may yield additional novel compounds to be added to our list of osmotic solutes used by oxygenic phototrophs.

3.1. DIMETHYLSULFONIOPROPIONATE

Dimethylsulfoniopropionate (DMSP), a zwitterionic compound produced from methionine as a precursor, is widespread as a compatible solute in marine algae (Reed, 1983). Species that accumulate DMSP include macroalgae such as *Ulva* and *Enteromorpha* and unicellular marine bloom-forming algae such as *Phaeocystis*, *Ceratium*, *Cyclococcus*, and *Gyrodinium* (Dickson et al., 1980, 1982; Edwards et al., 1987; Gibson et al., 1990; Bischoff et al., 1994). Osmotically stressed *Ulva* and *Platymonas* appear to increase their DMSP content in reaction to an increased salinity of their environment (Dickson et al., 1980; Kirst, 1990); however, in *Enteromorpha* there was little evidence of salinity-regulated DMSP synthesis (Young et al., 1987). In Antarctic intertidal green macroalgae, on the other hand, the high DMSP concentrations are strongly regulated under changing salinities (Karsten et al., 1991).

In addition to an osmotic function, it has been suggested that DMSP acts as a cryoprotectant. Evidence for this comes from a comparison of the DMSP content of polar macroalgae to temperate and tropical species. Not only did polar species consistently contain higher DMSP concentrations, their content of DMSP increased two- to fourfold when they acclimated to grow at 0°C as compared to 10°C, an effect shown for the Antarctic macroalgal species *Acrosiphonia arcta*, *Enteromorpha bulbosa*, and *Ulothrix subflaccida* (Karsten et al., 1990). The cryoprotective function of DMSP has been experimentally proven (Nishiguchi and Somero, 1992).

The literature about the possible occurrence of DMSP in cyanobacteria is somewhat contradictory. Visscher and van Gemerden (1991) reported between 36.4–38.1 µmol DMSP per gram protein in pure cultures of the benthic marine and hypersaline mat-forming *Microcoleus chthonoplastes*, a species that is otherwise known to produce glucosylglycerol as its osmotic solute (Zavarzin et al., 1993; Oren et al., 1994; Oren, 2000b). Karsten et al. (1996) also list detection of DMSP in *Synechocystis*, *Lynghya*, *Nostoc*, and *Anabaena* isolates. However, concentrations are low, between 0.1 to 6.28 mmol/kg fresh weight, when compared with values of up to 48.7, 63.1, and 290 mmol/kg recorded in temperate, tropical, and polar Chlorophyceae, respectively.

DMSP is the precursor of dimethylsulfide (DMS), a water-soluble gas that diffuses from the marine environment into the atmosphere and has a profound impact on the properties of the atmosphere and the global climate. For that

reason, DMSP and DMS were among the parameters monitored during the experiments in which patches of ocean water were fertilized with iron to encourage algal growth with concomitant scavenging of inorganic carbon in an attempt to eventually reduce atmospheric CO₂ and to decrease the global greenhouse warming. Significant increases in DMSP were recorded during such experiments, followed by an increase in DMS (Turner et al., 1996).

3.2. COMPATIBLE SOLUTES OF ALGAE IN HYPERSALINE ENVIRONMENTS

The most salt-tolerant of all oxygenic phototrophs are species of the unicellular green alga genus *Dunaliella*. *Dunaliella salina*, generally colored red due to a high content of β-carotene, is found worldwide in brine pools, in hypersaline lakes, and in saltern crystallizer ponds. Green *Dunaliella* cells even occasionally produce dense blooms in the Dead Sea when the upper water layers become diluted as a result of rain floods during exceptionally rainy winters (Oren, 2002). At these salinities even the best salt-adapted cyanobacteria are unable to grow: salt concentrations around 200–250 g/l appear to be the upper limit for massive development of cyanobacteria (Oren, 2000b).

Glycerol is the compatible solute of *Dunaliella* (Ben-Amotz and Avron, 1973; Borowitzka and Brown, 1974). To some extent glycerol may be considered the ideal osmotic solute, as it is easy to synthesize, its biosynthesis is energetically less expensive than the production of the other solutes found in other organisms (Oren, 1999), it is only a little inhibitory, if at all, to “nonhalophilic” enzymes, and it can be mixed with water in any desired ratio, so there is no real limit to the concentration at which it can be accumulated inside the cell. Cells of *D. salina* grown at 5 M NaCl were found to contain up to 7 M glycerol, equivalent to a concentration of 644 g/l. The question why this seemingly ideal compatible solute is not used much more extensively by a wide variety of taxonomic groups that have to cope with salt stress will be addressed in the final section of this chapter.

The importance of glycerol in *Dunaliella* was first indicated in 1964 (Craigie and McLachlan, 1964): when *D. tertiolecta* was incubated at increasing NaCl concentrations in the presence of ¹⁴CO₂, and the cell components then fractionated, a dramatic increase was found in the amount of label located in glycerol. Glycerol is produced by reduction of dihydroxyacetone phosphate, an intermediate of the Calvin cycle of autotrophic CO₂ fixation, to glycerol-3-phosphate, which is then dephosphorylated to glycerol. Excess glycerol (e.g., following salt downshock) may be removed by the oxidation to dihydroxyacetone, followed by phosphorylation to form dihydroxyacetone phosphate, which can then be converted into osmotically inactive starch (Ben-Amotz, 1975). The NAD-dependent glycerol-3-phosphate dehydrogenase is located in the chloroplast, while the NADP-specific glycerol dehydrogenase is found in the cytosol. Thus, the cells can adjust their glycerol content by biosynthesis and degradation of the solute. This, together

with the rapid swelling and shrinking of the cells as a reaction to sudden changes in salinity described in the introduction section, allows a high level of flexibility to the cells, enabling adaptation to a wide range of salt concentrations.

Dunaliella is not the only alga that uses glycerol for osmotic balance: high concentrations of glycerol have also been reported in *Asteromonas* (Prasinophyceae), a wall-less unicellular flagellate found in salt marshes and brine ponds, as well as in oceans and brackish water (Ben-Amotz and Grunwald, 1981).

4. Epilogue

As shown in the preceding sections, many types of osmotic solutes have been detected in salt-adapted oxygenic phototrophs, and these belong to different chemical categories: polyols, amino acids and derivatives, sugars, heterosides, and others. We know little why certain compounds are preferred by different organisms. A few generalizations can, however, be made:

1. Phototrophic microorganisms growing at the highest salt concentrations in brines at or approaching saturation accumulate low molecular weight compounds that are highly soluble in water (glycine betaine) or can be mixed with water in any desired ratio (glycerol). At these salinities the larger osmotic solutes such as heterosides, disaccharides, etc. are probably unsuitable as they cannot be accumulated at concentrations sufficient to balance the molar salt concentration in the environment.
2. Glycerol, the simplest of all organic compatible solutes, is found in the eukaryote world only. Brown (1990) has stated that glycerol "may be regarded as God's gift to solute-stressed eukaryotes." The reason why glycerol is not universally used as the ideal osmotic solute for all stressed micro- and macroorganisms should probably be sought in the high permeability of most biological membranes to glycerol. It has been ascertained that *Dunaliella* possesses highly unusual cell membranes that are several orders of magnitude less permeable to glycerol than most other membranes from both prokaryotes and eukaryotes (Brown et al., 1982; Gimmler and Hartung, 1988). The reason for the low glycerol permeability of the *Dunaliella* membranes has never unequivocally been established, but it was suggested that it may be correlated with a high content of sterols in the membrane. With very rare exceptions sterols are lacking in prokaryotic membranes, and this may explain why no prokaryote, photosynthetic or nonphotosynthetic, appears to use this simple compound.
3. Particularly polyols are important stress metabolites, because they exhibit multiple functions in metabolism – osmolytes, compatible solutes, rapidly available respiration substrates (important under desiccation), antioxidant (e.g., mannitol), etc. Therefore, they are mainly produced by algae from extreme environments not only in terms of salinity, but also concerning desiccation (e.g., supralittoral and aeroterrestrial taxa).

4. Most organic compatible solutes in oxygenic phototrophs contain only carbon, oxygen and hydrogen. The marine system is generally nitrogen-limited, and therefore nitrogen containing solutes (amino acids and derivatives) appear to be either found in organisms adapted to low salinity only, or, in the case of glycine betaine, in organisms found predominantly in dense microbial mats in hypersaline systems which are not known to be nitrogen-stressed. On the other hand, seawater contains as much as 23 mM sulfate, and therefore the choice of many marine micro- and macroalgae to use DMSP for osmotic balance (and possibly for other purposes as well) appears to make sense in view of the chemical composition of the environment.

In summary, while we still lack a unifying concept why certain organisms chose certain compounds and what the specific advantages are of each of the many osmotic solutes identified thus far in the world of algae and cyanobacteria, it is clear that these solutes are as varied as the algal world itself. It is even highly probable that more organic osmotic solutes will be identified in the future. The above overview of the distribution and metabolism of organic compatible solutes in oxygenic phototrophs, prokaryotes as well as eukaryotes, shows that the problem of how to cope with elevated salt concentrations can be solved in many different ways.

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PART 7:
ADAPTATION OF ALGAE
TO CHANGING ENVIRONMENTS

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Henley
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Biodata of **Lucas J. Stal**, author of the chapter “*Cyanobacteria: Diversity and Versatility, Clues to Life in Extreme Environments.*”

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CYANOBACTERIA:

Diversity and Versatility, Clues to Life in Extreme Environments

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1. Introduction

Bacteria have inhabited Earth for 3.8 billion years and life on our planet was microbial for 3.2 billion years (Schopf, 1994). During this long period, microorganisms have evolved an incredible diversity, although a major part of this diversity may have already existed in the Archean. Cyanobacteria and, hence, oxygenic photosynthesis evolved 2.7–2.2 billion years ago and had therefore ample time to diversify and adapt to newly evolving niches that emerged on Earth (Schopf et al., 2002; Blank, 2004; Tice and Lowe, 2004). Through the advent of oxygenic photosynthesis (Blankenship, 1992), cyanobacteria were responsible for the oxygenation of the Earth's atmosphere (Buick, 1992), thereby allowing the evolution of plants and animals 0.6 billion years ago and eventually were shaping the present biosphere.

Cyanobacteria combine the fixation of CO₂ and N₂, the two most important biogeochemical processes on Earth. They are globally important primary producers and contribute greatly to the global nitrogen budget (Karl et al., 2002). Cyanobacteria are essential players in the Earth's present and past ecosystems. For any understanding of the evolution of life and of the biogeochemical cycles on Earth, knowledge about the ecology and evolution of the cyanobacteria is a prerequisite.

Cyanobacteria colonized successfully almost any illuminated environment on Earth, many of which are considered to be hostile for life. Cyanobacteria play a prominent role in many of these extreme environments. This chapter attempts to find clues explaining the evolutionary and ecological success of cyanobacteria.

2. Cyanobacteria: Diverse Oxygenic Phototrophic Prokaryotes

Cyanobacteria are the only prokaryotes that perform oxygenic photosynthesis. Cyanobacteria were formerly known as blue-green algae but this term has been abandoned when their prokaryotic nature became apparent (Stanier and van Niel, 1962). As a monophyletic group within the Kingdom *Bacteria*, cyanobacteria are remarkably diverse, both with respect to their morphology as well as to their physiology and metabolism.

Cyanobacteria are also exceptional because some representatives are capable of cell differentiation which is unique among prokaryotes. Many species reveal a remarkable flexibility and adapt to a wide range of environmental conditions, which is attributed to their metabolic versatility (Stal, 1991). We have only begun to unravel the enormous genetic diversity of cyanobacteria, to understand how it is generated and maintained, to realize its importance and how it influences ecosystem function.

2.1. COLORFUL DIVERSITY

The blue-green appearance of cyanobacteria is caused by the two photopigments, chlorophyll *a* and phycocyanin. The phycobiliproteins form structures on the photosynthetic membranes (thylakoids) that are called phycobilisomes which serve as the light-harvesting antennae for photosynthesis. Light harvested by the phycobiliproteins is transferred to the photosynthetic reaction centers and used to drive photosynthetic electron transport.

The harvesting of light is an essential and critical process in the vast majority of cyanobacteria, and phycobiliproteins play therefore a central role. Cyanobacteria have gone through a tremendous diversification with respect to their light-harvesting properties, and the term “blue-green bacteria” does not give justice to the plethora of colors that characterizes this group of organisms. The array of different colors allows the occupation of a range of different niches by different pigmented cyanobacteria and allowing their coexistence in the same habitat (Stomp et al., 2004).

There are basically three types of phycobiliproteins: allophycocyanin, phycocyanin and phycoerythrin, which are organized in stacks that form the phycobilisome (Grossman et al., 1993). Allophycocyanin (absorption maximum 650 nm) forms the basis of these stacks and is present in all phycobilisomes, albeit in low amounts. Phycocyanin (absorption maximum 620 nm) is linked to allophycocyanin. The chromophore of phycocyanin is the blue phycocyanobilin (PCB). In one case, phycocyanin has been shown to carry an additional phycoeruthrobilin (PEB) chromophore, which otherwise is only found in phycoerythrin (absorption maximum 565 nm) (Swanson et al., 1991). The red chromophores of phycoerythrin are PEB and phycoerythrobilin (PEB). Phycocyanin can be largely replaced by phycoerythrin, causing cyanobacteria to appear red. Cyanobacteria that contain both pigments in equal amounts may appear brownish or almost black. Some cyanobacteria are capable of complementary chromatic adaptation and can actively change the proportion of phycocyanin and phycoerythrin, depending on the spectral quality of the light that they receive (Tandeau de Marsac, 1977). They make more of the blue phycocyanin when exposed to red light and more of the red phycoerythrin when exposed to green light. Phycoerythrocyanin (absorption maximum 570 nm) is another phycobiliprotein that has been detected in a few cyanobacteria but is comparably rare and contains an unusual chromophore (Bryant et al., 1976).

Phycoerythrins exhibit a considerable amount of diversity. They contain either PUB and PEB, or only PEB. Depending on the chromophore composition, phycoerythrin possesses different spectral properties. Moreover, the relative amounts of PUB and PEB may vary in order to optimize light harvesting. Phycoerythrin containing a high proportion of PUB would be better adapted to blue light, such as is present in the open ocean (Palenik, 2001), while PEB-rich cyanobacteria are better adapted to the light that prevails in coastal waters. This type of chromatic adaptation is fundamentally different from the complementary chromatic adaptation mentioned earlier. Chromatic changes in cyanobacteria may be controlled by plant type phytochromes that act as photoreceptors and sense changes in the prevailing light climate (Kehoe and Grossman, 1996). The PUB chromophore transfers the light energy to PEB (Swanson et al., 1991), although in the oceanic diazotrophic cyanobacterium *Trichodesmium* this transfer is inefficient and it has been suggested that the PUB:PEB ratio serves as a switch that modulates the energy transfer to photosystem II, thereby protecting nitrogenase from (photosynthetic) O₂ inactivation (Subramaniam et al., 1999).

Some cyanobacteria (*Prochlorococcus*, *Prochloron*, *Prochlorothrix*) lack phycobilisomes and phycobiliproteins and possess a quite different pigment composition. The unicellular *Prochloron didemni* (Lewin and Withers, 1975) and the filamentous cyanobacterium *Prochlorothrix hollandica* (Burger-Wiersma et al., 1989) contain chlorophyll *a* and *b*. *Prochlorococcus* contains divinyl derivatives of chlorophyll *a* and *b* (Goericke and Repeta, 1992). *Prochlorococcus* are tiny unicellular cyanobacteria known as the most abundant phototrophic organism in the ocean (Chisholm et al., 1988). Some strains of *Prochlorococcus* may produce a tiny amount of phycoerythrin (Hess et al., 1996). The function of this phycoerythrin is not clear but it could be involved in light harvesting although it has also been proposed that it could serve as a photoreceptor (Mullineaux, 2001).

The phycocyanins of thermophilic cyanobacteria may be different from those of mesophilic organisms (Samsonoff and MacColl, 2001). Phycocyanins of thermophilic cyanobacteria resist thermal denaturation better than the pigments from mesophilic strains. The phycocyanin of the thermophilic *Synechococcus lividus* has a blue shifted absorption spectrum (absorption maximum 608 nm instead 620 nm). The shifted absorption maximum might be the consequence of the structural changes of the protein that renders it a higher thermostability. Thermophilic cyanobacteria with lower temperature optima such as *Oscillatoria terebriformis* contain phycoerythrin. The blue shift in the absorption spectrum may therefore be a partial compensation for the lack of phycoerythrin in *S. lividus*.

2.2. SIZE AND SHAPE MATTERS

Cyanobacteria exhibit a remarkable morphological diversity that has always been the basis for the taxonomy of this group of organisms. On the basis of their morphology and cell division characteristics, cyanobacteria are subdivided into five subsections that are largely supported by the phylogeny of the 16S rRNA gene

(Wilmotte and Herdman, 2001). The unicellular cyanobacteria are grouped in the subsections I and II. The unicellular *Gloeobacter violaceus* is a special case because it is the only cyanobacterium known that lacks thylakoids (Rippka et al., 1974) and roots phylogenetically deeply with subsection I. Cyanobacteria in subsection I divide by binary fission while those in subsection II can also undergo multiple divisions. Cyanobacteria belonging to subsection II can form a large number of very small daughter cells, baeocytes, which subsequently grow out to normal-sized cells. The filamentous cyanobacteria are grouped in subsections III–V. Subsection III comprises all filamentous cyanobacteria that are composed of one cell type (Oscillatoriales). The subsections IV and V comprise filamentous cyanobacteria that exhibit cell differentiation, a rare phenomenon among prokaryotes. All cyanobacteria belonging to subsections IV and V are capable of fixing atmospheric N₂. The fixation of N₂ takes place in special differentiated cells, heterocysts, which comprise 5–10% of the cells. Heterocysts do not grow or divide. Many species belonging to these subsections also differentiate akinetes, cells that are capable of surviving extended periods of desiccation, cold, nutrient deprivation and other unfavorable conditions (Adams and Duggan, 1999) and hormogonia, short motile non-heterocystous trichomes with tiny cells that aid in the dispersal of the organism. Akinetes germinate when conditions allow the proliferation of the cyanobacterium. It is not precisely known for how long akinetes can survive, but viability after several decades in desiccated condition has been reported. The cyanobacteria of subsection V are distinguished from IV by true branching of the trichomes, resulting from the division of cells in more than one plane.

The cell size of cyanobacteria covers a range of two orders of magnitude. The unicellular picocyanobacteria (*Prochlorococcus* and *Synechococcus*) are among the smallest cyanobacteria. *Prochlorococcus* have cell diameters smaller than 0.5 µm (Chisholm et al., 1988). Picocyanobacteria are usually defined as organisms with cell diameter <2 µm but in the practice this size limitation is artificial and not always in agreement with 16S rRNA gene phylogeny, and organisms with larger cell size are conveniently also grouped with the picocyanobacteria. These organisms are abundant in the plankton. *Prochlorococcus* occurs only in oligotrophic marine environments (oceans, Mediterranean Sea, Red Sea), while *Synechococcus* are found both in freshwater and marine habitats and also in nutrient-rich environments.

Picocyanobacteria have also been found in the benthic environment of a hypersaline microbial mat (Abed et al., 2002). These uncultivated “picobenthic” cyanobacteria are 0.75–1 µm and occur in *Gloeocapsa*-like colonies embedded in mucilaginous investments but seem to be not closely related to any other cyanobacteria. To date it is unknown how widespread picobenthic cyanobacteria are and what their function is in microbial mats.

Unicellular cyanobacteria with cell diameters of more than 30 µm exist. Cyanobacteria of subsection II (Pleurocapsales) such as *Dermocarpa* and *Hyella* can reach very large cell sizes, particularly when they are about to divide multiple

times to produce baeocytes. These tiny cells could be confused by microscopic observations for picocyanobacteria.

Picocyanobacteria have small genomes and are specialists. Particularly the smallest of the picocyanobacteria, *Prochlorococcus*, with genome sizes varying from 1.6 to 2.4 Mbp, are tuned to the specific environmental conditions in the habitats where they thrive, whereas the slightly larger *Synechococcus* spp. may be more versatile. An important advantage of the small cell size is the large surface to volume ratio increasing the affinity for nutrients and can be seen as an adaptation for living in low-nutrient environments.

Many cyanobacteria show an incredibly high level of organization, and both unicellular and filamentous species show features of multicellular behavior. The subsection II euendolithic (boring) cyanobacterium *Hyella* forms very complex and large structures with pseudofilaments inside coastal carbonate limestone and dolomites as well as in ooid sand grains (Lukas and Golubic, 1983). The function of this complex multicellular structure is unclear, but one could speculate that it could serve the contact of the innermost cells with the environment outside the rock. For instance, baeocytes are produced and released outside the pit, ensuring the dispersal of the organism. *Hyella* has very well-preserved Precambrian (Meso-Proterozoic) fossil counterparts with identical morphology.

Merismopedia is characterized by well-organized platelet-like colonies containing 8–64 cells, related to single-celled *Synechocystis* (Palinska et al., 1996). Although in culture the property of platelet forming is often lost, it seems an important and stable phenomenon in natural environments. The colonies are produced by binary fission of the cells in two planes at right angles to each other. *Eucapsis* is a genus that differs by cell division in three planes and forms cubic aggregates. *Merismopedia* occurs frequently in benthic environments, particularly in microbial mats. The formation of platelet colonies may have ecological advantages. The platelets position themselves actively with respect to the direction of the light so that they receive more or less light. The related genus *Microcystis* is a unicellular colony-forming planktonic cyanobacterium. Cell division in this organism is in three planes forming large irregular colonies. *Microcystis* possesses gas vesicles that make them buoyant. When this organism is mixed deep into the water column it will float to the surface. This strategy is advantageous because it will allow the organism to receive more light than those that are lacking gas vesicles, even when the synthesis of gas vesicles comes at a considerable metabolic cost (Walsby, 1994). The speed of floatation is a function of the cell (colony) size. It would take the small single cells of *Microcystis* much longer to migrate a certain distance in the water column than the colonies that contain several 10s to 100s of cells. In addition, the size of the colonies may also protect the organism from grazing. The same advantage could be attributed to aggregates of picocyanobacteria although this may be the result of the copious production of extracellular polymeric substances (EPS) that stick the cells together. EPS exudation is often triggered by unbalanced growth caused by nutrient (nitrogen) deficiency. Other unicellular cyanobacteria that form well-structured colonies

include *Gloeocapsa* and *Gloeothece*. The former is often found in microbial mats while the latter forms biofilms on carbonate cave walls. The formation of aggregates with the cells embedded in a mucilaginous matrix may be an important adaptation for living in such benthic or terrestrial environments. The EPS matrix may retain water and allow the organism to survive periods of dryness (Potts, 1994). Also, EPS bind nutrients, immobilize toxic compounds, protect to high irradiances and may have a variety of other benefits for the organism.

Filamentous cyanobacteria range also almost two orders of magnitude in trichome width that varies from less than 1 μm to more than 50 μm . Among the thinnest cyanobacteria are *Halomicronema*, halophilic, moderate thermophilic cyanobacteria, *Pseudanabaena*, known from pelagic and benthic habitats, *Schizothrix* from calcifying marine microbial mats and other, *Phormidium*-like, cyanobacteria. *Lyngbya* are among the largest cyanobacteria and specimens of 50 μm width have been reported. These cyanobacteria occur mostly as benthic organisms, forming microbial mats in a wide range of coastal sediments. They have also been reported as epiphytes and attached to corals. Trichomes of such large cyanobacteria can be several millimeters or even centimeters in length. Many *Lyngbya* possess very thick, multilayered and well-structured mucilaginous investments. *Microcoleus chthonoplastes* is a cosmopolitan mat-forming cyanobacterium that is characterized by the formation of bundles that are enclosed by well-structured polysaccharide sheath (Garcia-Pichel et al., 1996). One bundle may contain from several up to almost 100 individual trichomes and those large bundles may be compartmentalized. The function of these *Microcoleus* bundles is not clear and in culture this property is often lost. Most likely these bundles provide a microenvironment that retains water and nutrients and protects the organism from desiccation and high light. In a number of cases, filamentous anoxygenic phototrophic bacteria *Chloroflexus* have been found inside the bundle (Ley et al., 2006). The role of these bacteria, whether beneficial or not, is not precisely known. The individual trichomes of *M. chthonoplastes* are motile by gliding movement and it has been observed that they glide out of the common sheath. Whether they can actively move into an existing bundle is not known. Other examples of cyanobacteria that form conspicuous structures include *Rivularia*, subsection IV cyanobacteria that form calcified benthic structures in coastal marine and lotic environments (Whitton, 1987). Terrestrial *Nostoc* is another example of subsection IV cyanobacteria with macroscopic structures that allow them to live and survive under extreme conditions in arid environments (Mollenhauer et al., 1999).

Many planktonic filamentous cyanobacteria organize themselves into colonies or sheaths. This is the case for oceanic N₂-fixing cyanobacterium *Trichodesmium* that is found as “puffs” (round aggregates) or “tufts” (elongated aggregates) (Davis and McGillicuddy Jr., 2006), and similar aggregates are produced by the brackish heterocystous cyanobacterium *Nodularia* and the freshwater *Aphanizomenon* (Walsby et al., 1997). The function of these aggregates is similar as described earlier for the unicellular *Microcystis*.

2.3. METABOLIC VERSATILITY, FLEXIBILITY AND REACTIVITY

The success of cyanobacteria in many past and modern environments can be attributed to a large extent to their metabolic versatility, flexibility and reactivity. Cyanobacteria have a choice of different metabolic pathways and physiologies. They have a wide range of tolerances of environmental conditions that make them flexible with respect to their responses. Important is that cyanobacteria may be very reactive, responding instantaneously to changing conditions. Cyanobacteria have answers to each of the six combinations of environmental conditions of light, dark, aerobic and anaerobic (Stal, 1995).

Cyanobacteria are phototrophs, and in illuminated environments they have access to an easy and available energy source. They use water as an electron donor, which is in aquatic environments also not a limiting factor. Cyanobacteria are autotrophic, that is they use CO_2 as carbon source. Under conditions that prevent oxygenic photosynthesis, many species can switch to an anoxygenic mode of photosynthesis using sulfide as the electron donor (Cohen et al., 1986; Garcia-Pichel and Castenholz, 1990). This is for instance the case when in hot springs or other types of sulfureta high concentrations of sulfide inhibit photosystem II activity. There is also some evidence that ferrous iron can be used as electron donor in anoxygenic photosynthesis in cyanobacteria. Whether the switch to anoxygenic photosynthesis needs induction or not is strain specific and depends on the particular environmental conditions.

In the dark, cyanobacteria switch instantaneously to aerobic respiration, using intracellular storage carbohydrate and oxygen as terminal electron acceptor. Under anaerobic conditions some cyanobacteria are capable of fermentative metabolism. The intracellular storage carbohydrate is fermented via a variety of pathways, depending on the species. They can optimize the energy yield by using elemental sulfur (producing sulfide) (and perhaps ferric iron, producing ferrous iron) or protons (producing hydrogen gas) as electron sinks. Enzymes for fermentation are constitutively expressed so that the switch to fermentation can be instantaneous (Stal and Moezelaar, 1997).

For dark metabolism, cyanobacteria are not necessarily dependent on the limited storage carbohydrates. Several species have been shown to be able to exhibit heterotrophic growth on a number of organic compounds. Moreover, many more species are capable of uptake of organic compounds when light is available. This mode is called photoheterotrophic growth.

Cyanobacteria produce a variety of different storage compounds that allow them to survive often prolonged conditions of starvation. Glycogen is a glucose polymer and the main energy and carbon storage. It is synthesized from photosynthate in the light and mobilized in the dark, either for aerobic respiration when oxygen is present or it is fermented. The amount of glycogen can be considerable when growth is unbalanced, for example under nitrogen limitation. Some cyanobacteria also accumulate polyhydroxyalkanoates (PHAs), mostly polyhydroxybutyrate (PHB) (Stal, 1992). The function of these typical bacterial reserve

compounds is not completely clear but it has been suggested that they are used as typical carbon storage or as redox buffer. Cyanophycin is a non-ribosomal polypeptide with an aspartic acid backbone and side chains of arginine. Both amino acids occur in equimolar amounts in this storage polymer. The function of cyanophycin is probably nitrogen storage, although energy storage has also been proposed (Smith, 1982). Phycobiliproteins may also serve as storage for nitrogen. Phycobiliproteins make up an important part of the total cell protein (as much as 50%). When cyanobacteria become nitrogen (or sulfur) limited there first response is to degrade to a great extent their phycobiliproteins (bleaching). It has been proposed that the function of phycoerythrin in a marine *Synechococcus* was mainly nitrogen storage (Wyman et al., 1985). Finally, polyphosphate is accumulated by many cyanobacteria when phosphate is in excess and used as storage (Healey, 1982).

A very important property of many cyanobacteria is the capacity of the fixation of atmospheric dinitrogen (N_2). Nitrogen is an essential element for the synthesis of biomass. It is incorporated in amino acids, the building stones of proteins and other polypeptides, nucleic acids, cell wall components and in chlorophyll. The atmosphere contains 78% N_2 and this is the most abundant form of nitrogen on Earth. However, it is unavailable for most organisms except for specialized prokaryotes that are capable of synthesizing and maintaining nitrogenase. This enzyme is sensitive to oxygen and therefore nitrogenase can only function in an anaerobic environment. Many cyanobacteria are capable of N_2 fixation but this seems contradictory with the oxygenic nature of cyanobacteria. Diazotrophic (N_2 -fixing) cyanobacteria have to cope with O_2 in the surrounding medium but even more with the intracellular photosynthetically produced O_2 (Gallon, 1992).

Cyanobacteria have developed various strategies to cope with the problem of the incompatibility of photosynthesis and N_2 fixation, contributing further to their diversity. Roughly 50% of the cyanobacteria are unable to fix N_2 . It is not clear whether they have lost this capacity or whether the diazotrophic cyanobacteria have acquired it later. Non-diazotrophic cyanobacteria do not contain nitrogenase genes. Many potential diazotrophic cyanobacteria express nitrogenase and fix N_2 only under anaerobic conditions when oxygenic photosynthesis is inhibited. Examples of such conditions include sulfureta where sulfide inhibits photosystem II, or in the anoxic lower part of microbial mats where only far red light is available, exciting only photosystem I. This strategy has been called “avoidance.”

The most conspicuous adaptation is represented by the heterocystous cyanobacteria (subsections IV and V). These organisms have separated the incompatible processes of N_2 fixation and oxygenic photosynthesis in two different cell types, the heterocyst and the vegetative cell, respectively. Heterocysts have lost the capacity of oxygenic photosynthesis and CO_2 fixation, but have retained photosystem I (Adams, 2000; Golden and Yoon, 2003). Heterocysts possess a thick glycolipid cell envelope that serves as a gas diffusion barrier (Walsby, 1985). Heterocysts provide a virtually anaerobic environment for the oxygen-sensitive

nitrogenase. This strategy is also known as “spatial separation” and these cyanobacteria fix N₂ in the light simultaneous with photosynthesis. Heterocystous cyanobacteria are also capable of dark N₂ fixation, albeit usually at lower rates. There is a considerable diversity among heterocystous cyanobacteria. Apart from an astonishing large morphological diversity, heterocysts have for instance different cell envelopes which make them more or less adapted to ambient O₂ levels, temperature and salinity. Heterocysts may also actively adapt their cell envelope in response to changing environmental conditions. In addition, heterocysts may chromatically adapt for optimal light harvesting for N₂ fixation (Staal et al., 2003b). The location of the heterocyst in the trichome (terminal and/or intercalary), its frequency, size and shape are species specific. Almost certainly all of these morphological differences have important ecological functions, albeit in most cases they have not been experimentally identified. Heterocystous cyanobacteria are known from freshwater, brackish and terrestrial environments and as symbionts with a variety of other organisms. Remarkably, free-living heterocystous cyanobacteria are absent from the marine plankton and rare in marine microbial mats. The reason for this is unclear.

There are a few unicellular and filamentous cyanobacteria that are capable of aerobic N₂ fixation (Bergman et al., 1997). The majority of these strains would fix N₂ during the night when photosynthesis does not take place. This strategy is known as “temporal separation.” This strategy is the least understood. When these cyanobacteria are grown in the laboratory under alternating light–dark cycles they usually confine N₂ fixation to the dark phase. However, all of the known organisms in this group are capable of diazotrophic growth under continuous light, and under certain conditions N₂ fixation can be forced to the light phase of a light–dark grown culture (Ortega-Calvo and Stal, 1991). Moreover, when the dark phase is very short a considerable part of N₂ fixation occurs during the light. The many different daily patterns of N₂ fixation reported from cultures and natural environments suggest that this group of cyanobacteria is diverse, utilizing a variety of delicate tuned adaptations.

Trichodesmium is a non-heterocystous, filamentous diazotrophic cyanobacterium and an important organism in the tropical oceans. It is different from other non-heterocystous cyanobacteria because it fixes N₂ during the day and it resembles in this respect the heterocystous cyanobacteria (Church et al., 2005). Nitrogenase is present in a subset of cells in the trichome, and in analogy with heterocysts these cells have been termed “diazocytes” (Fredriksson and Bergman, 1997). However, it is uncertain whether diazocytes are terminally differentiated cells like the heterocysts. More likely, these cells turn temporally into N₂-fixing cells and therefore this strategy has been considered as a combination of spatial and temporal separation (Berman-Frank et al., 2001). Contrary to heterocystous cyanobacteria, *Trichodesmium* does not fix N₂ during the night. Nitrogenase synthesis starts at the end of the night until midday. Thereafter, nitrogenase starts to decline and is rapidly and irreversibly inactivated in the dark. The N₂-fixing cells are kept anoxic by a high rate of respiration combined with a low dissolved O₂ in

the warm ocean water. This limits the geographic distribution of *Trichodesmium* to oceanic areas with water temperatures above 25°C. Under such conditions a heterocyst is not required (Staal et al., 2003a). There is perhaps another diazotrophic cyanobacterium active in the tropical ocean that potentially fixes N₂ during the day. This putative unicellular cyanobacterium is only known by its *nifH* sequence and designated as “Group A” (Church et al., 2005).

Many but not all cyanobacteria are motile. The vast majority of motile cyanobacteria move by gliding movement (Häder, 1987). Gliding movement is confined to filamentous cyanobacteria that move through self-propulsion over a solid substrate or along other trichomes. In *Oscillatoria* and *Spirulina*, gliding is accompanied by the rotation of the trichome along its axis. Heterocystous cyanobacteria are usually immotile but they produce motile hormogonia. Baeocytes are also motile and have the same function as hormogonia in filamentous species (Al-Thukair and Golubic, 1991). A marine unicellular *Synechococcus* has been described that is motile by a swimming mode, although it lacks flagella (Waterbury et al., 1985). This mode of motility is poorly understood and its function is obscure (Brahamsha, 1996).

Cyanobacteria produce a large variety of secondary metabolites, some of which are toxic for higher organisms. The ecological function of these secondary metabolites is in most cases unknown. However, some of these secondary metabolites may find important applications in medicine.

3. Diversity and Ecosystem Function

In recent years the idea emerged that diversity is important for ecosystem function but research that aims at elucidating the mechanisms is scarce, particularly in the case of cyanobacteria-dominated ecosystems. Likewise, very little is known about the mechanisms that generate and maintain diversity in a fluctuating ecosystem. Any ecosystem will experience changes in the long or in the short term. Due to the short generation times of many microorganisms, changes in environmental conditions will affect the function of microbial ecosystems. Such ecosystem fluctuations may occur regularly at a seasonal or even on a daily basis as well as episodically or erratically. Intuitively, one would expect that an ecosystem that possesses higher species richness would be more resistant towards perturbations. When the environmental conditions of an ecosystem change and result in a lesser function of a certain organism, another type or species that carries out the same ecosystem function but that is better adapted to the new situation will take over. By maintaining a certain redundancy in the diversity, the ecosystem insures itself for the continuous and optimized performance of all its functions, together which defines that particular ecosystem. The insurance hypothesis (Yachi and Loreau, 1999) is defined as “any long-term effects of biodiversity that contribute to maintain or enhance ecosystem function in the face of environmental fluctuations.” The insurance hypothesis predicts that a more diverse community is better able to resist an external stress (Boles et al., 2004).

The question of how diversity is generated and maintained in a microbial community is difficult to answer. Point mutations lead to the inactivation, modification or change in regulation of genes and generate diversification on an evolutionary timescale. The vast majority of these mutations are either neutral (do not lead to a change in function) or purifying (are lethal and are therefore eliminated from the population). In addition, gene duplication may result in a new function of the duplicated and initially presumably redundant gene. Eventually, these mechanisms lead to microdiversity and to the appearance of organisms that might perform better than its ancestor, but it is unlikely that it would give an organism the ability to gain completely new metabolic capabilities, necessary to exploit other environments. In order to achieve this, organisms have to acquire genetic material from another by lateral gene transfer and recombination of genetic information. There are basically three mechanisms for lateral gene transfer: transformation (uptake and recombination of environmental DNA), transduction (transfer of DNA through a virus) and conjugation (transfer of DNA through physical contact of two cells). These mechanisms result in both the acquisition and loss of genes and counterbalance these processes rather than accumulating genetic information, although some organisms specialize and decrease the size of the genome (e.g., in the case of picocyanobacteria in the oceans), while others are generalists with large genomes (such as the filamentous diazotrophic cyanobacterium *Lyngbya aestuaria* in coastal microbial mats). These processes obscure the phylogeny of microorganisms, and make it difficult to delimit species. Even when the sequence of the ribosomal gene is identical, bacteria may have highly divergent genomes and ecophysiologies (Jaspers and Overmann, 2004).

Another explanation for the distribution and exploitation of new (and extreme) environments is the axiom that “everything is everywhere, but the environment selects” (Baas-Becking, 1934). Microorganisms are always in large numbers and are so small that they are easily transported and their distribution is therefore unlikely to be restricted by geographic boundaries (Finlay, 2002). However, extreme environments require specific adaptations from the microorganisms that occupy these environments that may prevent them from living under other conditions. Because extreme environments are often distant and isolated from each other, microorganisms in these environments are unlikely to escape from there and will evolve in these discontinuous environments. Thermophilic cyanobacteria with a growth optimum at 70°C often don't grow below 50°C and are unlikely to be able to survive longer periods at lower temperatures (Allewalt et al., 2006). Recent analyses of the genetic diversity of microbial communities in extreme environments have demonstrated that each of these were inhabited by specific genotypes that did not occur elsewhere (Foti et al., 2006) and similar biogeographic distributions have been observed for marine bacteria (López-López et al., 2005). Many sea-ice bacteria have maximum growth temperatures of below 10°C and will die above that temperature (Staley and Gosink, 1999). Hence, there are limited possibilities for the dispersion of such bacteria, although it could be possible that they are transported between the poles through deep water currents. Although members of the same genera are found at both poles, cosmopolitan

species of sea-ice bacteria have not been identified. This does not necessarily mean that they are not there. A huge diversity may be present in any ecosystem but at such low frequency that we cannot access it with the current methodologies (Pedrós-Alió, 2006). However, this does not explain how this seed bank of hidden diversity survives extreme conditions and remain viable, unless all this diversity may enter a state of dormancy or produce resting cells such as spores that prevent them from dying.

There are also examples of the cosmopolitan distribution of microorganisms. This is, among others, the case with the mat-forming cyanobacterium *M. chthonoplastes* (Garcia-Pichel et al., 1996), with some extreme halophilic cyanobacteria (Garcia-Pichel et al., 1998) as well as with some hot spring strains (Ward et al., 1998). Are these cyanobacteria being distributed and transported crossing the borders of these discontinuous environments or have they always been there and adapted optimally to their environment, eliminating the necessity of further evolution? It has been proposed that cyanobacteria are “living fossils” and have evolved very slowly since they became abundant during the Archean (hypobradytic) (Schopf, 1994).

Do cyanobacterial communities insure themselves against environmental stress and ecosystem change by maintaining a redundant diversity? Many cyanobacterial communities are composed of several species that can readily be distinguished on the basis of their morphological characteristics. Basically, all cyanobacteria fulfill the same ecosystem function: they are photoautotrophs. Nevertheless one can distinguish different functional groups within the cyanobacteria that are not redundant. For instance, some cyanobacteria are diazotrophs while others are not. Some cyanobacteria perform anoxygenic photosynthesis or are capable of fermentation others are not. Other non-redundant functions include pigmentation and other differences in physiological and metabolic capacities. Undoubtedly, there remains a certain redundancy among the functional groups with subtle adaptations and differences in the performance of specific tasks.

The conditions in microbial ecosystems that are exposed to daily and seasonal variations are rarely constant due to the changes in light, temperature, water potential, salinity and tidal inundation. The flexibility and reactivity of microorganisms as well as their functional redundancy will overcome short-term changes in ecosystem conditions but for long-term changes such as those caused by different seasons the whole community structure may alter and functional groups may disappear and others will appear. The generally large population size and short generation times of microorganisms involves their potential to genetically adapt to long-term changes.

4. Extreme Environments

Extreme environments are at the edge of habitability and the organisms that inhabit them are termed “extremophiles.” Life depends on the availability of liquid water and conditions that ascertain the stability of macromolecules such

as polypeptides and nucleic acids. Typical examples of extreme environments include those that are exceptionally dry, hot, cold, salty, acid or alkaline. However, whether environmental conditions are really extreme depends on one's point of view. For organisms living in extreme environments conditions may not be experienced as such. These organisms are usually well adapted to the conditions and would therefore experience a temperate environment as extreme and would probably die. Observed from that point of view, extremophiles do not exist because they will either not grow or perform poorly and therefore they would be outcompeted by organisms that are better adapted to these extreme conditions.

Life may have originated from hot springs (Hartman, 1998) but eventually it moved to environments with lower temperatures. Eventually, life colonized every habitable place on Earth. Of course these changes were not abrupt and allowed sufficient time for life to adapt. The oxygenation of the atmosphere at the end of the Proterozoic can be considered as the most dramatic global change event in the history of Earth. O₂ is a hazardous gas and toxic to all life unless it has developed detoxifying mechanisms. O₂ forms oxygen radicals that cause oxidative stress and cause mutations in DNA. During the Archean most environments were anoxic and organisms were obligate anaerobes for which an aerobic environment became an extreme environment. O₂ is also a powerful oxidant allowing the energetically highly efficient aerobic respiration. Aerobic respiratory and photosynthetic electron transport chains are sites where oxygen radicals are formed. Aerobic organisms evolved with efficient mechanisms to protect themselves from the toxic O₂ and that became dependent on it. For these organisms anaerobic environments became extreme environments. The possibility of using O₂ as an electron acceptor for the highly efficient aerobic respiration has led to an explosive evolution of life on Earth during the Phanerozoic. Possibly, oxygen radicals have increased the rate of evolution through their mutagenic activity. Hence, life as it evolved on Earth with all its biodiversity and adaptations to a plethora of environmental conditions, might not have been possible without environmental factors that promote DNA damage and mutations.

The temperature limits of life are roughly between 0 and 120°C. The pH limits of life are 1–12. Salinity does not exclude life and this is possible from freshwater to saturated salt. Liquid water must be available but some organisms can survive prolonged periods of time completely desiccated. Cyanobacteria are known to inhabit hot and cold deserts and other extreme arid environments. These organisms survive prolonged periods of complete desiccation. Although cyanobacteria occupy various extreme environments, they are not the most extreme extremophiles. The highest temperature at which cyanobacteria may grow is slightly above 70°C (Ward et al., 1998) and the maximum salinity is about 25% (Garcia-Pichel et al., 1998). The lower pH limit is close to 6 but they tolerate very alkaline conditions of pH 11 (Gerasimenko et al., 2003, Kazmierczak and Kempe, 2004). Apart from these “traditional” extreme environments, other environments exist that are extreme and exclude therefore many organisms. Such

environments include environmental conditions that fluctuate strongly; such is the case with intertidal microbial mats, environments with high levels of toxic compounds such as sulfide (sulfureta), such that are exposed to very high light and UV irradiances or polluted environments (e.g., oil or heavy metals).

Cyanobacteria in coastal microbial mats have adapted in various ways to the extreme fluctuations in these environments (Stal, 2001). They are generalists and possess a wide range of metabolic capabilities. With their pigments and gliding motility, mat-forming cyanobacteria can position themselves optimally in the light gradient. In many cases mats are perennial, showing the limited abilities of these cyanobacteria to cope with too large changes in the environmental conditions.

Cyanobacteria adapt to the salinity of the environment by the synthesis and accumulation of compatible solutes. The type of compatible solute determines the upper salinity limit which can be tolerated by the cyanobacteria. At increasing environmental salinities cells tend to lose their turgor pressure because water will pass through the cytoplasmic membrane out the cell. In order to maintain its turgor pressure, the cell needs to increase the concentration of osmotic active compounds. Upon a sudden increase in salinity a series of processes will be activated. In most organisms Na^+ ion is actively extruded, which is exchanged by K^+ . Subsequently, the cell starts synthesizing low-molecular weight organic compounds that are compatible with the cell's metabolism. These compounds are therefore also called "compatible solutes" (Welsh, 2000). Cyanobacteria accumulate a variety of different compounds, depending on their salinity tolerance which are synthesized upon salinity stress. Freshwater, salt-tolerant cyanobacteria accumulate small carbohydrates such as sucrose and trehalose. Marine and halotolerant cyanobacteria produce glucosyl-glycerol as compatible solute and extremely halotolerant cyanobacteria use the quaternary ammonium compound glycine betaine, which can either be synthesized or taken up from the medium (Moore et al., 1987). To this latter group belongs *Halothecae* that may have evolved only once from unicellular cyanobacteria by obtaining the property of the synthesis of glycine betaine as osmolyte. Glycine betaine occurs only in the most extreme halotolerant cyanobacteria and is required for growth at the highest salinities (Garcia-Pichel et al., 1998).

Cyanobacteria of the genus *Chroococcidiopsis* are known from hot and cold deserts and other extreme arid environments. These organisms are capable of surviving prolonged periods of extreme desiccation or frozen. Cyanobacteria belonging to this genus are extremely resistant to X-ray irradiation (Billi et al., 2000). The basis of the resistance against X-ray irradiation was the capability of *Chroococcidiopsis* to very effectively and rapidly repair DNA damage. To date, very little is known about the molecular mechanisms that underpin the capability of organisms to withstand desiccation (Billi and Potts, 2002). Certainly, the thick multilayered extracellular sheaths of these organisms are important as the hydrated polysaccharides may serve as a water buffer and protect the organisms from too rapid and complete dehydration (Ophir and Gutnick, 1994). A possible

new type of taxis has been proposed for cyanobacteria that track water thereby avoiding desiccation (Garcia-Pichel and Pringault, 2001).

The upper temperature limit at which cyanobacteria in hot springs grow is perhaps a little over 70°C (Ward et al., 1998). It has been shown that different isolates occupy well-defined niches in the temperature range of 50–72°C (Allewalt et al., 2006). Strains occurring at a certain temperature appear to be better adapted to that temperature and show higher growth rates than other isolates. The photosynthetic apparatus is probably most sensitive to high temperature, but the physiological background of temperature adaptation in thermophilic cyanobacteria is unknown.

Light is obviously essential for phototrophic organisms but at very high intensities an unbalanced absorption and utilization of the energy may occur. For instance, exposure to full sunlight at midday may cause over-excitation of the photosynthetic apparatus and cause damage to it as well as to other cell components. Triplet chlorophyll molecules and oxygen radicals will cause photoinhibition and lead to oxidative damage. This is especially the case when the light includes UV wavelengths. These high-energy short wavelengths are deleterious and lead to oxidative stress, DNA damage and mutations. Since any phototrophic organism may be exposed temporally to high light conditions and UV stress, protective mechanisms must be present. Phototrophic microorganisms have evolved a variety of different mechanisms to acclimate to high light conditions. Motile cyanobacteria might use phototaxis as a behavior to avoid exposure to high light intensities. Photophobic responses help the organism to reverse the direction of movement and migrate away from it. In case of longer-term exposure to high light, microorganisms may produce photoprotective pigments and/or alter the composition and activity of the photosynthetic apparatus. Repair mechanisms are activated in case any photooxidative damage occurred. Cells sense high light using photoreceptors such as phytochromes that trigger alterations in the gene expression profiles (Mullineaux, 2001). Phytochromes are biliproteins that sense light quality, quantity and direction, have been found in cyanobacteria and probably evolved early in the evolution (Montgomery and Lagarias, 2002). Cyanobacteria produce small polypeptides known as “high-light-inducible proteins” (HLIPs) as a response to high light (He et al., 2001; Salem and van Waasbergen, 2004). HLIPs show sequence similarity to “early light-inducible proteins” (ELIP), found in higher plants. Although it is clear that HLIPs are important in the protection of phototrophic microorganisms, their precise function is not known.

5. Summary

Cyanobacteria are oxygenic phototrophic prokaryotes that appeared on Earth 2.7–2.2 billion years ago and they were responsible for the oxygenation of the Earth's atmosphere that eventually triggered an explosion in the evolution of life.

As judged from microfossils, the morphology of cyanobacteria has changed little from the moment that they appeared. This has been taken as evidence for the success of cyanobacteria in occupying available habitats and niches. Cyanobacteria are among the most important primary producers and are the only oxygenic phototrophs that combine the fixation of CO_2 and N_2 . They occupy almost any illuminated environment on Earth and are very well equipped to live under extreme environmental conditions such as hypersaline, alkaline, high or low temperatures, dryness, high light, etc. Contrary to the general belief, many cyanobacteria are not generalists. Many species appear to be highly specialized organisms, adapted to a narrow set of environmental conditions, particularly in the case of extreme environments. However, our knowledge about the nature of these adaptations is fragmentary. The success of cyanobacteria is not exclusively found in their metabolic diversity, flexibility and reactivity. Cyanobacterial communities can be diverse and evolve rapidly when conditions change but the mechanisms involved are largely unknown.

6. References

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LIFE IN A HYPERVARIABLE ENVIRONMENT:

Algae of the Great Salt Plains of Oklahoma, USA

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1. Hypervariable Terrestrial Saline Environments

The Great Salt Plains (GSP) spans approximately 65 km² in northwestern Oklahoma, USA. Although soil on the flats consistently retains about 10–20% water by weight, the flats are largely devoid of macroscopic plants due to seepage and evaporation of subterranean NaCl-dominated Permian brine at 15–25% salinity. Except following infrequent heavy rain, a thin (<1–10 mm) variable salt crust persists over much of the flats, and the interstitial water is often near NaCl saturation (>25%). However, several intermittent and a few permanent freshwater streams traverse the flats, providing localized lower salinity niches. Episodic heavy direct rainfall and associated flooding of these streams inundates vast areas of the flats with fresh or low salinity water, which quickly recedes. The flats then return to the more typical salt crust over a period of days to weeks.

Other than the GSP, virtually nothing is known about algae of hypersaline intermittent lakes, even in Australia which has countless such lakes (Timms, in press). Algae are ubiquitous on the GSP flats, although chlorophyll biomass is typically very low, and diversity appears to be restricted to a small subset of genera in the divisions Cyanophyta, Chlorophyta, and Bacillariophyta (Major et al., 2005; Kirkwood and Henley, 2006). Chlorophyll biomass is correlated with interstitial dissolved inorganic nitrogen (DIN), particularly ammonium, but not soluble reactive phosphorus (Kirkwood and Henley, 2006). However, given the dynamic salinity and temperature conditions on the flats surface (Major et al., 2005; Kirkwood and Henley, 2006), nutrient availability may be a comparatively trivial problem for physically stressed algae and cyanobacteria. Indeed, most GSP algal isolates are unable to grow in the laboratory at the high interstitial salinities typically found *in situ*, yet direct soil samples invariably yield viable algae when exposed to reduced salinities in the laboratory (Major et al., 2005; Kirkwood and Henley, 2006). Thus, most or all of the algae of the GSP must tolerate high salinities that will not support growth.

With respect to hypervariable physical and chemical conditions, we believe that the GSP is distinct from the aquatic hypersaline systems that have been commonly studied. The GSP, with relatively rapid salinity fluctuations from NaCl saturation to freshwater and diel soil temperature fluctuations of 20–30°C (Major et al., 2005; Kirkwood and Henley, 2006), is likely to be representative of terrestrial hypersaline habitats such as the largely dry Australian saline playas (van de Graaff et al., 1977). Large permanent saline water bodies such as the Dead Sea, Great Salt Lake, and Mono Lake do not normally exhibit the same extremes and diel amplitudes of temperature as arid zone shallow playas such as in the Australian interior (Timms, in press) and the GSP flats. Large lake salinities are much more stable, changing gradually and within a relatively narrow range. Even with large hydrodynamic changes at Mono Lake, its salinity has (so far) remained between ~50 and 100 g/L over the last one or two centuries (Jellison et al., 1996). GSP soil temperatures in summer (Major et al., 2005) routinely exceed potentially lethal temperatures based on lab experiments (Henley et al., 2002). Thus, terrestrial hypersaline environments are physiologically more challenging than aquatic saline habitats. Another interesting question is whether algae adapted to this hypervariable environment can grow over a broader salinity range than relatives from marine and freshwater habitats. Here we present comparative growth responses to salinity for a GSP chlorophyte and its relatives isolated from marine and freshwater habitats.

Although salinity is an important environmental variable controlling algal growth and biomass at the GSP, resident algae also must be adapted to the other extreme conditions that occur, including long periods of desiccation and UV exposure. The GSP can experience month-long periods without precipitation, and daily summer soil temperatures exceed 50°C (Kirkwood and Henley, 2006). In conjunction with osmotic desiccation at the GSP, the combined lack of precipitation and high temperatures likely promotes a severely desiccating environment. Although water is probably almost always available to algae within the soil due to the upward wicking of groundwater brine, algae growing on the soil surface would be exposed to both desiccation processes and full sunlight/UV exposure.

Here we provide the first experimental evidence that many GSP chlorophyte and cyanobacterial isolates survive desiccation in saturated brine. In addition, we have discovered a number of *Dunaliella* (normally a unicellular flagellate) strains that maintain a colonial or palmelloid morphology. Masyuk (1973) reported such forms and for Great Salt Lake Brock (1975) stated "... as a palmelloid form it is found coating rocks and solid substrates throughout the lake." Lerche (1937) reported palmelloid stages at <1% salinity, but the ecophysiological significance has never been demonstrated. Interestingly, two of our colonial *Dunaliella* strains have the ability to grow in freshwater, as well as a range of salinities up to saturation (Kirkwood and Henley, 2006). This is a highly unusual form of euryhalinity, and the first to be reported as far as we know. We also provide evidence to show that, in GSP chlorophyte algae, a colonial morphology can be more beneficial than a motile, unicellular morphology under desiccating conditions.

Although it appears that the colonial morphotype is perhaps less important in UV tolerance, it may have other important benefits to life at the GSP. Thus, it appears likely that the harsh conditions of the GSP have selected for the colonial morphology and induced this trait in some GSP *Dunaliella* spp. to grow under freshwater conditions as well as to survive desiccation and freshwater shock.

2. Algae of the Great Salt Plains

Algal isolation, identification, and growth rate screening procedures have been described elsewhere (Major et al., 2005; Kirkwood and Henley, 2006). Diatom collection and analyses are detailed in Potter (2006) and Potter et al. (2006).

The isolated strains from the GSP belong to the Cyanophyta, Chlorophyta, or Bacillariophyta. We have deposited 51 morphologically, genetically, and physiologically unique strains in the UTEX and CCMP national cultures collections. All of these plus a few additional strains were screened for salinity tolerance (Kirkwood and Henley, 2006) and their 16S or 18S rRNA genes sequenced. The vast majority of them have now been accessioned and cryopreserved by one or both culture collections. In February 2006, our working collection at OSU included 135 strains inclusive of those deposited to the national collections (an earlier total of 177 isolates were summarized in Major et al., 2005); not all of these 135 are necessarily genetically unique. Note that most of these morphology-based identifications of the culture collection taxa are tentative, and we encourage taxonomic experts to characterize them more rigorously. This is particularly true of the diatoms, which were identified only from live material. We have also noticed some inconsistencies between morphological identifications and 16S rRNA gene sequence-based phylogenies of some cyanobacteria isolates, thus their genus assignments may be incorrect (Kirkwood et al., unpublished results). We also recognize that ideally morphological variability with environmental conditions should be taken into account in assigning isolates to existing taxa and in circumscribing new taxa.

It is important to note that our isolations were not intended to be an exhaustive collection of all taxa present, rather a representative sampling of the common types of halotolerant algae at the Salt Plains. Many of the ~24 genera and 107 putative species of diatoms carefully identified using cleared frustules from field samples (Potter, 2006; Potter et al., 2006) have no counterpart in the culture collection. For example, broadly distributed and moderately abundant taxa such as *Psammodicyon constrictum* and *Achnanthes* spp. are not represented in our culture collection. This is likely due both to the unavoidable selective aspects of algal isolation and to the major differences in the microhabitats sampled in our earlier algal isolation project (2002–2003) and the diatom survey of Potter (2006). Samples collected for algal isolation were randomly collected from mostly salt-saturated pool and soil microenvironments all over the GSP. The diatom survey of Potter (2006) sampled along transects representing salinity gradients (i.e., freshwater to salt flat microenvironments). However, the overwhelmingly abundant and species-diverse genera in field samples (*Navicula*, *Nitzschia*, and *Amphora*)

are also well represented in the culture collection. The low-salinity pool sites included in the diatom survey highlighted the localized importance of species such as *Mastogloia pumila* and *Cymbella pusilla* (Potter, 2006; Potter et al., 2006).

Similarly, 16S rRNA gene environmental clone libraries from field samples reveal a wide diversity of cyanobacteria (Kirkwood et al., unpublished results). Within some phylogenetic clades (e.g., *Geitlerinema*), multiple culture isolates are interspersed with multiple clone sequences; in other clades, clone sequences have few (*Komvophoron*) or no (*Microcoleus*) closely related isolates. In contrast, clades such as *Phormidium* are well represented among culture isolates, but largely absent from the clone library. The clone library also detected many diatom plastid 16S rRNA genes (unpublished data), which we will be able to compare to cultured isolates. Preliminary phylogeny shows most of the environmental clones to be allied with *Navicula*, *Nitzschia*, and *Amphora*, consistent with the morphological survey.

Based on cultured isolates, direct observations of field samples, and environmental clone libraries to date, algal flora at the GSP is generally comprised of relatively few genera from only three divisions. However, there is considerable species and ecotype diversity within those few higher taxa (Kirkwood and Henley, 2006; Kirkwood et al., unpublished results). This leads us to conclude that the hypervariable conditions at the GSP have promoted algal diversification relative to stable hypersaline systems through a combination of hypermutation events and opportunistic temporal niche exploitation (Kirkwood and Henley, 2006). The latter requires that all of the taxa can survive prolonged periods of salinities that are much higher than their growth limits.

An example of possible adaptive radiation is the typical halophilic chlorophyte *Dunaliella* that is commonly found at the GSP. We have 27 isolated strains of *Dunaliella* from the GSP, and of these we have sequenced one or more genes for 20 isolates. They form two or more robust clades that are distinct from most *Dunaliella* isolates from around the world (Buchheim et al., unpublished results), most or all of which are from aquatic habitats in contrast to the GSP soil-associated habitats. GSP *Dunaliella* isolates are also more genetically and morphologically diverse. Interestingly, they cluster with three new isolates from hypersaline soils in the Great Basin desert of the western USA, on the opposite side of the Continental Divide. As noted earlier, some of our isolates possess a novel nonmotile, colonial morphotype (Kirkwood and Henley, 2006), which is probably in response to suboptimal conditions (Kvíderová and Henley, unpublished results). These observations may reflect the distinct selective pressures in soil versus aquatic habitats, which again is likely driven by the hypervariable conditions in saline soil.

3. Chlorophyte and Cyanobacterial Response to Slow Desiccation

Given the discrepancy between the low to moderate salinities permissive for growth in the laboratory and typically high *in situ* salinities (Kirkwood and Henley, 2006), we investigated the ability of GSP chlorophytes and cyanobacteria to resume growth following evaporation of cultures and subsequent rehydration.

Chlorophytes and cyanobacteria were kept in 25-ml flasks containing 15 ml of sterile 1% or 5% salinity SP brine f/2 medium and air-dried at 20–25°C under 50 µmol m⁻² s⁻¹ cool white fluorescent illumination on a 14:10-h L:D cycle. Flasks were covered with loose glass caps or cotton plugs allowing gradual evaporation. After approximately 7 months, the cultures were dry (no visible water) with salt crystals, and we added 15 ml of fresh sterile 1% or 5% salinity SP brine f/2 medium to each culture according to their predrying salinity. The cultures were checked visually for growth over the next 60 days. Growing cultures were transferred to fresh liquid and solid media. If growth in at least one of these media was observed, the strain was considered as surviving desiccation. The effects of micro-habitat, nominal salinity at isolation site, and morphology on desiccation survival rates were evaluated by chi-square test.

Survival rates of individual genera are summarized in Table 1. Thirty two of the 69 cyanobacterial strains (46%) survived desiccation. Among the survivors, only *Cyanodictyon* (101-1) was colonial (cells embedded in mucilage); colonial

Table 1. Desiccation survival in GSP cyanobacteria and chlorophyte strains.

Genus	Number of strains		% survivors
	Before	After	
Cyanobacteria	69	32	46
Colonial			
<i>Aphanocapsa</i>	1	0	0
<i>Aphanothece</i>	2	0	0
<i>Cyanodyction</i>	2	1	50
Filamentous			
<i>Chlorogloeopsis</i>	1	1	100
<i>Geitlerinema</i>	20	9	45
<i>Halomicronema</i>	1	1	100
<i>Komvophoron</i>	2	1	50
<i>Leptolyngbya</i>	1	1	100
<i>Lyngbya</i>	1	1	100
<i>Phormidium</i>	26	10	38
<i>Pseudanabaena</i>	9	6	67
<i>Spirulina</i>	2	0	0
<i>Tychonema</i>	1	1	100
Chlorophyta	30	18	60
Unicellular			
<i>Chlorosarcinopsis</i>	1	0	0
<i>Dunaliella</i>	20	12	60
<i>Tetraselmis</i>	1	0	0
Colonial			
<i>Halosarcinochlamys</i>	1	1	100
<i>Dunaliella</i>	7	5	71
All strains	99	50	51

strains of *Aphanothecce*, *Aphanocapsa*, and one strain of *Cyanodictyon* did not recover. The moderate survival rate of cyanobacteria is consistent with the inability of most isolates to grow at salinities above 5% (Kirkwood and Henley, 2006). Most of these cyanobacteria strains are usually maintained at 1% salinity and only some grow well at 5% salinity; all of the latter survived. *Nodularia* sp. (157-1) (formerly identified as *Phormidium laetivirens*), *P. okenii* (177-1), and *Tychonema bornetii* (173-3) were isolated from dried mats, but *T. bornetii* did not survive desiccation in the laboratory. Because cyanobacterial mats at the GSP are usually found near low salinity creeks, it is possible that drying *in situ* is unaccompanied by high external salinity. In addition, the *in situ* mat matrix of polysaccharides may retain adequate moisture.

Eighteen of 30 (60%) chlorophyte strains (mostly *Dunaliella*) survived desiccation. Six of eight colonial strains (75%) and 12 of 22 unicellular strains (55%) were recovered. The high tolerance of *Dunaliella* and *Halosarcinochlamys* is expected. *Dunaliella* strains are found in hypersaline pools or soils and the strain *Halosarcinochlamys* (BS) was isolated from saturated brine stock solution (approx. 28% salinity). Our original GSP *Dunaliella* isolate is routinely maintained in saturated SP brine, where it is metabolically active (cells are motile as confirmed by confocal microscopy) inside of large crystals (Fig. 1), presumably in brine channels.

We categorized all desiccation-tested isolates variously according to micro-habitat of origin (brine pool, mat, soil, or unknown), nominal salinity class at the isolation site (average near saturation, ~half saturation, or unknown), and morphology (unicellular, colonial, or filamentous). Chi-square tests of survival rates among these categories were not significant (not shown), indicating no obvious correlation of desiccation tolerance with habitat or morphology.

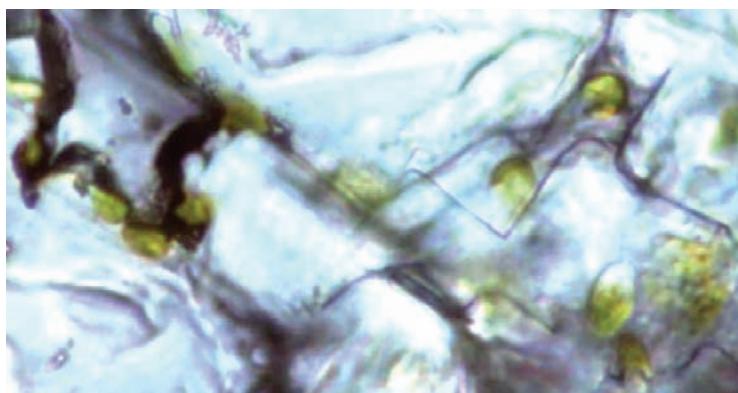


Figure 1. Photograph of GSP *Dunaliella* sp. inside of a large salt crystal formed during culture evaporation. Courtesy of Dr. Melanie Mormile, University of Missouri at Rolla. Cells (green) are approx. 10 µm long.

4. *Dunaliella* Response to Desiccation and UV-B

We assessed whether the colonial morphotype of some GSP *Dunaliella* strains improved their resistance or tolerance to desiccating conditions and UV-B. The rationale is that closely packed cells with copious amounts of mucilage are better suited than unicells to retain water as well as buffer the effects of increasing osmotic potential in the surrounding medium. Also, cells packed within the centre of the colony would be protected from UV-B rays absorbed at the surface of the colony. Therefore, even if the surface cells die from UV toxicity, the interior cells may remain viable and proliferate.

To compare the effect of desiccation on colonial versus unicellular chlorophyte strains, two parallel 24-well plates containing 1% Bacto agar with AS-100 medium were inoculated with each strain into triplicate wells (see Table 2 for media salinities). Both plates were covered with the plate lids, but only one was sealed with cellophane tape to minimize desiccation. Both sets of plates were incubated at $60 \mu\text{mol m}^{-2} \text{s}^{-1}$, 14:10 L/D at 25°C over the course of 96 h. Water loss was measured in blank plates over the experimental time period, by weighing control (i.e., sealed) and desiccation treatment plates each day. Final water concentration of desiccated 24-well plates was 22% (loss rate = 17% d⁻¹) of the initial 99% water concentration in agar. No measurable water loss occurred in the experimental controls. Chlorophyll biomass was measured daily using a custom-built fiber optic fluorometer (Henley et al., 1991; Kirkwood and Henley, 2006) modified with a volt meter for readout.

Table 2. Summary of growth responses to UV-B exposure and desiccation in GSP chlorophytes and CCMP 220, a close genetic relative from unknown origin.

Morphology/Genus	Strain ID	Salinity (%NaCl)	UV-B (% control)	Desiccation (% control)
Colonial				
<i>Dunaliella</i>	109-1	10	100 (17)	142 (20)
<i>Dunaliella</i>	112-2	10	20 (13)	71 (9)
<i>Halosarcinochlamys</i> *	BS	10	85 (49)	149 (92)
<i>Tetracystis</i>	176-2	1	25 (2)	67 (17)
Unicellular				
<i>Dunaliella</i>	112-3	10	25 (5)	0
<i>Dunaliella</i>	201-5	10	45 (23)	2 (1)
<i>Dunaliella</i>	CCMP 220	10	50 (3)	10 (2)

Values are means (standard error of the mean). UV-B treatment data are biomass of UV-B exposed relative to control cultures, 72 h after UV-B exposure (dose = 150 KJ m⁻²). Desiccation treatment data are biomass of desiccated relative to control cultures at the end of a 96-h desiccation period. Salinity is the initial NaCl concentration of the growth medium used in each experiment.

**Halosarcinochlamys* is a proposed new genus (Buchheim et al., 2006). It was first isolated from a 20-L carboy that stored brine-stock made from salts collected at the Great Salt Plains, OK.

The assessment of UV-B exposure on GSP chlorophytes was performed by first normalizing all culture densities ($\sim 10^4$ cells ml^{-1}), and then transferring a 20-ml subculture of each strain into a polystyrene petri-dish bottom for UV-B treatment (dose = 150 kJ m^{-2} applied over a 15-min exposure). We used Spectronics Corp. (Westbury, New York, USA) model XX-15B lamps with a peak emission at 302 nm, and measured UV with a UVP Inc. (Upland, California, USA) model UVX digital radiometer. Each culture was continuously swirled during UV-B exposure, and all control cultures were kept in the dark. Immediately after each UV-B treatment, both control and UV-B treated cultures were inoculated (in triplicate) into 24-well plates containing 1% Bacto agar with AS-100 medium (see Table 2 for medium salinities) and sealed with cellophane tape. Growth was measured daily as chlorophyll fluorescence using the same technique described above. Initial fluorescence readings (Day 0) were taken within minutes of each UV-B treatment. The plates were then incubated at $60 \mu\text{mol m}^{-2} \text{s}^{-1}$, 14:10 L:D at 25°C over the course of 72 h.

The chlorophyte desiccation and UV-B experiments were statistically tested for morphotype differences in two ways. Corresponding to results in Table 2, results for all four colonial strains were pooled and all three unicellular strains were pooled. Separate unpaired, two-tailed Student's t tests were used to test for different response of morphotypes to UV-B and desiccation. For the time-course results in Fig. 2, we pooled the results for the two colonial GSP *Dunaliella* strains and the two unicellular GSP *Dunaliella* strains by day and used repeated measures ANOVA to test for difference between morphotypes.

By comparing a number of colonial and unicellular chlorophytes, representing *Dunaliella*, *Tetraclysis*, and *Halosarinochlamys* genera, it is apparent that the colonial morphology is likely beneficial under desiccating conditions (Table 2). All colonial chlorophytes tested had notably higher biomass levels (relative to their controls) than unicellular types, and pooled results for unicellular versus colonial strains differ significantly (two-tailed, unpaired Student's t test, $p < 0.01$). In fact, *Dunaliella* 109-1 and *Halosarinochlamys* BS had $\sim 1.5\times$ the biomass of their controls at the end of the desiccation period. In contrast, all of the unicellular types were $< 10\%$ of their control's biomass. Although it is clear that the colonial morphotypes did quite well under desiccating conditions, the increased biomass levels may have been a consequence of increasing nutrient concentration with evaporation over time. With respect to UV-B tolerance, the benefits of a colonial morphology are less striking and not statistically significant (Table 2; two-tailed, unpaired Student's t test, $p > 0.05$). Interestingly, *Dunaliella* 109-1 and *Halosarinochlamys* BS showed the greatest resistance to UV-B exposure, as they did with desiccation. However, the other colonial morphotypes varied in their response to UV-B, and were within the same range of values determined for the unicellular forms. When comparing the growth response of GSP *Dunaliella* strains to a single UV-B dosage over time, it is apparent that the colonial morphology may buffer the initial effects of UV-B (Fig. 2; repeated measures ANOVA, $p < 0.01$), but this was not necessarily the case for all colonial types. *Dunaliella* (112-2) had a delayed negative response to UV-B, compared to the

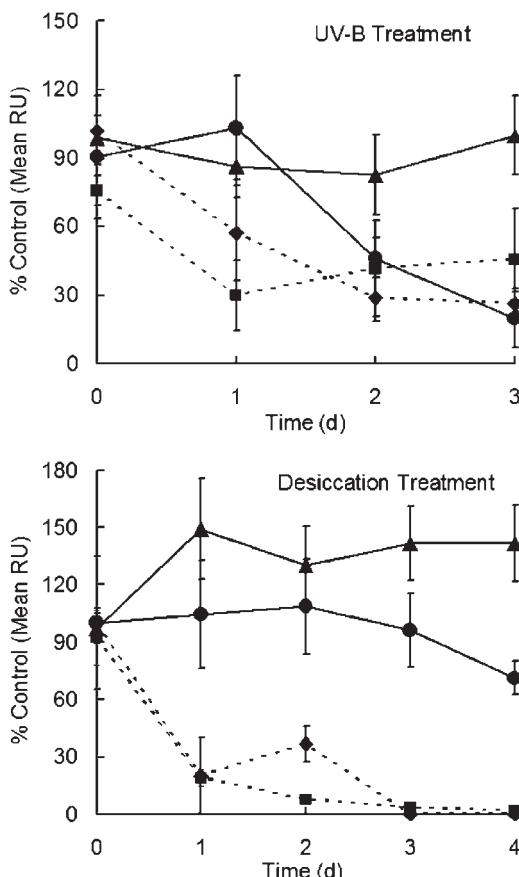


Figure 2. Growth responses of GSP *Dunaliella* strains 112-3 (diamonds), 201-5 (squares), 109-1 (triangles) and 112-2 (circles) to UV-B and desiccation treatments over time. Morphotypes are distinguished by solid lines (colonial) and dashed lines (unicellular).

other *Dunaliella* strains, but had the lowest relative biomass at the end of the experiment. Over time, it is clear that the colonial forms of *Dunaliella* tolerate desiccating conditions better than their genetically related unicellular counterparts (Fig. 2; repeated measures ANOVA, $p < 0.01$).

5. Salinity Tolerance Within a Phylogenetic Clade

The salinity-growth response of GSP isolate *Picochlorum oklahomensis* UTEX 2795 (=CCMP 2329) was compared with phylogenetically related taxa isolated from marine and freshwater habitats. Freshwater taxa ("Nannochloris" spp. ANR-9 and AS 2-10, *Gloeotila* sp. JL 11-10 and *Marvania* sp. JL 11-11) were

isolated as described in Phillips and Fawley (2000). They are all in the ambiguously resolved “*Nannochloris*-like” freshwater clade that is sister to the marine *Picochlorum* clade (Henley et al., 2004). Marine taxa included *Picochlorum* sp. UTEX 2491 from the Salton Sea (California, USA), which has an identical 18S rRNA gene sequence to GSP *P. oklahomensis*. *Picochlorum* sp. UTEX 2378 is also closely related based on sequence. *P. oculata* UTEX 1998 from the Virginia coast (USA) is more deeply branching within the *Picochlorum* clade. “*Nannochloris* sp.” UTEX 2055 from Singapore coastal waters has not been sequenced, but we presume it to be allied with *Picochlorum* based on the clear phylogenetic delineation of marine and freshwater taxa (Henley et al., 2004). All taxa were grown in triplicate cultures in artificial AS-100 medium at a series of NaCl-salinities: 0, 1, 2.5, and 4% for freshwater strains and 0, 2.5, 5, and 7.5% (plus 10% for three isolates in a preliminary experiment) for marine strains. Here we present qualitative comparative growth results.

GSP isolate *P. oklahomensis* UTEX 2795 is able to grow at about half-maximal rate at 10% salinity, and very slowly at 15% salinity (Henley et al., 2002). Other closely related isolates of nominal *Picochlorum* spp. from other saline/marine habitats also grow at near maximal rates from freshwater to 7.5% salinity, or approximately double seawater salinity (Table 3). Thus, broad halotolerance appears to be a general trait within marine/saline isolates of this genus, and growth in freshwater is not a problem. However, based on limited evidence it appears that the GSP isolate is more tolerant of extremely high salinities (>7.5%). In contrast, freshwater isolates from a sister clade to *Picochlorum* are capable of maximal growth only up to 1% salinity, and grow only minimally or not at all at

Table 3. Qualitative growth rates of *Picochlorum oklahomensis* UTEX 2795 and marine and freshwater isolates at a series of salinities.

	Salinity (weight percent)						
	0%	1%	2.5%	4%	5%	7.5%	10%
Marine/hypersaline							
<i>P.</i> sp. UTEX 2378	++	nt	++	Nt	++	++	nt
<i>P. oculata</i> UTEX 1998	++	nt	++	nt	++	++	–
<i>Nannochloris</i> sp. UTEX 2055	++	nt	++	nt	++	++	–
<i>P.</i> sp. UTEX 2491	++	nt	++	nt	++	++	nt
<i>P. oklahomensis</i> UTEX 2795	++	nt	++	nt	++	++	+
Freshwater							
“ <i>Nannochloris</i> sp.” ANR-9	++	++	(+)	–	Nt	nt	nt
“ <i>Nannochloris</i> sp.” AS 2-10	++	++	–	–	Nt	nt	nt
<i>Gloeotila</i> sp. JL 11-10	++	++	–	–	Nt	nt	nt
<i>Marvania</i> sp. JL 11-11	++	++	(+)	–	Nt	nt	Nt

++, near-maximal growth rate; +, suboptimal growth; (+), marginal growth; -, no growth; nt, not tested

2.5% (Table 3). Thus, consistent physiological differences between freshwater and marine taxa within the “*Nannochloris*-like” clade correspond to clear genetic divergence and possible differences in cell division (Henley et al., 2004). The main distinction is intolerance of moderate to high salinities in freshwater taxa, while marine taxa have no problem growing in freshwater. With the exception of the terminal *Picochlorum* clade, the class Trebouxiophyceae is predominantly or exclusively freshwater. Together these observations suggest that the ancestral forms inhabited freshwater, and one lineage acquired the ability to tolerate high salinity without losing tolerance for freshwater. The nature of the molecular mechanism(s) necessary to achieve broad halotolerance is unclear.

6. Acknowledgments

This chapter is based upon work supported by the National Science Foundation under Grant Nos. MCB-0132097, 0334508, and 0514652 (WJH). JK was further supported by the Academy of Sciences of the Czech Republic project AV0Z60050516 and by Ministry of Education and Youth of the Czech Republic project IM6798593901. Growth data at 10% salinity for UTEX 1998, 2055, and 2795 were determined by Allyson Fry.

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PART 8: OTHER MICROORGANISMS AND EXTREME HABITATS

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Bermejo

Knoll

Edgcomb

Bernhard

Jeon

Onofri

Zucconi

Selbmann

Hoog

Los Rios

Ruisi

Grube

Altermann

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THE FATE OF BIOLOGICAL MATERIALS IN ACIDIC ENVIRONMENTS OF THE RIO TINTO, SOUTHWESTERN SPAIN

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1. Introduction

Earth is a planet that records its own history, including aspects of its biological history (Knoll, 2003). The long-term evolution of life is recorded by fossils, molecular biomarkers, biogeochemically informative isotopic abundances, especially of carbon and sulfur, and sedimentary textures that reflect biological activity. One might reasonably expect that other planets have recorded their histories as well, encrypting evidence of past tectonics, climate, atmospheric composition, and, if present, life in the physical and chemical properties of sedimentary rocks. For most of Earth history, the biota was microbial, and it is likely that microbial signatures will be the astrobiological targets of any planetary sediment we will be privileged to examine close at hand.

We know this is the case for Mars, where both orbital (Malin and Edgett, 2003) and rover-based observations (Grotzinger et al., 2005) document a stratigraphic record. At our present level of understanding, however, that record differs substantially from ancient terrestrial rocks that record life's history on our own planet. For example, on Earth the two principal rock types that contain well preserved Precambrian fossils are early diagenetic cherts precipitated by ground waters percolating through carbonate successions and fine-grained (clay-rich) siliciclastic rocks that accumulated under reducing conditions. Global mineralogical surveys have failed to detect widespread carbonate rocks on the martian surface (Bandfield et al., 2003), although trace amounts have been identified in martian meteorites (McKay et al., 1996; Bridges and Grady, 2000). In contrast, oxidation of surficial materials is widespread (Klingelhöfer et al., 2004; Ming et al., 2006). This suggests that the standard paleobiological exploration program that has proven so successful on Earth cannot easily be exported to Mars. Rather than search on Mars for rocks like those that have yielded biological signatures on Earth, we may, for the moment, at least, do better to search on Earth for rocks similar to those found at Meridiani Planum and elsewhere, asking whether such deposits are likely prospects for astropaleobiological exploration.

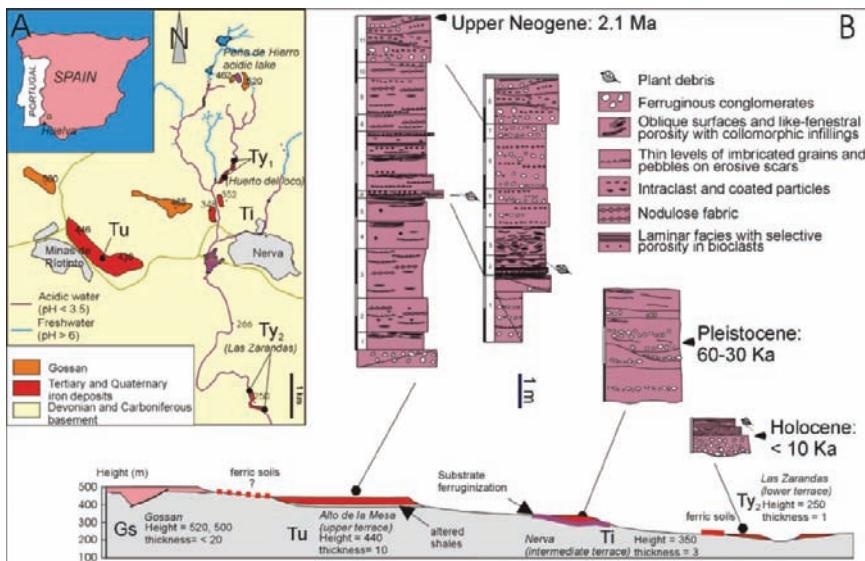


Figure 1. General geology of the Rio Tinto headwater area showing: (A) the outcrop distribution of Tertiary and Quaternary ferric rocks and sediments deposited by the acidic river during different terrace-forming events; (B) stratigraphic columns of the three river terraces ranging from the Upper Neogene to Holocene. These have yielded a moderately diverse fossil record including microbial filaments, plants, and insects.

In this paper, we summarize ongoing research on organic matter and fossil preservation in the acidic, sulfate-rich deposits of Rio Tinto, southwestern Spain (Fig. 1). Rio Tinto sediments contain a suite of minerals comparable to those formed at Meridiani Planum, including jarosite and ferric iron-bearing minerals (Fernández-Remolar et al., 2005a, b). Moreover, exposed terraces preserve a record of diagenetically stabilized Rio Tinto sedimentary rocks to complement currently forming deposits (Fernández-Remolar et al., 2005a, b). Understanding the diagenetic fate of biological materials in the Rio Tinto depositional system will help us to evaluate the astropaleobiological potential of acid-sulfate deposits on Mars.

2. Organic Matter in Rio Tinto Waters and Sediments

The geochemical properties of the Río Tinto acidic waters (Fernández-Remolar et al., 2005a, b) strongly suggest an aggressively oxidizing fate for organic molecules introduced into the environment by biological activities. Solution pH ranges from ca. 0.8 to 3 and the redox potential from 350 to 600 mV. The river water arises from several acidic springs that are sourced in an aquifer that flows through hydrothermal pyrite formed during the last stages of Hercynian orogenesis

(Leistel et al., 1998). Oxidation of sulfides via biotic and abiotic processes produce subsurface solutions that become iron- and sulfate-enriched (Fernández-Remolar et al., 2005a, b). Moreover, sulfide weathering that produces sulfate and ferric iron (as well as the buffering effect of the ferric iron) also releases protons to the water. Perhaps surprisingly, given the environment, organic geochemical analyses (Figs 2 and 3) of acidic and oxidizing Río Tinto solutions and jarosite sediments precipitated under highly acidic conditions ($\text{pH} < 1.5$) indicate that this environment can favor the preservation of diverse organic molecules during early diagenesis.

Organic analysis was performed by solid phase microextraction (SPME) coupled with GC-MS. Finely ground solid sample was heated in a vial closed with a septum at 170° for 30 min. A $100\ \mu\text{m}$ polydimethylsiloxane (PDMS) fibre was then exposed to the headspace, keeping the sample at the same temperature for a further 30 min. Analytes on the fibre were then thermally desorbed in the injection port of a Perkin Elmer Autosystem XL-Turbomass GC-MS instrument at 270° for 4 min (splitless mode). The analysis was performed using a capillary column (5% diphenyl-95% dimethylpolysiloxane, $30\ \text{m} \times 0.25\ \text{mm ID.}, 0.25\ \mu\text{m}$ film). Temperature was raised from 40° (4 min) to 150° at a rate of $10^\circ/\text{min}$, held for 2 min, 150° to 255° at $5^\circ/\text{min}$, held for 5 min, and 255° to 300° at $10^\circ/\text{min}$, and held for 20 min. The mass spectrometer was operated under EI mode, at ionization energy 70 eV, m/z range 30–600, transfer line at 300° . The aqueous samples were extracted by SPME using a $65\ \mu\text{m}$ PDMS-DVB fibre in the headspace of a 20 ml vial filled with 10 ml sample and heated at 70° for 30 min. 2 g NaCl were added to the sample prior to extraction. The analytes extracted by the fibre were analyzed by GC-MS as described above. Reference spectra included in the NIST library were used for the molecular characterization of analytes. Organic compounds were considered to be determined only when the correlation between the sample compound spectra and the reference spectra exceeded 85%.

Some organic molecules may have contaminated samples during collection, transportation, and preparation. For example, phthalate-derived compounds (13 and 14 in Fig. 2A; 21, 29, and 31 in Fig. 2B; 30, 32, and 36 in Fig. 3) and PVC-derived contaminants (6 in Fig. 2A and 3 in Fig. 2B), organic compounds in the plastic containers used for sampling, have been detected as abundant compounds. Silicon-bearing organics, artifacts that originated in the GC column, appear in the water chromatograms (see Fig. 2A–B). However, the quantity and diversity of organic molecules found in these first results strongly suggests a predominant organic input from organisms living in and adjacent to the Río Tinto.

Organic analyses of different water samples show manifest differences in the diversity of compounds detected. As seen in Fig. 2A, acidic solutions sampled in stream B are dominated by low molecular weight aldehydes and ketones (compounds 1–10 in Fig. 2A, excepting 6, which is PVC-derived). Moreover, above 13 min of retention time, two compounds (11 and 12 in Fig. 2A) have been identified as possible long-chained hydrocarbons with aldehyde and carboxylic groups, possibly branched, at least in part. A comparable organic association has

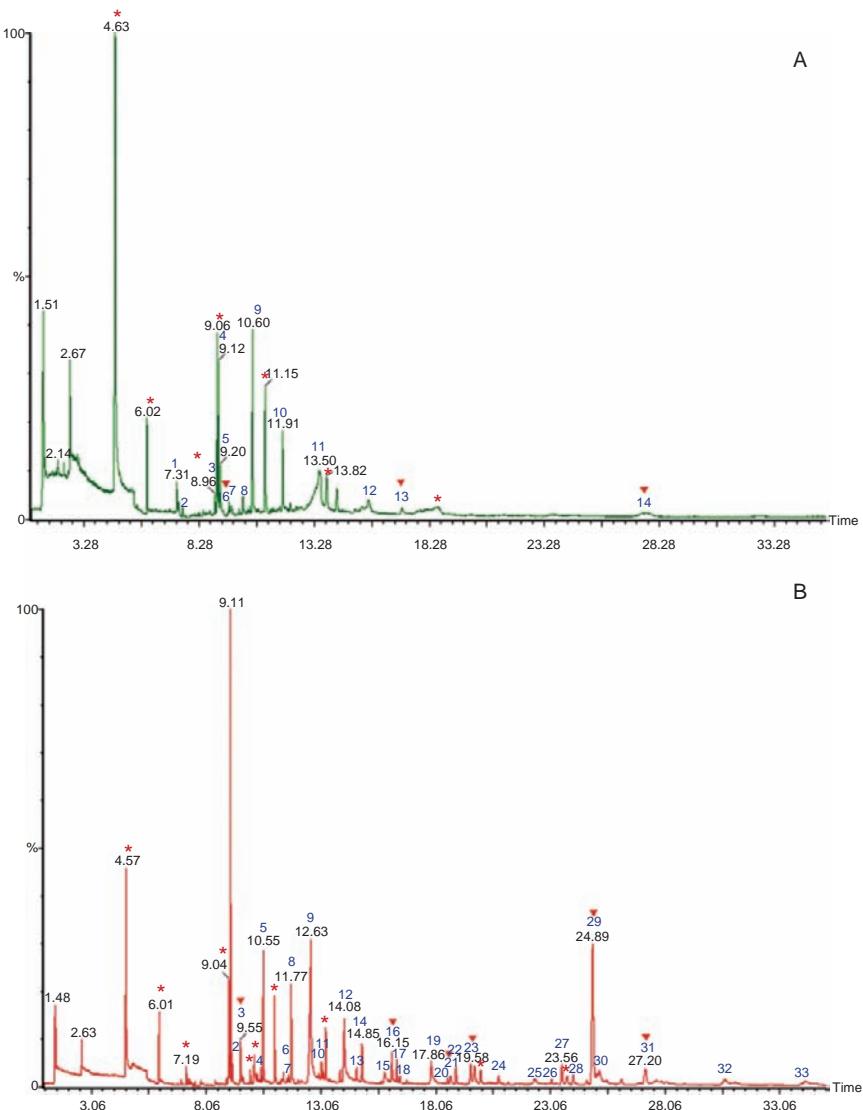


Figure 2. GC-MS organic analysis of water samples obtained in (A) headwater stream B (see Fernández-Remolar et al., 2005a, b) and (B) the Berrocal sampling area, showing qualitative differences in organic composition. In both chromatograms, the red asterisks indicate silicon-derived compounds that originated by bleeding of chromatographic columns; red triangles mark contaminants sourced in PVC and plastics. The following 14 compounds have been identified in: (A) 1 – 3-heptanone; 2 – heptanal; 3 – 3-octanona; 4 – decane; 5 – octanal; 6 – 1-hexanol, 2-ethyl (contaminant sourced in PVC); 7 – 3-nonenone; 8 – 5-nonenone; 9 – nonanal; 10 – decanal; 11 – unidentified long-chain carboxylic acid; 12 – unidentified long-chain aldehyde; 13 and 14 – phtalates (contaminant sourced in plastics); (B) has yielded ca. 33 compounds, including the following: 1 – decane; 2 – octanal; 3 – 1-hexanol, 2-ethyl (organic contaminant sourced in PVC); 4 – undecane; 5 – nonanal;

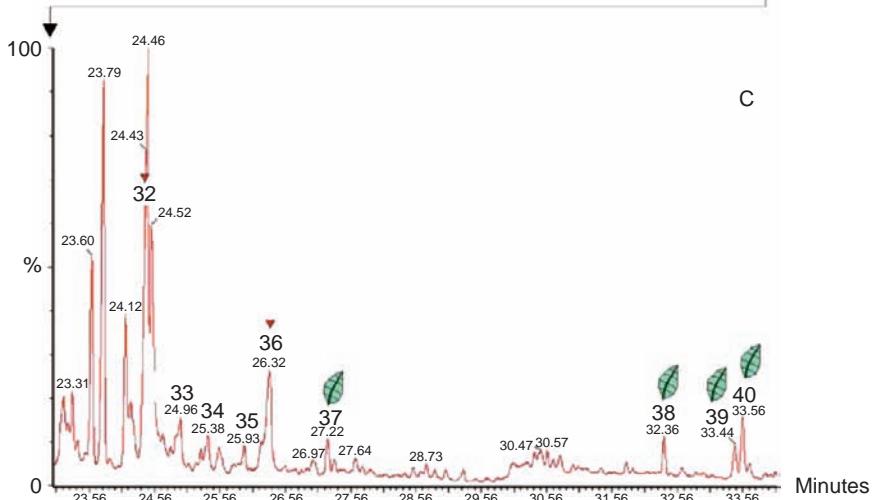
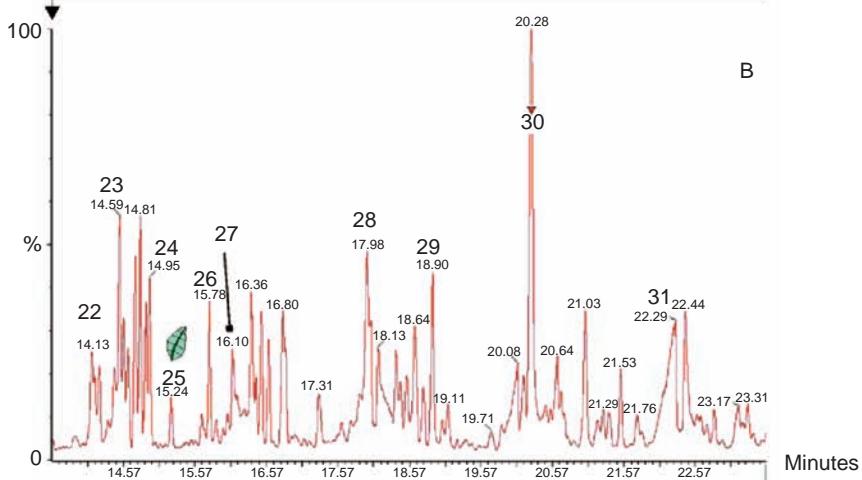
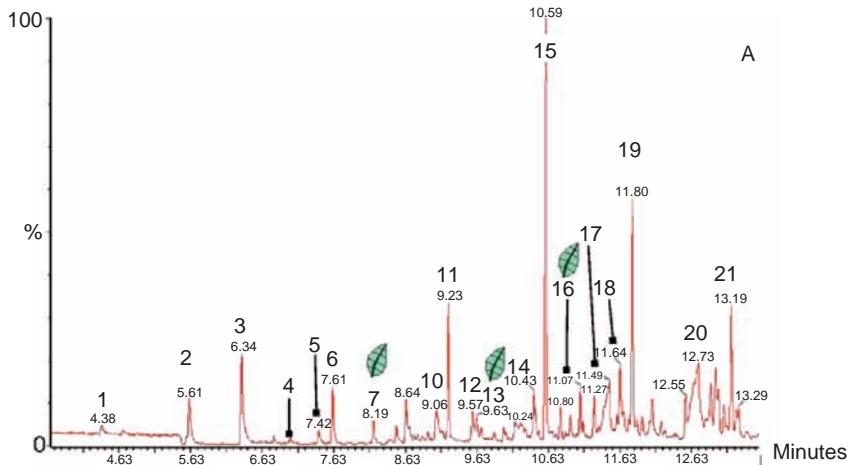
been recognized in the Berrocal sample, obtained 20 km downstream of the headwaters; it also contains low molecular weight aldehydes and carboxylic acids (compounds 1 through 15, and 19– 24 in Fig. 2B, excepting 3, 16, 21, and 23, which are plastic-derived). The Berrocal sample, however, has provided a wider abundance of simple long-chain hydrocarbons and hydrocarbon bearing distinct aldehyde, carboxylic, ester, and amine groups (compounds 24–33 in Fig. 2B, but not 23, 29, and 31, sourced in plastics).

Preliminary analyses of modern jarosite sediments show an analogous array of compounds, with the addition of several molecules of unquestionable plant origin (see Fig. 3 for molecular characterization of plant-sourced organics) and a higher abundance of compounds derived from humic complexes (compound 3 in Fig. 3A), lipids and their degraded by-products, and fatty acids (i.e., 31 in Fig. 3B). The jarosite sample also contains dimethyl disulfide, which occurs as the first eluted molecule in the GC chromatogram (Fig. 3A). Other compounds have been recorded as weaker peaks not easily identifiable at low concentration. Low peaks ranging between 23 and 24, 27 and 31, and 37 and 38 may correspond to carotenoid degradation products, saturated and unsaturated hydrocarbons, and/or sterol derivatives.

The GC results reported here are preliminary, and further research is needed to understand the interactions of biological and physical processes involved in the production and diagenesis of Río Tinto organic matter. Nonetheless, comparisons among the three sample groups strongly suggest environmentally specific sources and fates of biomolecules. For example, water analyses are dominated by short and long-chain aldehydes, ketones and, to a minor extent, carboxylic compounds that likely originated during membrane degradation. Given the high abundance of microbial films in the river, it may be that these organic analytes relate to the decay of microbial membranes and exo-polysaccharides (EPS). This interpretation may be especially relevant for the sample water obtained in stream B in the headwaters of the river, which is directly sourced by an acidic spring. As expected, the downstream Berrocal samples show a stronger influence of plant and humic substances washed into the river from the surrounding ecosystem.

The jarosite sample is a sulfate-rich sediment that precipitated in an acidic pool abandoned by stream B during the dry season (Fernández-Remolar et al., 2005a, b). These briny stagnant waters received appreciable input of plant materials as they dried up. Additionally, ferric iron-rich precipitates formed within the

6 – allylheptanoate; 7 – dodecane; 8 – decanal; 9 – nonanoic acid; 10 – 4-*t*-butylcyclohexylacetate; 11 – undecanal; 12 – decanoic acid; 13 – tetradecane; 14 – dodecanal; 15 – undecanoic acid; 16 – 2,5-cyclohexadiene-1,4-dione-2,6-bis (1,1-dimethylethyl) (contaminant sourced in plastics); 17 – unidentified long-chain alkane; 18 – unidentified long-chain aldehyde; 19 – dodecanoic acid; 20 – hexadecane; 21, 29, and 31 – phthalates (same as in 15); 22 – pentadecanal; 23 – unidentified di-*t*-butyl benzene (same as in 15); 24 – eicosane; 25 – unidentified long-chain carboxylic acid; 26 – unidentified long-chain alkane; 27 – hexadecanal; 28 – unidentified long-chain alkane; 30 – unidentified long-chain amine; 32 – unidentified long-chain amine; 33 – unidentified long-chain ester.



drying pool may have veneered biological materials, preserving them during early diagenesis. The presence of dimethyl disulfide may result from microbial interaction with the aquifer hosted in the pyritic ore that sources the acidic springs that feed the river. The dimethyl disulfide is related to dissimilatory sulfate reduction under aerobic and anaerobic conditions in sulfate-rich waters (Iverson et al., 1989; Bashkin, 2002, p. 139). Therefore, surface and subsurface Río Tinto acidic sulfate-rich solutions can be a source of biogenic short-chain S-bearing organic compounds.

Jarosite and other iron-bearing sulfates like schwertmannite may play a particular role in organic preservation at Rio Tinto. Like clay minerals and iron oxides (Eusterhues et al., 2003; Kennedy et al., 2006), precipitating jarosite crystals provide a protective matrix against the oxidative processes. A precursor stage in jarosite precipitation is the formation of ferric iron-sequestering polymers (Lazaroff et al., 1982) that strongly diminish the oxygen content of ambient water and, hence, the oxidizing power of ferric, acidic solutions. Under this low ferric iron activity, iron-respiring microbes have limited biochemical access to Fe³⁺, which has been observed for neutral pH iron-reducing bacteria and thermophilic iron-oxidizing bacteria (Bridge and Johnson, 1998; Jones et al., 2006). Forty million year old evaporite deposits contain jarosite materials that demonstrate net organic preservation (Aubrey et al., 2006). Very preliminary results also show the presence of organic molecules in diagenetically stabilized Rio Tinto sedimentary rocks 10,000 to about two million years old. Whether these represent long-term preservation, initiated under conditions like those observed in the modern system, or organic contaminants delivered by ground water percolation through porous sediments has yet to be determined. ¹⁴C measurements, especially on the older



Figure 3. GC-MS chromatogram obtained during the analysis of H-Jarosite sampled in stream I from the headwater region of the Rio Tinto Basin. Given the high diversity of the organic constituents, the chromatogram has been split into three parts (A) through (C) to facilitate the reading of results. The leaf symbol indicates molecules with a clear plant origin, whereas red triangles mark contaminants sourced in plastics. (A) has yielded 21 compounds: 1 – dimethyl disulfide (secondary product of bacteria oxidation on S-bearing organics resulting during the degradation of metallic sulfides); 2 – hexanal (caproic aldehyde resulting from lipid oxidation); 3 – furfural (degradation of humic acids and cellulose); 4 – 4-methyl heptanona; 5 – 4-methyl heptanone; 6 – heptanal (enantol sourced in lipid degradation); 7 – α -pinene; 8 – 6-methyl-2-heptanona; 9 – benzaldehyde; 10 – unidentified compound; 11 – octanal (lipid degradation); 12 – 1,4-diethyl-benzene; 13 – Eucalyptol; 14 – nonanona; 15 – nonanal; 16 – isopinocarveol (sourced in the *Pinus aciculas*); 17 – fatty acid (possible caprylic acid); 18 – 2-decanona; 19 – decanal; 20 – pelargonic acid (early degradation of lipids); 21 – undecanal. (B) has at least 10 organic compounds: 22 – undecenal; 23 – tetradecene; 24 – tetradecanol (mystic alcohol); 25 – longifolene (formed in the pine aciculas and resine); 26 – n-alkane (C₁₅); 27 – tridecanol; 28 – phytanal; 29 – hexadecanal (palmitaldehyde); 30 – phtalate (organic contaminant sourced in plastics); 31 – unidentified fatty acid. (C), provides 9 identifiable organics: 32 and 36 – phtalates (see 30); 33 – icosadiene; 34 – methyl ester in tridecanoic acids; 35 – hexacosadiene; 37 – manolic oxide (1H-nafto [2,1b]pirane that derive from the degradation of pine resine); 38 – ethyl pinmarate (component in conifer resine); 39 – abietic acid (as 38); 40 – methyl-dihydroabietic acid (product of the abietic acid degradation).

terraces, should tell more about the long-term preservational potential of biomolecules in Rio Tinto and, by implication, Meridiani Planum-like environments.

3. Fossil Preservation in Rio Tinto Deposits

Independent of organic preservation, precipitated minerals can preserve a morphological record of organisms, including microorganisms. On Earth, as noted above, the precipitates most commonly associated with fossil preservation are silica (e.g., Barghoorn and Tyler, 1965; Knoll 1985) and carbonates (e.g., Knoll and Semikhatov, 1998), but oxidized iron minerals (Stein et al., 1982) and sulfates (Bonny and Jones, 2003) can also preserve details of cell morphology and tissue structure. Jarosite and oxidized iron precipitates in the Rio Tinto basin preserve a range of biological structures (Fernandez-Remolar et al., 2005a, b), including coccoidal and filamentous bacteria (Fig. 4A–B), clusters of filamentous fungi

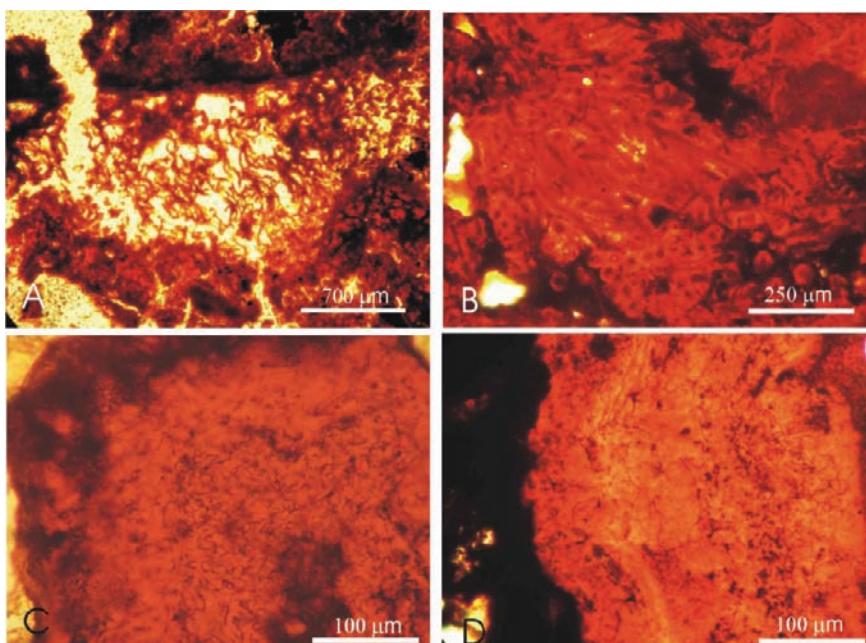


Figure 4. Microbial filaments within modern ferric sediments and lithified Río Tinto ancient deposits. (A) Shows filamentous fungi in modern ferric sediments that grew vertically inside a microcave possibly formed via the decay of plant remains. (B) Preserved filaments in ca. 2.1 million year old Upper Terrace deposits, showing a micron-thick ferric oxide coating that covers organic filaments. (C) Shows filaments included within a cryptocrystalline ferric infill occupying a pore primarily formed by a cubic pyrite grain, which were obtained in sediments of the young Holocene terrace. (D) Bacteria remains trapped during the deposition of ferric oxide cement laminae obtained in the old terrace.

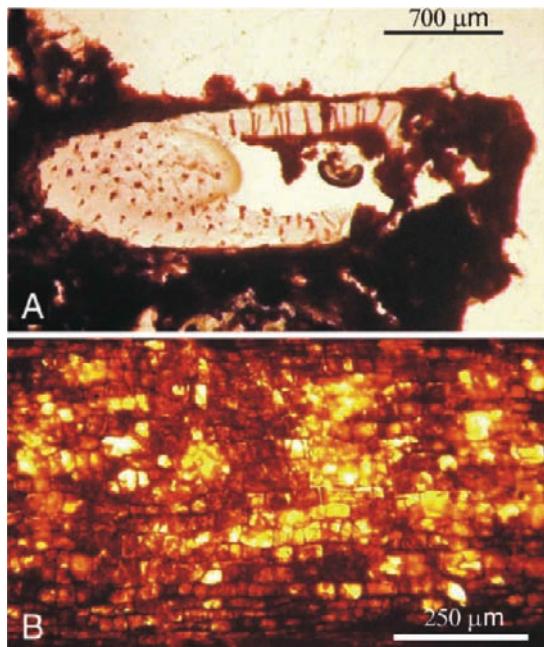


Figure 5. Macroorganisms preserved in the Holocene Lower Terrace sediments at Las Zaradas. (A) Insect organic cuticle conserved inside a cryptocrystalline ferric cement – note spiracles preserved by ferric molds. (B) Fine cellular preservation in plant tissue imparted by submicron-scale mineral coating.

(Fig. 4C–D), insect cuticle (Fig. 5A), and plant tissues (Fig. 5B). In all cases, fossilization began with relatively decay-resistant portions of organisms – extracellular sheaths of iron bacteria, chitinous walls of fungal hyphae, cuticles of insects, and the cellulosic cell walls of plants. Bacterial and fungal decay of cytoplasm began before fossilization and, judging from the abundance of fossils preserved as hollow casts, continued afterward. Biological surfaces clearly formed favorable sites for mineral precipitation, with sub-micron mineral laminae preserving morphology in remarkable detail – note, for example, the spiracles preserved in insect cuticle by diagenetic mineral infilling (Fig. 3A). Diagenetic growth of hematite at the expense of goethite appears to decrease the fidelity of preservation, but interpretable fossils do occur in two million year old hematite beds in the uppermost terrace of the Rio Tinto (Fig. 1).

The quality of preserved morphology seems to depend on the primary composition of fossils. That stated, schwertmannite and nanophasic iron oxides (Fernández-Remolar et al., 2005a, b) are stable in a higher pH range and may confer a greater chemical resistance to mineralogical changes that occur when acidic sediments are exposed to neutral rainwater. These compounds usually coat biological remains under the acidic aqueous matrix (Fig. 6A–B), forming a thin

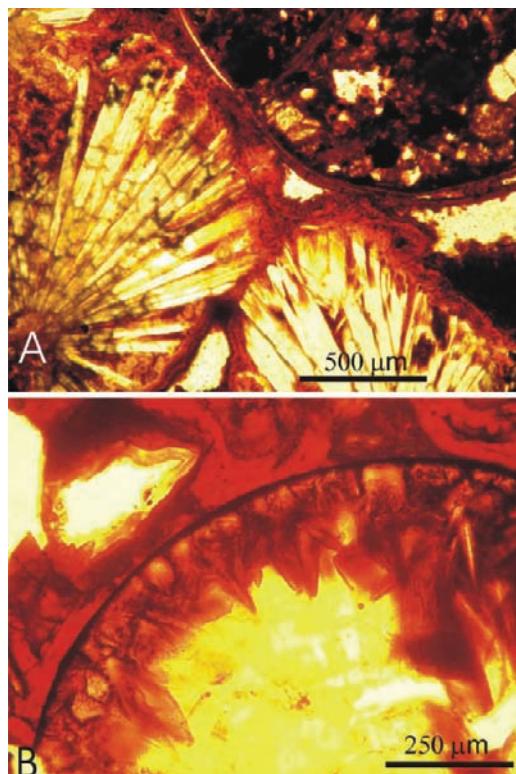


Figure 6. Ferric iron precipitates protecting sulfates against dissolution under neutral waters sourced by rainfall during the wet season. (A) Fibro-radiate crystals coated by laminar ferric deposit, from modern sediments of the Río Tinto headwater area. (B) Possible plant remains of the young terrace that were primarily filled by sulfates and coated by ferric iron deposits.

ferruginous film that can protect incipient fossils when exposed to less acidic waters. Once the most labile organic components have been degraded by bacteria and fungi, the remaining hollow casts may eventually be filled by gypsum (Fig. 6A). Nanophase iron oxides provide the best materials for fossil preservation, given that they will change mineralogical structure only by recrystallization to goethite, which currently occurs under neutral meteoric water. Other compounds in Río Tinto sediments undergo a desulfation process that implies reorganization of the mineral structure due to loss of sulfates transported into solutions with a higher pH, where goethite rapidly oversaturates. Sulfate efflorescences and jarosite precipitates have a preservation potential much lower than sediments precipitated from acidic waters as schwertmannite and nanophase iron oxides. As sulfates are highly soluble when exposed to rainwater or neutral streams they can hardly preserve the preserving biological structures that were trapped inside efflorescences.

In some cases, sulfate relicts can be preserved intact or as simple pseudomorphic structures replicated by iron oxyhydroxides. Both may represent different preservational stages associated with diagenesis. Modern internal molds filled with sulfates can be protected by an iron oxyhydroxide coating against the dissolution by undersaturated and neutral waters (Fig. 6A). Older remains found in the Young Terrace deposits (Fernández-Remolar et al., 2005a, b) show evidence that sulfate infillings were eventually replaced by iron oxyhydroxides (Fig. 6B). These fossilization products acquire a higher resistance to physical and chemical attack in an aggressively oxidizing environment and thus may provide a useful analogy to sedimentary rocks on the Meridiani plain.

4. Conclusions

Acidic and oxidized waters of the upper Rio Tinto precipitate a range of minerals that includes iron oxides/hydroxides and sulfates comparable to those identified in outcrop rocks at Meridiani Planum, Mars. Because the Rio Tinto system includes both modern precipitation, where relevant biological and physical parameters can be observed directly, and diagenetically stabilized sedimentary rocks, where one can study how a record of those parameters can be encrypted in the geologic record, this basin provides unusual insights into the potential for fossil and molecular biomarker preservation in ancient martian sediments. We do not, of course, know whether Mars ever had organisms to be preserved, but the test of that hypothesis will lie in direct paleobiological and biogeochemical investigations of Noachian and Hesperian rocks. Fossil and organic preservation at Rio Tinto suggests that mixed iron oxide-sulfate deposits like those at Meridiani Planum constitute compelling targets for in situ paleobiological analysis and sample return (Knoll et al., 2005).

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DEEP-SEA MICROBIAL EUKARYOTES IN ANOXIC, MICROOXIC, AND SULFIDIC ENVIRONMENTS

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1. Introduction

Measuring the extent of eukaryotic microbial diversity is essential to our understanding of eukaryotic evolution and the structure and function of microbial food webs. In the past several years, molecular approaches have been used to address an increasing interest in the diversity of microbial eukaryotes, particularly that of protists from various marine environments. These have included pelagic environments (e.g., Moon-van der Staay et al., 2001; Massana et al., 2002), deep-sea environments (López-García, 2001), the ocean surface (Díez et al., 2001; Moon-van der Staay et al., 2001), coastal environments (Massana et al., 2004), and a river (Berney et al., 2004), as well as extreme environments, including acidic and iron-rich rivers (Amaral Zettler et al., 2002), deep-sea hydrothermal vents (Edgcomb et al., 2002; López-García et al., 2003), microoxic (<10 µM oxygen) and anoxic waters and sediments in salt marshes (Stoeck and Epstein, 2003), permanently anoxic deep-sea waters (Stoeck et al., 2003, 2006), anoxic shallow sediments of marine and freshwater (Dawson and Pace, 2002; Bernhard et al., 2006). These studies have revealed an extraordinary diversity of previously undetected eukaryotic lineages based on small-subunit ribosomal RNA (SSU rRNA) sequences. For a recent overview of higher level classification of eukaryotes that emphasizes the protists, see Adl et al. (2005). Anoxic (lacking dissolved oxygen) environments have been present throughout Earth's history, and sulfide-rich conditions are likely to have existed in the deep oceans into the late Proterozoic (Canfield, 1998; Shen et al., 2002), during the origin and early diversification of eukaryotes when atmospheric oxygen concentrations were about 1% of present day levels (Schopf and Klein, 1992). Indeed, the sulfur cycle has been implicated in the origin of eukaryotes (e.g., Searcy, 1992; Martin and Müller, 1998; Moreira and López-García, 1998; Gray et al., 1999). While the details of eukaryogenesis remain debatable and it is impossible to re-enact the origin and early diversification of Eukarya, additional insights into the origin and diversification of eukaryotes can be gleaned from the study of extant anaerobic and microaerophilic

benthic microbial communities that may harbor eukaryotic taxa that retain ancestral characteristics.

Methodological difficulties have hampered the study of the structure and function of anaerobic microbial communities that can be inhibited or killed by trace amounts of oxygen, and isolation attempts do not necessarily recover the most abundant microbes. We have relatively little information about the phenotypic and evolutionary diversity of free-living protists from anoxic and microoxic marine environments. Most studies of protistan diversity have relied upon morphological characters to differentiate between genera and species. Microscopic approaches that have aimed to document and describe the diversity of protists in marine environments have contributed significantly to our current knowledge, however, they have been restricted to relatively large ($>20 \mu\text{m}$) cells with conspicuous morphologic characteristics. Most protists have not yet been cultivated with present techniques. Consequently, culture-based studies and microscopic criteria cannot provide quantitative measures of genetic diversity because they frequently do not provide comprehensive profiles of community composition. Phylogenetic analysis of SSU rRNA gene sequences directly amplified and cloned from environmental samples has provided great insight into studies of microbial diversity, ecology, and evolution. Surveys of eukaryotic microbial diversity based on comparisons of SSU rRNA genes from marine pelagic environments reveal a microbial world with few taxonomically assignable representatives (e.g., López-García et al., 2001; Moon-van der Staay et al., 2001; Stoeck et al., 2006). In contrast to the extensive application of SSU rRNA-based approaches for the analysis of prokaryotic diversity, this approach has only recently been applied to the microbial eukaryotes in deep-marine microoxic and anoxic habitats (e.g., Edgcomb et al., 2002; López-García et al., 2003; Stoeck and Epstein, 2003; Stoeck et al., 2003, 2006). The results of molecular surveys that rely on PCR amplification of selected genes must be interpreted with caution because of amplification biases with each primer set, which almost certainly miss an unknown portion of the microbial community. As a result, differences in representation of particular groups in clone libraries must be interpreted with caution. When surveys are conducted in different environments using different nucleic acid isolation techniques and sets of primers for PCR amplification it is difficult to interpret the significance of minor differences in results. Some standardization of methodological approaches will make such comparisons more meaningful. Nonetheless, when one considers the results of different studies of microoxic and anoxic marine environments, some striking variation in taxonomic representation is apparent. For example, when comparing the results of a survey of deep-sea hydrothermal vent sediments (Edgcomb et al., 2002), an anoxic marine water body (Stoeck et al., 2006), and anoxic sediments around fumaroles on a submarine caldera floor (Takishita et al., 2005), it is apparent that certain taxonomic groups are likely to play a significant role in each individual environment (Fig. 1). For example, in the Guaymas sediments and in the Cariaco water column,

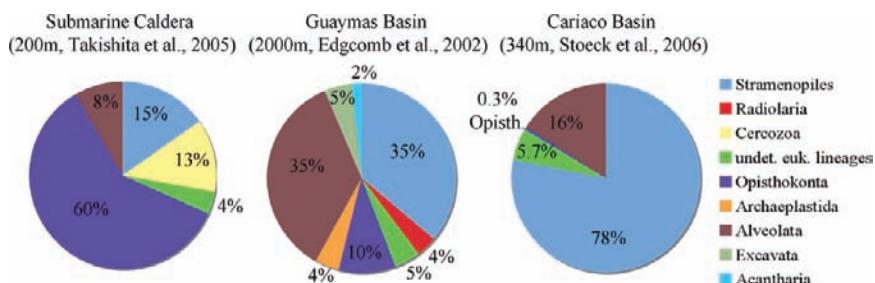


Figure 1. Representation of different taxonomic groups present in clone libraries from three different SSU rRNA-based surveys of marine anoxic or microoxic environments.

stramenopiles and alveolates represented significant portions of each of these clone libraries, and the Kagoshima Bay (submarine caldera sediments) clone libraries were dominated by the opisthokonts. Conspicuous differences, such as the greater diversity of taxonomic groups in the deep-sea hydrothermal vent sediment clone libraries is notable, but it is not possible to draw any further conclusions from this observation at this time because each of these surveys was conducted using different primer sets. It is interesting to note that unlike the other two studies selected for this comparison, the deep-sea vent survey was conducted using only a single set of primers (Edgcomb et al., 2002), so in light of the more restricted primer sampling, perhaps the increased diversity detected in this environment relative to the other two is noteworthy. It has been argued that certain microaerophilic parasites represent early diverging lineages in the eukaryotic line of descent (Sogin et al., 1989). Because they do not require atmospheric levels of oxygen for growth, these organisms lack two of the oxygen-consuming organelles: mitochondria and peroxisomes. The absence of these features in the most phylogenetically divergent eukaryotic lineages suggests that anoxic and microoxic marine environments and the thiobios might support novel and diverse communities. Considering the thermophilic phenotype of the deepest prokaryotic branches, sulfidic, microoxic, and anoxic habitats, including those within deep-sea hydrothermal vent environments, are of interest for studying eukaryotic microbial diversity. This is supported by the discovery of clones affiliated with jakobid flagellates in an anoxic water layer below the chemocline in a super-sulfidic (6 mM) anoxic fjord (Behnke et al., 2006). Jakobids are free-living heterotrophic flagellates that have bacterium-like mitochondrial genomes, suggesting they might represent one group of extant representatives of primitive eukaryotes. Although this is a shallow water study, it highlights the importance of examining anoxic and sulfidic environments for novel taxa of evolutionary interest. Since the taxon sampling of free-living protozoa is particularly deficient, protistan phylogenies almost certainly exclude clades of principal ecological and evolutionary importance (Baldauf, 2003). This underscores the importance of expanding of

our knowledge of protistan diversity in general, and in particular, of protists in deep-sea anoxic and microoxic environments.

The terminology “deep sea” can have slightly different meanings to different readers. “Deep sea” is interpreted here as below the photic zone. In many cases, this is usually well below ca. 200 m, but a few studies referred to here are of shallower sites (ca. 200 m) that are considered aphotic due to local water turbidity. Although aphotic sulfidic sediments were much more widespread in the geological past (e.g., Canfield, 1998), they are still relatively common. Sites where local organic enrichment leads to the development of sulfide-enriched habitats include hydrothermal vents and sediment-rich “cold” seeps, fjords, silled basins such as Cariaco Basin, Black Sea, and Santa Barbara Basin (SBB), whale falls, and open ocean margins with well-developed oxygen minimum zones (OMZ), such as, Monterey Bay, California. In fact, the eastern edges of most of the world’s oceans have well-developed OMZ. Although hydrogen sulfide is “toxic” (i.e., inhibits respiration) at concentrations known to exist in marine oxygen-depleted environments, many of these sites have high densities of eukaryotic microorganisms. During the late Archaean or Proterozoic, when eukaryotes originated, these deep-aphotic environments may have played an important evolutionary role during a time when the risk of DNA damage due to UV radiation was high (e.g., Sagan, 1973; Towe, 1996; Cockell, 1998). Bernhard et al. (2000) suggested that an aphotic environment similar to SBB, a basin between the California mainland and the northern Channel Islands where bottom waters are microoxic and periodically anoxic, may have served as the site of primeval eukaryotic diversification. Such deep, aphotic basins may have provided critical refugia from harsh surface conditions. Richards and Bass (2005) performed a comprehensive analysis of available environmental sequences and identified several sequences that are almost identical from different oxygen-depleted environments that often cluster together to the exclusion of sequences from oxygen-rich sites. This observation supports the idea that these lineages represent truly anaerobic or anoxic-tolerant organisms, which are less likely to be aerobic organisms introduced into those environments through deposition. More studies of microoxic and anoxic habitats are needed to determine if anaerobic or anoxic-tolerant lineages have cosmopolitan distributions. This paper addresses the available information on protist diversity from several categories of “deep” marine environments, with a special focus on anoxic, microoxic, and sulfidic habitats.

A detailed discussion of photosynthetic organisms in these environments is beyond the realm of this manuscript, although it has been suggested that photosynthesis may occur in the deep ocean, for example, at hydrothermal vents (e.g., Van Dover, 1994) by means of black body radiation. Recent molecular studies in photic oceanic regions reveal a large diversity of photosynthetic picoeukaryotic heterokonts (see review by Moreira and López-García, 2002). Such surveys are expanding our previous knowledge of picoeukaryotic marine algae and suggest these small photosynthetic organisms are widespread and likely play an important role in oceanic primary production.

2. Silled Basins

Silled basins create an environment where ocean circulations between oxygenated surface waters and oxygen-depleted bottom waters are reduced or eliminated entirely due to local bathymetry and circulation. Data are discussed here from sites below the photic zone in three silled basins that were sampled at the anoxic/oxic interface or below. As noted, the SBB is a depression between the California mainland and the northern Channel Islands (USA), and has a maximum depth of ca. 600 m, a sill depth of ca. 425 m, and microoxic bottom waters that rarely exceed 5 μM oxygen (Bernhard et al., 2003). The Cariaco Basin is located off coastal Venezuela, has a maximum depth of ca. 1,400 m, and a sill depth of 146 m (Lin et al., 1997). This basin represents the world's largest, truly marine permanently anoxic basin. It exhibits vertical chemical gradients influenced by the limited physical transport of oxygen downward and reduced compounds upward in the water column, both of which are countered by biological demands. In Cariaco the oxygen concentration decreases to 0 μM between 250 and 350 m depth; with deeper waters that have remained anoxic and sulfidic for centuries to millennia (Richards and Vaccaro, 1956). The Soledad Basin is located off the western coast of Baja California, has a maximum depth of ca. 540 m, and a sill depth of ca. 290 m (van Geen et al., 2003). A study of SBB eukaryotes within microbial mats of sulfide-oxidizing bacteria (in situ oxygen concentration 0.1 μM , but bottom-water oxygen and hydrogen-sulfide concentrations vary considerably within the basin both spatially and temporally) established that an abundant and relatively diverse eukaryotic community occurs in sediments in the vicinity of the oxic/anoxic interface (Bernhard et al., 2000, 2003; Bernhard and Buck, 2004). The laminated SBB sediments had much greater abundance and biovolume of eukaryotes than Soledad Basin sediments, and even though flagellates were the most abundant microbial eukaryotes present in both SBB and Soledad Basin (quantitative data not available for Cariaco), foraminifera contributed the greatest biovolume at both sites (Bernhard et al., 2000; Bernhard and Buck, 2004).

The identity of the primary microbial mat bacterium at the sediment/seawater interface differed in SBB and Soledad Basin. The Soledad Basin site was dominated by the sulfide-oxidizing bacterium *Thioploca*, while the SBB sediments were overlain with a mat of *Beggiatoa* (Bernhard and Buck, 2004). In spite of this difference, the two environments are not far apart, and one would expect more similarity in eukaryotic microbial community composition than what was observed. This supports the notion that different protistan communities can be unique and communities can be very diverse. Most species of flagellates and ciliates from deep-sea sedimentary environments are known by their SSU rRNA gene sequences only, however a few species were confidently identified in Bernhard and Buck (2004) based on microscopy. Consistent with results from Guaymas Basin, the anoxic specialist ciliate genus *Metopus* occurred at all three sites (SBB, Soledad, Cariaco) as did *Paralepharisma* ciliates (Bernhard and Buck, 2004).

Based on morphological criteria, Bernhard and Buck (2004) did not observe any other positively identifiable common forms of ciliates or flagellates that were observed to occur in all three sites (Soledad, Cariaco, SBB). A molecular analysis would almost certainly have revealed phylotypes that occurred in all three locations. Euglenoids were the most abundant protist observed in SBB sediments, exceeding 6×10^5 cells mL⁻¹ in the top centimeter. Both Soledad and SBB communities supported the foraminifer *Nonionella stella*, and the flagellates *Calkinsia aureus* and *Sphenomonas* sp. (Buck and Bernhard, 2001). In Cariaco, SBB, and Soledad Basin, the foraminifera were dominated by a single species: *Bolivinia subadvena* in the Soledad, *Virgulinella fragilis* in the Cariaco, and *N. stella* in SBB (Bernhard and Buck, 2004).

Associations between single-celled eukaryotes and prokaryotes are common in anoxic and sulfidic environments, most notably among flagellates and ciliates (Fenchel and Finlay, 1995; Epstein et al., 1998; Bernhard et al., 2000; Vannini et al., 2003). Prokaryotic partners of these protists include endosymbiotic purple non-sulfur photosynthetic bacteria (Fenchel and Bernard, 1993a, b), methanogenic archaea (e.g., van Bruggen et al., 1983), as well as putative ectosymbiotic sulfate-reducing bacteria (Fenchel and Ramsing, 1992), although these identifications were obtained from associates of shallow water anaerobic or microaerophilic hosts. Endosymbiotic methanogens have been found in ciliates, and epibiotic hydrogen-sulfide oxidizers on euglenoids (Buck and Bernhard, 2001; see also discussion of Monterey Bay cold seeps later). In the case of the foraminiferan protists, it has been established that they have non-phototrophic putative symbionts (e.g., Bernhard, 1993, 2003; Bernhard and Alve, 1996; Bernhard et al., 2000, 2006), yet their exact identity is still unknown. Data from Percoll density gradient and DAPI staining of SBB samples show that the majority of the eukaryotes observed (24 morphotypes), many of which belong to taxa that are new to science, harbor prokaryotes in a putative symbiotic association (Bernhard et al., 2000) either as endobionts (living inside the host) or ectobionts (living on the host). However, at present, little is known about the identity of these symbionts or about many of the protistan hosts in SBB, and even less is known about the nature of the protist–prokaryote associations. It is possible that some of these associations are predator–prey relationships instead of symbiotic relationships. Fluorescent in situ hybridization (FISH) studies could be used to resolve this question. The consistent lack of ectobionts on certain taxa is an indication that associated bacteria are not merely using the exteriors of eukaryotes as substrates in that environment. In the SBB, ciliates were the most diverse group of symbiont-bearing taxa observed (Bernhard et al., 2000). The sulfur spherules were not observed in any of the ciliate epibionts in SBB. The dominance of ciliate and flagellate specimens with associated putative symbionts observed in both Cariaco (based on a non-quantitative sample) and in SBB, was not observed in Soledad Basin (Bernhard and Buck, 2004).

The sub-millimeter distributions of eukaryotic nanobiota and meiofauna, plus co-occurring prokaryotes was examined in laminated, microoxic sediments

of the SBB using the fluorescently labeled embedded core (FLEC) technique (Bernhard et al., 2003). This approach combines epoxy embedding with fluorogenic probes and hot-knife microtomy to visualize live cells in serial sections of ca. 0.1 mm, and allows associations between prokaryotes and eukaryotes to be observed within undisturbed sediment samples. Unprecedented associations were noted. For example, at depths in the sediment where high sulfide concentrations were expected, intimate associations between flagellates and *Beggiatoa* were observed. Additionally, whereas ciliates were usually solitary, flagellates were often observed in aggregates of single morphotypes ($>3 \times 10^4 \text{ mm}^{-3}$). Such aggregates have the potential to significantly impact pore-water chemistry at scales $<1 \mu\text{l}$ (Bernhard et al., 2003). All of the identifiable flagellates in these swarms had ectobionts, suggesting the hosts are anaerobes (Bernhard et al., 2003). The biotic distribution patterns observed using FLEC were complex, with aerobes and anaerobes occurring along the same horizon and aerobes commonly occurring deeper in the sediments. This is an indication of complex pore-water chemistry. Non-quantitative observation of FLEC sections from a sample when bottom-water oxygen concentration was $2.4 \mu\text{mol L}^{-1}$ shows a peak in flagellate abundance at a depth of 2–3 mm below the sediment surface (flagellates were the most numerically dominant eukaryotes observed), a peak in foraminifera at the surface of the sediments, and a peak in ciliate density at 1–2 mm depth into the sediments. Foraminifera and flagellates were observed down to 9–10 mm depth, and ciliates down to 8 mm depth. In a second sample taken when bottom-water oxygen was nearly undetectable ($0.1 \mu\text{mol L}^{-1}$), protists in all three groups were less abundant, and were found mostly in the top few millimeter of sediment (Bernhard et al., 2003). These authors only analyzed the top 1 cm of sediments at these sites with FLEC, and there are no doubt, ciliates and flagellates and other microbial eukaryotes below this depth.

An unusual observation was that each of the major eukaryotic biomass contributors in the SBB (*N. stella*) and in Cariaco (*V. fragilis*) sequesters chloroplasts in spite of living in well below the euphotic zone (Bernhard and Bowser, 1999; Bernhard, 2003). It has been proposed that the sequestered chloroplasts play a role in the dominance of these organisms in these two sulfide-enriched environments (Grzymski et al., 2002).

The Black Sea is the largest known anoxic sulfide-enriched basin on Earth yet little is known about the microbial community inhabiting the sediments in the vicinity where its anoxic/oxic interface impinges the seafloor. The Black Sea differs from other well-studied marine oxygen-depleted, sulfidic sites in that there is little, if any, coexistence of oxygen and sulfide in the water column (Bernhard, pers. comm.). It is not yet known whether this community differs from that of other sulfidic environments. Initial studies have been conducted by Bernhard (unpublished data) of five samples collected along a transect from 147 to 2,025 m water depth, including the region where the redox boundary impinges the seafloor. The exact sulfide concentration of these samples was not measured, but at the redox boundary and below they smelled of hydrogen sulfide. The eukaryotic community in the

>63 µm fraction of these samples was described in terms of general taxonomic composition and abundance. Filamentous bacteria similar in morphology to the sulfide-oxidizing bacterium *Beggiatoa* were observed in the three samples that were collected between 147 and 196 m water depth. These filaments were not abundant, and did not form cohesive mats, as in other deep-water sulfidic marine settings. A planktonic diatom, similar in appearance to *Skeletonema*, was the most abundant eukaryote in samples from 159 to 196 m. Representative specimens of this diatom appeared viable, but are assumed to have recently settled to the seafloor from the overlying water column. Other protists included euglenoid flagellates, testate and non-testate amoebae, and allogromid foraminifers (Fig. 2). At 330 m, far below the expected maximum penetration of dissolved oxygen, the only eukaryotes observed to date are large (~250 µm) ciliates, possibly of the anaerobic genus *Paralepharisma* (Bernhard, unpublished data).

Stoeck et al. (2003) sampled the water column of the Cariaco Basin at two depths below the oxic/anoxic interface (270 m): 340 m and 900 m. The maximum density of protozoa was detected immediately below the oxic/anoxic interface,

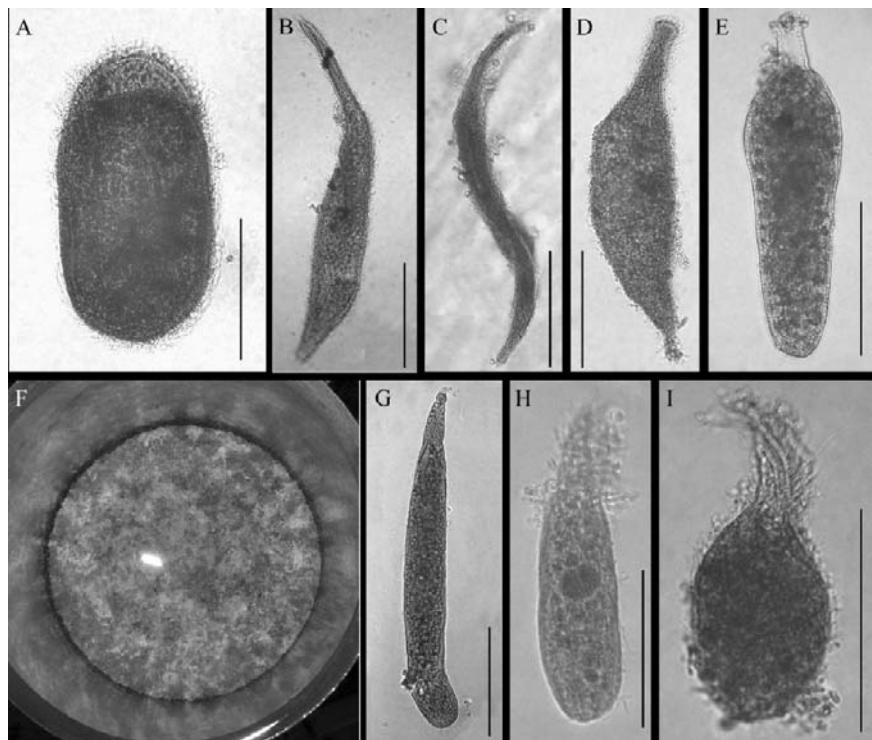


Figure 2. Examples of Black Sea eukaryotes. Individuals in top row are all from a core taken at 330 m depth, all in bottom row are from a deep core taken at 2,025 m. Scale bars are ~100 µm.

just below a peak in heterotrophic bacterial production (Taylor et al., 2001, Stoeck et al., 2003). The closely juxtaposed peaks in density of these two populations suggest that heterotrophic protozoa may exert demographic controls on bacterial populations in the Cariaco water column, and that heavy grazing of bacterial populations may impact nutrient cycling in this part of the ocean. In addition to novel lineages within recognized protistan clades, Stoeck et al. (2003) detected several phylotypes that may represent novel deeply branching lineages, however they note that since they are not related to any other recognized eukaryotic clade or to each other, their basal position may instead be a result of their divergent nature or a result of insufficient taxon sampling. Similar to other deep-marine molecular surveys, Stoeck et al. (2003) recovered a large number of sequences that affiliated to the alveolates, some within the uncultured marine alveolate Group I, including a clade of sequences that appear to be ancestral to this group, and some that formed a new deeply branched alveolate lineage that they termed uncultured marine alveolate Group III. Related sequences to those within their Group III were recovered by Edgcomb et al. (2002) from Guaymas Basin hydrothermal vent sediments, suggesting that they are at least ubiquitously distributed in deep-marine environments. This is supported by the observation of a large number of divergent alveolate lineages detected in molecular surveys of a wide range of environments including deep-sea plankton, hydrothermal vents and sediments, and marine pelagic environments as discussed above.

An in-depth 18S rRNA gene sequence analysis of eukaryotic microbial diversity in the water column of the Cariaco Basin is presently under way. To date we have surveyed over 10,000 clones obtained from three different stations within the basin at four discrete water depths corresponding to positions within, above, and below the oxycline, and from 900 m depth (significantly below the oxycline). As such, this phase of our project represents the most comprehensive survey of this type of marine environment to date. Nucleic acids were isolated as described previously (Stoeck et al., 2003). We amplified 1,000–1,300 bp fragments of the 18S rRNA gene using three primer sets, consisting of a single forward primer E528F (5' -CGGTAATTCCAGCTCC-3') (Edgcomb et al., 2002) and three different universal reverse primers Univ1391RE (5'-GGCGGTGTGTACAARGRG-3') (Dawson and Pace, 2002), Univ1492RE (5'-ACCTTGTACGRCTT-3') (Edgcomb et al., 2002), and Univ1517 (5' -ACGGCTACCTGTTACGACTT-3' modified from Univ1492RE). The PCR protocol utilized HotStart Taq DNA polymerase (Qiagen, Valencia, CA), an initial hot-start incubation for 15 min at 95°C, followed by 30 amplification cycles (denaturing at 95°C for 45 sec, annealing at 55°C for 1 min, and extension at 72°C for 2.5 min), and final extension at 72°C for 7 min. The PCR products were cloned separately for each primer set, using the TA cloning kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Clones were sequenced in 384-well plates using an ABI 3730XL capillary sequencer. Editing of sequences, assembly of contigs, and BLAST analyses were performed using a series of UNIX shell scripts that incorporate PHRED and PHRAP (assembled by Hilary Morrison and Rich Fox at the Marine Biological

Table 1. Cariaco basin clones from microoxic and anoxic water column samples with 91–96% sequence identity with nearest match in GenBank.

Clone identifier ^a	Water depth (m) ^b	BLAST score	Query/subject ^c	Closest match (accession nos) ^d	Taxonomic group
BCI5F13RM2C07	256–270	817	467/483	Chain-forming colpodid ciliate (AY398684.1)	Colpodea
BCB5F13RM1A06	326–340	1,112	794/865	Uncultured eukaryote isolate H52 (AY256215.1) ^e	
BCI5F15RM2F08	256–270	1,225	768/816	Uncultured marine eukaryote clone p14C4 (AY882493.1) ^f	
BCA5F15RM1A02	216–230	1,253	783/830	Uncultured marine colpodean ciliate DH147-EKD23 (AF290076.2)	Colpodea
BCA5F13RM1H07	216–230	1,055	741/789	Uncultured marine colpodean ciliate DH147-EKD23 (AF290076.2)	Colpodea
AA5F15RM4A10	185	1,202	589/610	Uncultured eukaryote isolate E187 (AY256275.1) ^f	
AI5F13RM1F06	225	1,828	1,044/1,082	Uncultured eukaryote isolate C1_E045 (AY046642) ^g	
A95F15RM3H02	900	1,988	1,165/1,215	Uncultured marine eukaryote clone p14C4 (AB179734.1)	Spumellaria
A95F15RM3G06	900	880	573/612	<i>Sticholonche</i> sp. JJP-2003 (AY268045.1)	Taxopodida
A95F15RM1B05	900	1,957	1,162/1,215	Uncultured marine eukaryote clone p14C4 (AY882493.1) ^f	
A95F15RM2B03	900	1,582	1,112/1,214	Uncultured marine eukaryote clone p14C4 (AY882493.1) ^f	
BCI5F15RM1D02	256–270	815	466/483	Uncultured marine colpodean ciliate DH147-EKD23 (AF290076.2)	Colpodea

^aFirst letter of identifier indicates sample location: Station A, or a combined sample from Stations B and C.^bRanges given for samples combined from Stations B and C. [O₂] = 0 at 190 m Station A, and at 226 and 240 m for Station B and Station C, respectively.^cRatio of number of matching bases between query and nearest match to the total length of matching sequence.^dClosest match in GenBank as of May 15 2006.^eSequences of environmental clones from tank bromeliads.^fSequences of environmental clones from Cariaco Basin (Stoeck et al., 2006).^gSequences of environmental clones from Guaymas Basin hydrothermal vent sediments.

Laboratory). Final sequences were checked for chimeras using the bioinformatics tools Check_Chimera (Cole et al., 2003) and Bellerophon (Hugenholtz and Huber, 2003) and by manual inspection of alignments in ARB (Ludwig et al., 2004). The phylogenetic analyses of the surveyed community are in progress, but preliminary results reveal surprising insights into the extent of novel protistan lineages at all taxonomic levels (unpublished data). For example, Table 1 presents the BLAST results for selected clones from 8 of our 48 clone libraries whose identity to the nearest match in GenBank was less than 97%. First analyses indicate that many of the novel phylotypes represent ciliates, and that these new sequences will contribute to our understanding of the true extent of diversity within this relatively well-studied group of protists. The ciliates represent one of the best-described protistan groups, which led some researchers to believe that most, if not all, ciliate species had been described (Finlay et al., 1996; Finlay, 2002), although not all researchers agree (Foissner, 1999; Richards and Bass, 2005). Stoeck et al. (2003) recovered a clade of ciliate SSU rRNA gene sequences from clone libraries of anoxic Cariaco waters at 900 m depth that appears to be a sister group to the established ciliate classes. If this new clade turns out to deserve class-level designation, then this candidate ciliate class, named CAR_H by Stoeck et al. (2003), represents the first new class of ciliates discovered in decades. Research is currently under way to obtain representatives of these ciliates in culture for microscopy and further studies. One goal of our current work in Cariaco is to obtain complete, or nearly complete, coverage of protistan diversity in selected samples. This will allow for the comparison of experimentally determined – as opposed to statistically calculated measures of protistan local species richness. FISH studies using probes designed from this large database along with culture-based studies will allow us to describe community dynamics over time and across environmental gradients.

3. Hydrothermal Vents and Cold Seeps

3.1. HYDROTHERMAL VENTS

The first survey of eukaryotic community SSU rRNA gene composition of the sediments/seawater interface proximal to a deep-sea hydrothermal vent was conducted in the Everest Mound area in the southern Guaymas vent field of the Guaymas Basin, Gulf of California (Edgcomb et al., 2002). The temperature gradient for the top 3 cm of the sampled cores from which sequences were obtained ranged from 3°C to 65°C. A thick (up to several centimeter) layer of flocculent mat of the sulfur-oxidizing bacteria *Beggiatoa* overlaid one core, whereas only a very thin layer (approximately 2 mm thick) was evident at the surface of a second core studied. This hydrothermally active environment includes vent plumes, seeps, and anoxic sediments, each exhibiting a wide range of temperatures, and supports surface-attached microbial mats, diverse prokaryotic and eukaryotic microbes, and symbiont-harboring invertebrates. Warm, sulfide- and hydrocarbon-rich,

anoxic sediments accumulate at a rate of 1–2 mm/year due to high biological productivity in the water column and a large terrigenous input from Baja and the Mexican mainland (Calvert, 1966). The approximately 100–500 m thick sediments release vast quantities of petroleum, short chain fatty acids and ammonia via pyrolysis of complex organic substrates (Von Damm et al., 1985; Bazylinski et al., 1988). The vent fluids that percolate through the organic-rich sediments have an unusual chemistry based on high carbonate content and near neutral pH, and release large amounts of methane (Welhan, 1988).

The amount of previously undescribed protist diversity detected in this environment is impressive. Culture-based studies on Guaymas Basin water column samples collected in close proximity to active hydrothermal vents indicate flagellates capable of living in the presence of very high hydrogen-sulfide concentrations (30 mM), suggesting that these taxa may be important components of deep-sea hydrothermal vent communities (Atkins et al., 2000, 2002). Since the sampling of clone libraries in studies of deep-sea microbial eukaryotes has not reached saturation and because of differences in PCR primers used here and in other studies, we do not know the full extent of diversity or the overlap between different marine environments. Two kinds of novel eukaryotic diversity were recognized in Guaymas Basin. The first corresponds to sequences that are unrelated to any other known eukaryotes, and some of which appear to represent early branches in the eukaryotic tree. Berney et al. (2004) suggest that on the basis of rare sequence signatures characteristic for particular groups, the last four of these clones may represent fast-evolving members of well-known groups instead of novel high-level taxonomic lineages. In a more recent study of shallow water anoxic sediments surrounding fumaroles in a submarine caldera (Takishita et al., 2005), novel eukaryotic lineages were also detected, but analyses did not include the partial Guaymas sequences, so a direct comparison of results is not possible. The incompleteness of molecular databases for known eukaryotes makes it probable that there will always be revisions to taxonomic classifications as new sequences are added to these databases. For example, the sequence C1_E027 can now be assigned as belonging to the clade including *Carpediemonas* + *Retortamonas* + diplomonads.

The second type of diversity revealed in the Guaymas survey corresponds to sequences that represent novel, deep branches within well-described eukaryotic clades including the stramenopiles, apicomplexa, dinoflagellates, ciliates, acantharea, and radiolaria. Their depth of branching is commonly comparable to class level differences. We also include within this category, lineages that are closely related to novel, uncultured alveolates (“OLI-” and “DH-” types) obtained in molecular surveys of marine pelagic environments (e.g., López-García et al., 2001; Moon-van der Staay et al., 2001; Moreira and López-García, 2002; Stoeck and Epstein, 2003; Stoeck et al., 2003; Massana et al., 2006; Stoeck et al., 2006).

DNA-based surveys of diversity may not be reliable indicators of viability because it is possible that they include inputs from dead or encysted forms, such as dinoflagellate cysts, that have been detected in marine sediments (Nehring, 1997). However, several groups of sequences affiliate with known specialists of

anoxic environments such as the anaerobic ciliates *Metopus contortus* and *Trimyema compressum*. This is consistent with published accounts of viable flagellate protists from several hydrothermal vent sites including Guaymas Basin (Atkins et al., 2000).

Rapid rates of reproduction allow these small flagellates to colonize temporally and spatially variable habitats such as these fine-particle, hydrothermally heated sediments. Atkins et al. (2000) isolated 18 strains of flagellated protists representing nine species from four Pacific deep-sea hydrothermal vents: Juan de Fuca Ridge (2,200 m depth), Guaymas Basin (2,000 m depth), 21° N (2,550 m depth) and 9° N (2,000 m depth). These vent flagellates belonged to six different taxonomic orders: the Ancyromonadida, Bicosoecida, Cercomonadida, Choanoflagellida, Chrysomonadida, and Kinetoplastida and many are ubiquitous members of marine, freshwater, and terrestrial ecosystems worldwide. The most common kinetoplastids isolated from two vent sites (9° N and Juan de Fuca) were *Rhynchomonas nasuta* and *Bodo saliens*. The only cercomonad isolated was *Massisteria marina*, and this organism was recovered from all four vent sites. The bicosoecids *Cafeteria* sp. and *Caecitellus parvulus* were isolated from 9° N, and the ancyromonad *Ancyromonas sigmoides* was isolated from 9° N. The recovery of many of these organisms from diverse marine habitats suggests that their adaptability makes them relatively easy to grow in the laboratory, and hence their recovery in culturing efforts cannot necessarily be interpreted as indicators of dominant active populations at these sites. However, many of these species are known to be tolerant of a wide range of environmental conditions (Patterson, 1993) including some extreme conditions (e.g., high hydrostatic pressure, and high sulfide and heavy metal concentration) common to hydrothermal vents (Atkins et al., 1998, 2002). Similarly, the Guaymas SSU rRNA gene survey (Edgcomb et al., 2002) recovered sequences encompassing the majority of described lineages in the eukaryotic domain. In addition to anoxic specialists, there exists a diverse collection of eukaryotic microorganisms that is comparable in composition to benthic, pelagic and near surface water populations. These include certain fungi, radiolaria, acantharea, stramenopiles, and ciliates (Lee and Patterson, 1998). For example, in the seawater layer above one core we recovered a specific relative of the ciliate *Euplates aediculatus* (a solid surface-associated filter feeder), described from a variety of habitats including marine sediment interfaces and freshwater pelagic environments (Fenchel, 1987). Bacterivorous heterotrophic flagellates such as the stramenopiles are known to be widely distributed in marine surface waters and to contribute a significant fraction (up to 35%) of heterotrophic flagellates in many regions (Massana et al., 2006), so it is not surprising that they were present in significant numbers in the Guaymas clone libraries from sediment samples spanning the oxic/anoxic interface (Edgcomb et al., 2002).

Sequences of phytoplanktonic taxa, such as green algae and diatoms (Platt and Li, 1986), are from organisms that require solar radiation for active photosynthesis and are therefore not suited to this aphotic environment. However, diatom operational taxonomic units (OTUs) have been recovered in surveys of other

aphotic, deep-marine environments (e.g., Stoeck and Epstein, 2003; Stoeck et al., 2003; Stoeck et al., 2006), and although these sequences may represent dead or dying sinking cells, some of them may represent anaerobic heterotrophic diatoms. Heterotrophy has been observed for some diatoms, and live diatoms have been observed in high abundance in anoxic marine sediments (Hellebust and Lewin, 1977; Admiraal and Peletier, 1979; Li and Volcani, 1987). In SSU rRNA gene clone libraries from Mid-Atlantic Ridge sediments (López-García et al., 2003), phototrophs were not detected, possibly a result of the relatively oligotrophic overlying water column. Some protist groups however have representatives that have been described from both benthic and pelagic environments, making it difficult to distinguish *in situ* populations at deep-sea vent sites in a rRNA-based survey. For example, benthic dinoflagellates are known, but a planktonic origin for some species or contributions of microaerophilic and aerobic particle-attached organisms from the water column cannot be ruled out.

In microoxic deep-marine environments, alveolates seem to dominate the eukaryotic microbial community. This group encompasses the apicomplexa, dinoflagellates, ciliates, and the taxonomically unassigned marine alveolates. The first two groups (Group I and Group II) of uncultured marine alveolates was described by López-García et al. (2001) and SSU rRNA sequences that affiliate with Group I have since been recovered from the Guaymas Basin (Edgcomb et al., 2002), the Mid-Atlantic Ridge (López-García et al., 2003), the coastal Pacific (Moon-van der Staay et al., 2001), and in the Cariaco Basin (Stoeck et al., 2003, 2006). The uncultured marine alveolate Group I contains no named species. Compared with data from the Pacific Guaymas Basin, some protist lineages seem ubiquitous in hydrothermal areas, whereas others, notably kinetoplastid lineages, have so far been detected only in Atlantic systems. Group II is also ubiquitously distributed and may be important ecologically, but it is difficult to interpret the role that these organisms play in the environment because the group is comprised almost exclusively of environmental sequences, having only one cultured representative, *Amoebophrya*, that has tentatively been assigned to this group.

The community structure of the oxic/anoxic interface at deep-sea hydrothermal vents likely includes both microaerophilic and facultative anaerobes, some of which may migrate into and out of the anoxic sediments. The active members of the eukaryotic community likely feed on the abundant bacteria sustained by hydrothermal activity and sedimentary input of organics from the Mexican mainland. López-García et al. (2003) deployed colonization devices containing different organic, iron-rich, and porous mineral substrates that were exposed to a hydrothermal vent fluid source for 15 days at the Mid-Atlantic Ridge. The primary pioneers of the colonization process were bodonids and ciliates 18S sequences have been detected that probably belong to parasitic protist (alveolate) lineages including the Perkinszoa, Apicomplexa, dinoflagellates and ciliates in Pacific (Edgcomb et al., 2002), and Atlantic deep-sea vents (López-García et al., 2003). Moreira and López-García (2003) suggest that this diversity of parasitic protists could be hosted by the relatively abundant animal populations that are

found in close proximity to these deep-sea vent sites, and may be implicated in observed episodes of sudden hydrothermal vent mortality. For example, *Perkinsus* ssp. are known to parasitize a variety of bivalves (Perkins, 2000) and one clone of *Perkinsus* ssp. was recovered from a colonization substrate placed next to a colony of *Bathymodiolus azoricus*, making it likely that these mussels are parasitized by this organism. Additionally, abundant sequences from Apicomplexa, including Gregarines, which are known to be parasites mostly of the digestive tract or body cavities of invertebrates have been detected in these surveys (Edgcomb et al., 2002; López-García et al., 2003; Moreira and López-García, 2003) suggesting that parasitization of different deep-sea hosts plays an important role in deep-sea vent ecology. High numbers of sequences from Group I and Group II marine alveolates in Mid-Atlantic Ridge (López-García et al., 2003), Pacific (Edgcomb et al., 2002), surface Atlantic, Pacific and Mediterranean waters (Díez et al., 2001; Moon-van der Staay et al., 2001), and deep-sea Antarctic plankton (López-García et al., 2001, 2003) 18S clone libraries indicates that these organisms are likely to be ubiquitous in marine environments.

In a study of anoxic sediments in a shallow water (200 m depth) caldera (“Tagiri” site) in Kagoshima Bay, Japan (Takishita et al., 2005) with similar chemical characteristics to the deep-sea hydrothermal vents of Guaymas Basin, a different distribution of SSU rRNA gene phylotypes was observed in spite of overlaps of a few sequences, particularly within marine alveolate Group I (Fig. 1). In spite of the difficulties associated with comparing the results of different molecular surveys as mentioned above, it is worth noting differences in clone library composition between the two different hydrothermal vent environments. In particular, the composition of the alveolates was different in the Kagoshima Bay samples in comparison to other deep-sea hydrothermal vents (Edgcomb et al., 2002; López-García, 2003), with no recovered phylotypes of ciliates, apicomplexans, or Perkinsozoa, and in addition, no kinetoplastids, Acantharia, or Polycystinea were recovered. The observed differences in alveolate community composition between the “Tagiri” site and the Guaymas site studied by Edgcomb et al. (2002) and Mid-Atlantic Ridge site studied by López-García (2003) make sense in light of the fact that Takishita et al. (2005) did not observe multicellular animals (potential hosts) at the “Tagiri” site. The abundance of stramenopile sequences in this and the Guaymas (Edgcomb et al., 2002) surveys (15% and 35% of clone libraries, respectively), some of which are related to planktonic diatoms, plus the detection of potential parasites of diatoms (see Takishita et al., 2005), suggests that these surveys are receiving input from sinking (dead) cells. The existence of encysted forms in the sediments however, cannot be ruled out. The sequences recovered by Takishita et al. (2005) from the “Tagiri” site closely affiliated with those of eukaryotic parasites, such as Phytomyxida and Ichthyosporea. Moreira and López-García (2003) have proposed that parasitic protists inhabiting areas around deep-sea hydrothermal vents are responsible for periodic episodes of massive mortality of dense animal populations at these sites. Since multicellular organisms were not observed in the “Tagiri” sediments based upon

microscopical observation, it is possible that the potentially parasitic forms detected there settled out from the water column above, or else survive in these sediments in a free-living state.

3.2. COLD SEEPS

Cold seeps are sites of low fluid discharge from sea floor sediments that share several attributes with deep-sea hydrothermal vents and with silled basins, including localized higher levels of released of methane and sulfide that usually lead to the development of high concentrations of mat-forming chemoautotrophic bacteria such as the sulfur-oxidizing bacteria *Beggiatoa* sp. and *Thioploca* sp. (Buck and Barry, 1998). High densities of chemosynthetic bacteria may stimulate populations of protists that graze directly or indirectly on these chemoautotrophs. Using a density-gradient isolation technique, five groups of eukaryotes were observed in both seep and control sediment samples in Monterey Bay: euglenoids, ciliates, atestate Foraminifera (allogromiids), testate Foraminifera, and nematodes (Buck and Barry, 1998). In these seeps, nematodes were the dominant eukaryote in terms of biovolume, followed by ciliates (Buck and Barry, 1998). Although the Foraminiferida are considered an aerobic taxon, the first confirmation of their inhabitation of cold seep microbial mats was demonstrated by two independent methods: ultrastructural analysis and adenosine triphosphate (ATP) analysis (Bernhard et al., 2001). Unlike the foraminifers from SBB, these did not exhibit endobionts.

Euglenozoa comprise a significant and diverse component of the protist population associated with the dense mats of chemoautotrophic bacterial mats found in the top 1 cm of sediments at deep-cold seeps in Monterey Bay, California (600–900 m) according to Buck et al. (2000). Many of these euglenozoans have rod-shaped epibiotic bacteria that cover almost all of their cell surface and that contain membrane bound translucent sulfur vesicles containing sulfur particles (Buck et al., 2000; Buck and Bernhard, 2001). Bernhard observed a variety of prokaryote–protist interactions in SBB (see discussion earlier), including the intimate association of flagellates with the sulfide-oxidizing *Beggiatoa*, possibly to gain access to a microhabitat devoid of potentially toxic hydrogen sulfide, as well as flagellate “swarms,” comprised almost exclusively of a single morphotype that harbored ectobionts (Bernhard et al., 2003, see discussion earlier). The tentative identification of the Monterey seep euglenoid ectobionts as sulfur oxidizers is supported by high in situ sulfide concentrations (ranging from undetectable at the sediment/water interface to a maximum of 350 µM) and co-occurrence of sulfur-oxidizing bacterial mats and endosymbiont-bearing bivalves (Buck et al., 2000). Complex associations between anaerobes and aerobes may play a significant role in expanding potential habitats of deep-marine protists.

A molecular survey of SSU rRNA gene amplicons present in the sediments (640 m depth) of the cold methane seeps of the Kuroshima Knoll in the southern Ryukyu Arc, revealed a eukaryotic community with extremely low diversity

relative to other marine environments previously reported, and indicated that the basidiomycetous fungus *Cryptococcus curvatus* was the dominant microbial eukaryote within this chemosynthetic ecosystem (Takishita et al., 2006). SSU rRNA gene sequences of several types of benthic foraminifers were recovered from the sediment surface, however in samples taken from the horizon just below the sediment surface to a depth of 9 cm, the only sequences recovered were associated with this fungus. Basidiomycetous yeasts are known to dominate yeast populations in oligotrophic oceanic waters, and appear to be ubiquitously distributed in freshwater, marine, and deep-sea environments (Nagahama, 2005). The dominance of this yeast in the microbial eukaryote community of any marine environment has not been previously reported, and may suggest a specific local association between this yeast and local seep fauna. The possibility is mentioned by Takishita et al. (2006) that this yeast may be an opportunistic pathogen of the local population of bivalves. The Kuroshima study illustrates the potential for unique protistan communities to be discovered in other deep-sea environments.

4. Concluding Remarks

Marine protists are one of the most abundant eukaryotic groups on Earth, and are thought to play major roles in global carbon and mineral cycles through their contributions to primary production and through grazing of prokaryotes and small phytoplankton (Fenchel, 1986; Caron and Goldman, 1990; Sherr and Sherr, 2000). Initial studies of deep-marine environments, including microoxic and anoxic habitats, support this notion. While reactions mediated by prokaryotes are undoubtedly crucial in redox processes, the role of eukaryotes in biotic redox reactions is also likely to be important. Although most eukaryotic taxa require O₂ to survive and hydrogen sulfide in micromolar concentrations is typically toxic to eukaryotes (e.g., National Research Council, 1979), certain protists and metazoans inhabit marine and freshwater redox zones (Fenchel and Finlay, 1995), often in high densities (e.g., Bernhard et al., 2000). These organisms may be important facilitators of redox reactions because they typically have prokaryotic associates and because most are mobile, enabling them to track shifting redox boundaries. Thus, their activities could affect pore-water solute transport (Pike et al., 2001), thereby affecting redox reaction rates and the mobility of eukaryotes may enable their associated prokaryotes to benefit from transport between oxidized and reduced zones.

Protistan diversity in anoxic environments may be significantly greater than previously thought. PCR-based approaches that utilize a combination of different primer combinations to capture the diversity of a microbial community minimize some of the limitations/biases of these methods mentioned above. The results of a multiple (three primer sets) PCR-primer survey of protistan diversity in the Cariaco Basin by Stoeck et al. (2006) suggest that molecular studies of diversity based upon single PCR-primer sets grossly underestimate the true extent of diversity. All the

sequences they recovered using multiple primer sets were unique. When a 99% sequence similarity cut-off was used to group sequences into OTUs, over 75% of these OTUs were novel, representing deep branches within described protistan groups, and novel lineages that appear unrelated to any known microbial eukaryote. However, analysis of their data suggests that even the multiple-primer approach missed a significant fraction of protistan diversity since only 4% of OTUs were shared between the three clone libraries (Stoeck et al., 2006). A similar result was obtained using a multiple-primer approach in a study of a shallow water supersulfidic anoxic fjord in Framvaren, Norway (Behnke et al., 2006). These studies demonstrate the importance of using a multiple-primer approach for future molecular surveys of diversity, and underscore the fact that continued efforts to sample diversity in different marine environments are essential.

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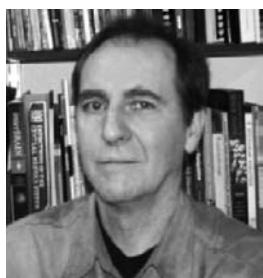
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FUNGAL ASSOCIATIONS AT THE COLD EDGE OF LIFE

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1. Introduction

Antarctica is the coldest, driest, and most isolated continent of our planet. The White Continent can be subdivided in several climatic zones (roughly sub-Antarctic, maritime Antarctic, and continental Antarctic) in which the possibility for life settlement strictly depends on the environmental conditions which gradually become harsher moving from maritime to continental Antarctica and, within the continental Antarctica, moving from the coast to the interior of the continent (Øvstdal and Lewis Smith, 2001). With only two phanerogams occurring at the edges of the continent, Antarctic terrestrial habitats are entirely dominated by lower organisms, including invertebrates, bryophytes, fungi, algae, and diverse prokaryotes. In continental Antarctica no vascular plants are present; the life of terrestrial ecosystems concentrates in the ice-free sites along the coastal areas where lichens, fungi, mosses, and algae grow abundantly; their occurrence decreases towards inland stations where isolated rocks occasionally present epilithic microorganisms, depending on the climate and the rock surface exposition and slope. In the ice-free areas of the McMurdo Dry Valleys (Southern Victoria Land), conditions become even more hostile. There, lichens occasionally colonize sheltered rock surfaces and life mostly withdraws inside porous rocks where milder nanoclimatic conditions are present. These life-forms, named cryptoendolithic, represent the predominant form of colonization of the Antarctic deserts (Friedmann and Ocampo, 1976; Friedmann, 1982; Wierzchos and Ascaso, 2002). The fissures and cracks of granitic rocks from this area are also colonized, by chasmoendolithic organisms (De los Ríos et al., 2004, 2005a, 2007). In these habitats, microbial life apparently meets in rather narrow niches and forms simple or more complex communities.

Lichen symbioses of fungi and algae are one of the most successful obligate associations in the Antarctic desert, and many other organisms like bacteria and nonlichenized fungi have been found to be associated with them. Even though the functional significance of these supplementary associated organisms is not clearly understood, this finding suggests that symbiotic life styles are more complex than previously thought. Among them, the ecological group of extremotolerant black meristematic fungi is always present. These heterotrophic organisms probably benefit to some extent from the adjacent primary producers in such an oligotrophic environment.

The low amount of available substrata, the extreme climatic conditions, the short period favorable for growing, may promote species with a certain degree of plasticity of life styles (Hawksworth, 2005). Black meristematic fungi are known for their ability of colonizing substrata and environments where other fungi fail to survive, such as salt pans, exposed monuments, and rocks (Wollenzien et al., 1995; Sterflinger and Krumbein, 1997; Zalar et al., 1999; Ruibal et al., 2005).

2. Lichen Symbioses in Antarctic Habitats

As a matter of fact, lichens are the visually prominent life forms in most Antarctic ecosystems, yet their diversity is generally lower than in temperate to tropical climates. Their distribution as far as is known was outlined in Øvstedral and Lewis Smith (2001), and reviewed in Kappen (2004). About 430 lichen species of diverse phylogenetic lineages are known so far to occur in Antarctica and South Georgia, and according to this compilation, 88 species of lichens can be found at the “cold edge of life” in Continental Antarctica, and two reach even beyond 86°S (Broady and Weinstein, 1998). Yet, some of the Antarctic species have a very wide geographic distribution, and can be found in habitats of the extreme North or in high mountains (Sancho et al., 1999; Øvstedral and Lewis Smith, 2001). Despite their photobiont species belong to widespread algal lineages (Romeike et al., 2002), the Antarctic strains seem to be adapted to low temperatures (Schofield and Ahmadjan, 1972; Aoki et al., 1998).

Apart from the widespread lichens, about 50% of the lichen mycobiont species is regarded as endemites in Continental Antarctica. This high number of presumed endemites should be interpreted with some caution. As we will see below, lichens show particular morphological adaptations to the hostile habitat and the genetic relationships with close Antarctic and non-Antarctic species still need to be tested in many cases (e.g., Ott et al., 2004). Some preliminary molecular data show that the infraspecific genetic diversity of Antarctic lichen mycobionts or their algae can be considerably low (Dyer and Murtagh, 2001; Wirtz et al., 2003; De los Ríos et al., 2005b).

The ecophysiology of Antarctic lichen has been studied thoroughly. In this chapter we will therefore not focus on ecophysiological studies and the reader is rather referred to the excellent review by Green et al. (1999). According to these authors and referenced articles, there is little evidence for particular adaptations

of Antarctic lichens to Antarctic environments. A general ability to photosynthesize at subzero temperatures, tolerance to low water potentials, the ability of lichens to utilize sublimed water from ice or air, as well as tolerance to high light levels seem key factors for the occurrence of different lichens along a gradient towards more continental conditions of the continent (Green et al., 1999).

2.1. ENDOLITHIC COLONIZATION BY FUNGI AND LICHENS IN THE ANTARCTIC DESERT

Rocks as prominent substrata (Nienow and Friedmann, 1993) appear rarely colonized at the surface of continental Antarctica, because the prohibitive conditions at the surface prevent microbial settlement. Only in maritime Antarctica and in protected niches (e.g., on mosses), substantial amounts of surface-covering lichens can dominate the vegetation. In such habitats, studies of initial stages of lichen colonization suggest that lichen algae can be acquired from free-living forms (see Schroeter and Sancho, 1996). If this is also the case under harsher conditions, has not been studied so far.

To escape the harsh conditions of the outside, microorganisms adopt a particular lifestyle, and live as so-called cryptoendolithic and chasmoendolithic (Golubic et al., 1981) communities (Fig. 1) in microscopic niches inside the rocks (Nienow and Friedmann, 1993; De los Ríos et al., 2002) (Fig. 1). The reasons why microorganisms colonize the inside of rocks rather than their surfaces lies in the significant nanoclimatic changes occurring over a distance of a few millimeters within the rock substratum (Friedmann and Weed, 1987; Nienow and Friedmann, 1993) and the formation of certain microenvironments associated to the endolithic growth (De los Ríos et al., 2003).

The temperatures at rock surfaces during winter – e.g. ranging in 1985 from -47.2°C to -19.4°C on Linnaeus Terrace in the McMurdo Dry Valleys - generally lies 2°C below the outside air temperature (Friedmann et al., 1987); in summer

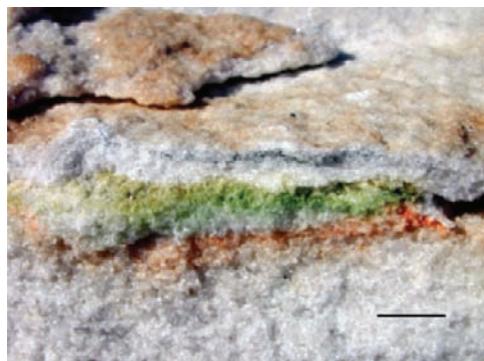


Figure 1. Cryptoendolithic community with well defined zonation. Bar = 1 cm.

the temperature at the sloped rock surface, which for instance in January 1985 on Linnaeus Terrace ranged from -14.1 to 17.2°C , may reach 20°C above that of outside air (Nienow and Friedmann, 1993; Friedmann et al., 1994). Large differences in microclimatic conditions are correlated with exposition. Wide thermal fluctuations at exposed sites contribute to the practically sterile conditions at the outside, while within the rock the nanoclimate provides milder and more stable conditions (Nienow and Friedmann, 1993).

Communities of chasmoendolithic lichens are also common in granitic rocks. Cells of both, algal and fungal symbionts occupy fissures and cracks of the lithic substrate with no clear heteromerous structure (De los Ríos et al., 2005a). This kind of growth has been recognized and characterized for two species of *Lecidea* in Granite Harbour area (Ross sea coast; De los Ríos et al., 2005b). The widespread distribution of this life-form in ice-free areas in Antarctica could be favored by the high frequency of rock-freeze-fracturing phenomena (Cowan and Tow, 2004).

In material from the McMurdo Dry Valleys, de la Torre et al. (2003) found close similarities of DNA sequence data suggesting the endolithic presence of *Buellia* spp., a genus that is also found in this habitat with epilithic ascomata. A closer assignment of this *Buellia* species is not possible due to the limited amount of data on this large lichen genus. Nonetheless, species of *Buellia* and other genera preserve the ability to form a thallus with characteristic morphology as soon as they reach protected niches (Fig. 2), such as small depressions, fissures or crevices, on rock surfaces, where epilithic structures can be observed.

Antarctic cryptoendolithic microorganisms apparently constitute very simple communities comprising only few species (Nienow and Friedmann, 1993). The most common and extensively studied is the “lichen-dominated community” in sandstone (Friedmann, 1982). This community colonizes porous rocks up to about 10 mm deep under the rock surface. Here the microbial growth leads to a typical stratification characterized by variously colored and biologically distinct bands



Figure 2. Superficial thalli of *Lecanora fuscobrunnea* in the McMurdo Dry Valleys.

(Fig. 1). This stratification is maintained because of the different physiological requirements (e.g., exposure to different solar radiation) and abilities (production of antimicrobial substances; Ocampo-Friedmann and Friedmann, 1993) of each microbial type. The community is usually organized as follows. A black zone is visible immediately under the reddish brown crust at the rock surface, followed by a white band, a green band, and sometimes a blue-green band. In the black and white zones, fungi and chlorococcal algae together form a lichen association. The fungal hyphae in the upper dark zone are melanized, while in the white zone they are colorless; they might also include different morphotypes of the same species with the phenotypic modification as a most likely answer to higher light intensities at the upper layers.

Other thick-walled and dark-pigmented, nonlichenized fungi, called rock black fungi, grow mixed with the lichen-forming fungi in the black zone. These fungi, often showing meristematic growth in vitro, are isolated in pure cultures as regular members of the lichen dominated cryptoendolithic community (Ruisi et al., 2007). These fungi will be discussed in more detail below. The green band is characterized by the presence of nonlichenized algae, among which the endemic chlorophycean alga *Hemichloris antarctica* is predominant, and several of the most extremophilic prokaryotes known to date, such as the cyanobacteria *Chroococcidiopsis* sp. and *Gloeocapsa* sp. (Friedmann, 1982). A further band has been occasionally observed when *Chroococcidiopsis* sp. forms a separate zone below *H. antarctica*. The leaching due to the acid production, particularly oxalic acid by fungi (Nienow and Friedmann, 1993) throughout the colonized zone, causes the accumulation of iron compounds in a red lower zone (Fig. 1).

Antarctic endolithic communities comprise microorganisms in different physiological state. The coexistence of living and dead microorganisms in these communities has been demonstrated by the use of diverse fluorescent probes with confocal scanning microscopy and the analysis by transmission electron microscopy (De los Ríos et al., 2004). When the biological activity in colonized rocks is interrupted, extinction and fossilization take over. A large percentage of endolithic colonization in the Antarctic deserts is dead and in various stages of fossilization. Depending on environmental conditions prevailing at the time of extinction, the process of fossilization can take two different paths, depending whether the organic material of the microorganisms has been preserved or not.

Presumably in the presence of liquid water, the microbial organic matter is decomposed but the leached colonized zone, as well as the characteristic weathering pattern on the surface are silicified and fully preserved (Friedmann and Weed, 1987). This is a typical trace fossil, easily identifiable by the color pattern on the surface of rock as well in the former colonized zone.

When extinction and fossilization takes place under dry conditions (like in the present), the process of fossilization is different. Organic structures collapse and become desiccated, but fine structural details, recognizable only with the electron microscope, are fully preserved. Some specimens show no trace of silicification, while others are silicified to varying degrees. This type of fossils has

been studied by transmission electron microscope (Friedmann and Koriem, 1989), by back scattered scanning electron microscopy combined with energy dispersive X-ray spectroscopy (Wierzchos and Ascaso, 2001; Ascaso and Wierzchos, 2003; Ascaso et al., 2005; Wierzchos et al., 2005) and by proton induced X-ray emission (Wierzchos et al., 2006), in view of their significance for Martian life investigations. In the last years, different inorganic deposits and/or physicochemical bioweathering mineral patterns have been also recognized as biomarkers of the previous presence of microbiota (Ascaso and Wierzchos, 2003; Wierzchos et al., 2003).

2.2. LICHENS AS EXTREMOTOLERANT ORGANISMS

In the Dry Valleys of Antarctica, low temperatures and the absence of water are the most important factors limiting lichen colonization. Endolithic lichens could have been able to tolerate the low temperatures of the Martian surface in the past, and especially the rock subsurface. Nevertheless, the lack of a ready supply of surface water could become a significant problem for the lichens. On the other hand, Ascaso et al. (2003) observed, through low temperature scanning electron microscopy (LTSEM), that ice crystals indicate the presence of water in the dehydrated hyphae of the fungal partner of epilithic lichens from Antarctica and suggested that under conditions of drought, the presence of small quantities of water in the apoplast may explain the survival of the dehydrated thallus. A mechanism of loosely-to-tightly bound water transfer was suggested as, at least partially, responsible for a freeze-protection of lichen thallus, without a concomitant increasing of cryoprotectants (Haranczyk et al., 2003). In addition, in regions of high light intensity, a lower amount of light reaches the lichen zone inside Antarctic rocks, while the dark-pigmented fungal layer screens the harmful UV, a process that would have been even more important in Martian rocks (Armstrong, 2003). Remarkably lichen associations are able to integrate photo-protective systems which provide higher resistance, by order of magnitude, to oxidative damages than both alga and fungus alone (Kranner et al., 2005). An increasing of phenolic compounds content under UV exposition, which both prevent UV penetration into lichen thallus and play a protective role as antioxidants, has also been observed (Buffoni Hall et al., 2002).

Lichens seem generally extremely resistant to high UV radiation and space vacuum. Intact lichens, even of temperate habitats, were extremely resistant to vacuum pressure and to UV radiation to doses up to 160 kJ m^{-2} , as assessed by confocal laser scanning microscopy using the LIVE/DEAD staining method (de Vera et al., 2003). Simulation of space conditions showed that lichens could withstand extended periods of outer space conditions. Even after 16 h, 50% of the *Xanthoria elegans* ascospores enclosed in ascocarps were able to germinate (de Vera et al., 2004a). A further investigation (de Vera et al., 2004b) demonstrated that the symbiotic state of the lichen thallus is more resistant as the isolated

bionts to UV radiation and vacuum space environmental parameters. They demonstrate the peculiar function of symbiosis in a lichen system to withstand extreme environmental conditions. On the basis of this high level of resistance, lichen symbiotic organisms may be also model organisms for an interplanetary transfer. In fact, the survival capability of lichens exposed to space conditions has been recently established during the first exobiology experiment carried out with lichens aboard BIOPAN 5 (facility of the European Space Agency located at the outer shell of the Russian Earth orbiting FOTON M2 satellite). After a space flight of two weeks, the bipolar species *Rhizocarpon geographicum* and *Xanthoria elegans* contained a majority of living cells and showed nearly the same photosynthetic activity as measured before the flight (Sancho et al., 2007).

Xanthoria elegans is a widespread and particularly extremotolerant lichen, that is also known from the ultimate altitudes of life (Khumbu Himal, 6,400 m, specimen in herbarium GZU, Institute of Plant Sciences, Graz). One other noteworthy species from Antarctica is also *Lecanora fuscobrunnea*, which seems to be one of the very few species that are able to form a superficial thallus in the McMurdo Dry Valleys (Fig. 2). The species belongs to the *Lecanora polytropa* group, which is generally well coping with extreme habitat conditions. In contrast to typical members of the *L. polytropa* group, *L. fuscobrunnea* is characterized by rather dark thalli and shows some peculiar anatomical adaptations. The hymenia are sometimes poorly developed in the ascomata, but also in cases where hyaline ascospores are readily formed, the tips of the paraphyses may become short-celled and constricted at the septa. Consequently, the hymenial surface can be covered by rounded melanized cells which could function as thallospores (i.e., mitotic spores; Poelt and Obermayer, 1990). Cultivation experiments with this lichen revealed another, distinct black fungus, which is identical in sequence to strains found in cryptoendolithic lichen-dominated communities in these environments. By scanning electron microscope observation, this supplementary fungus seems to be located as an endogenous associate within the lichen thallus (Fig. 3).

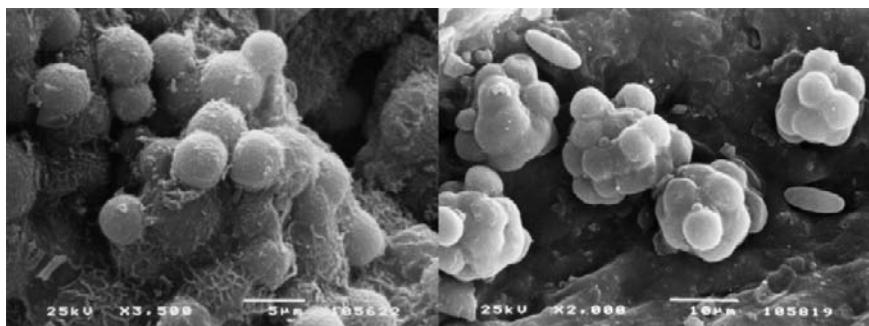


Figure 3. SEM observations of (left) a black fungus isolated from a cryptoendolithic community and (right) of an ITS identical strain within a *Lecanora fuscobrunnea* thallus.

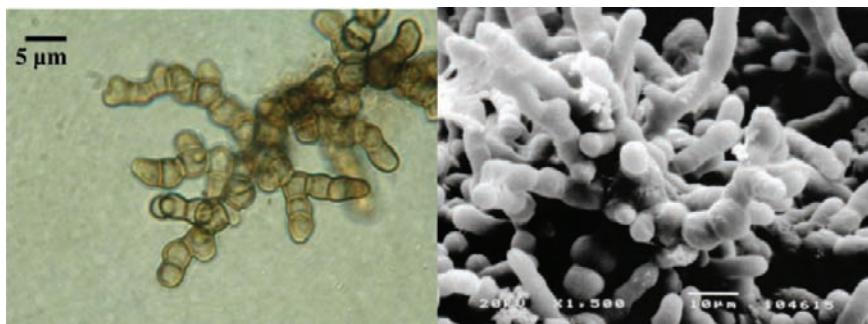


Figure 4. *Friedmanniomycetes endolithicus*. Light microscopic image (left) and scanning electron microscopic image (right) of hyphae.

3. Nonlichenized Fungi in Antarctic Habitats

Mycological studies in Antarctica have a centenary tradition. From the pioneering studies carried out at the beginning of the twentieth century (Bommer and Rousseau, 1900; Tsiklinsky, 1908; Gazert, 1912; McLean, 1918) up to date, with the last contributions provided by molecular studies (de la Torre et al., 2003; Clark et al., 2004; Peck et al., 2005; Selbmann et al., 2005), a huge amount of information on continental Antarctic mycodiversity has been collected. Continental Antarctic mycoflora is mainly composed of anamorphic fungi (filamentous fungi and yeasts), some *Zygomycetes*, very few species of *Ascomycetes* and very rare *Basidiomycetes*; 1,627 fungal or pseudofungal records, belonging to 140 genera and 247 species and infraspecific taxa, were reported (Onofri et al., 2007b). Microorganisms have been isolated from different substrata, mainly as saprotrophs: soil, animal dung, mosses, lichens (e.g., Möller and Gams, 1993), rocks; among these, rock is probably one of the less studied for searching non-lichenized fungal species. The diversity of microorganisms occurring on the rock surfaces or inside them is complex and not sufficiently investigated, but the increasing interest in these previously largely ignored fungi will probably demonstrate a much more marked presence in Antarctica (Selbmann et al., 2005).

3.1. ADAPTATIONS OF ANTARCTIC NONLICHENIZED FUNGI

One of the main characteristics of the Antarctic environment is represented by cold. Mean monthly temperatures in winter range from values below -15°C and -10°C in maritime Antarctica, to below -30°C in the slope zone of continental Antarctica, down to values lower than -50°C on the ice-plateau (Lewis Smith, 1984; Øvstdal and Lewis Smith, 2001). Large seasonal and annual variations in temperature also affect the Antarctic terrestrial communities. Temperature can

reach values allowing a fungal growth only during the summer period, although daily fluctuations, depending on cloudy or sunny days or presence of winds, frequently occur. Temperature growth tests revealed a large number of cold-tolerant mesophilic strains within the filamentous fungi (Kerry, 1990; Zucconi et al., 1996; Azmi and Seppelt, 1997), due to the more favorable microclimatic conditions occurring in protected niches, where temperature values may be much higher than the ambient ones. Moreover, fungi often show a wide temperature range for growth, possibly as a response to the unstable environmental conditions that characterize the Antarctic environment, with extreme temperature fluctuations.

When temperatures reach values low enough to reduce a normal growth rate but not enough to cause ice formation, cell membrane fluidity and transport of components through it decrease. A strategy, common to many organisms throughout the biological world, to maintain membrane fluidity, is the production of higher polyunsaturated fatty acids amounts with decreasing temperature. Moreover, if enzymatic activity is temperature dependent, at low temperatures metabolic activity can be enhanced by realizing either greater amounts of extracellular degradative enzymes or enzymes capable of catalyzing at low temperatures. As an example, an Arctic isolate of the bacterium *Colwellia* cfr. *psychrerythraea* synthesizes either greater abundance of proteases in cultures grown at -1°C than at 8°C, and proteases having lower temperature optima than previously expected (Huston et al., 2000). In some Antarctic fungi cold-active enzymes are produced, while other fungi show a wide enzymatic competence in order to enhance the kind of substrata possibly available (Fenice et al., 1997, 1998).

Most of the Antarctic freshwater is frozen as snow or ice, so Antarctic terrestrial microorganisms experience extreme desiccation, often enhanced by evaporation due to high winds. Presence of available melting water strongly influences fungal distribution over the continent, determining the prevalent fungal occurrence along the coastal sites, where milder conditions exist. Low water activity and osmotic stress often occur in the meantime. Several compounds, acting as osmoregulators, are intracellularly produced by xerophilic, xerotolerant, halophilic, and halotolerant microorganisms, as melanins, mycosporines, sugar, and sugar derivates (Volkmann et al., 2003; Grant, 2004; Kogej et al., 2004, 2006; Gunde-Cimerman et al., 2005). The most important ones among these are glycerol, trehalose, and mannitol (Brown, 1978; Feofilova et al., 1994; Weinstein et al., 2000). Trehalose, in particular, seems to be active against different environmental stress conditions, and its production seems to be induced by more stressors, as low and high temperatures, dehydration, desiccation, and freezing (Robinson, 2001). The polyols of lichen photobionts (e.g., ribitol) that are produced as transport sugars for the symbiotic partner are certainly also important osmolytes.

Low temperatures also affect the viscosity of extracellular solutions, which increases with temperature decreasing. Production of exopolysaccharides and their role as protective agents against damages caused by repeated freeze-thawing cycles was demonstrated in an Antarctic strain of *Phoma herbarum* Westend.

(Selbmann et al., 2002); a similar role can be played by the extracellular polymeric layer, probably polysaccharides, surrounding fungal structures (hyphae and conidia) common in the mycological Antarctic compartment. Moreover, exopolysaccharides seems to play an important role on extremotolerance enabling microorganisms to cope with different stressors; in fact, the ability to produce these substances is shared by microorganisms colonizing very diverse extreme habitats (Gunde-Cimerman et al., 2004). Alterations in the lipid compositions and intra- and extracellular soluble carbohydrates contents, have been observed in Antarctic strains of *Geomyces pannorum* (Link) Sigler and Carmichael, *Mortierella elongata* Linnem., and *Humicola marvinii* M.E. Palm and Weinst., grown at low temperatures (Weinstein et al., 2000).

When temperatures drop till inhibiting every metabolic activity, fungi presumably enter in a dormant state. Anhydrobiosis, that means survival in a complete dehydration state, enables to overcome the most unfavorable environmental conditions without metabolic activity, and strategies adopted to prevent ice formation mainly involve sugar production. Anhydrobiosis could also constitute a survival mechanism that allows wind dispersal of these fungi, as it does occur for other organisms in the McMurdo Dry Valleys (Nkem et al., 2006).

How terrestrial Antarctic fungi respond to enhanced UV-B radiation during ozone depletion in spring? Protection is achieved either by storing higher quantities of UV protective pigments or growing beneath soil surface particles, where shading reduces radiation flux. Pigmentation is universally recognized as an adaptive response to high solar radiation and many sun-screening pigments have been reported for fungi and lichens, as carotenoids and mycosporines (Young and Patterson, 1982; Arcangeli et al., 1997; Büdel et al., 1997; Arcangeli and Cannistraro, 2000; Torres et al., 2004). An adequate protection is often achieved by a combination of different pigments; they dissipate excessive energy from increased UV-B radiation and avoid the generation of toxic single oxygen (Wynn-Williams and Edwards, 2001). Also, biogeological strategies could be combined with the production of pigments. The displacement of potentially protective minerals onto the rock surface has also been detected in Antarctic endolithic communities (Villar et al., 2005).

4. Antarctic Rock Black Fungi

Black fungi – also called black meristematic fungi, black yeasts, or microcolonial fungi (Sterflinger, 2006) – living on or within the rocks are a taxonomic and phylogenetic heterogeneous group of fungi sharing similar morphological and physiological characteristics as response to a combination of similar stress factors (adaptive convergence), mainly represented by oligotrophic nutrient conditions, extreme temperatures, low water availability (due to temperature, wind, and extremely low precipitations), UV radiation, and osmotic stress. These environmental extremes act simultaneously in the Antarctic, but a combination of some of them can be experienced by organisms in other regions of the world.

4.1. DISTRIBUTION AND ADAPTATIONS OF ROCK BLACK FUNGI

Rock black fungi have been isolated from different environments, also from geographically very distant regions, as the Antarctic and the Mediterranean areas. Adaptive convergence involves different morphological and physiological features, such as a slow expanding cauliform-like colony, barely differentiated structures, and the presence of thick and heavily pigmented cell walls. Some rock black fungi have been isolated from the Antarctic cryptoendolithic lichen-dominated communities colonizing the inside of the sandstones in the Dry Valleys (Southern Victoria Land), and from different rocks in Northern Victoria Land.

Some of them have already been studied using both morphological and molecular approaches (Selbmann et al., 2005). Other strains have been isolated and wait to be studied, and always new strains are continuously isolated, from both Southern and Northern Victoria Land, suggesting that, in those specific niches, this group can be considered among the most commons and the absence of previous records of such peculiar group of fungi highly adapted to the Antarctic environment has to be mainly ascribed to the different kinds of substrata studied in mycological works up to date. Their extremotolerance, allowing their survival and growth in environments that are inhospitable for other microorganisms, suggested them as models to test their survival under space conditions.

Each of the morphological and physiological characteristics of the rock black fungi has an adaptive significance. Their morphological features make them suitable to withstand harsh environmental conditions; for instance, they show meristematic growth as stable character which allow them to keep optimal volume/surface ratio in order to minimize exchange with external stressors. Another remarkable character is the production of extracellular polymeric substances (EPS), possibly exopolysaccharides, which is involved in the protection against cycles of desiccation, freezing, and thawing (Selbmann et al., 2002, 2005); EPS surround fungal structures (hyphae and conidia), as observed both in nature and in culture, and create and maintain the proper microenvironmental conditions in the biofilms inside the rocks buffering pH and nutrient availability fluctuations (De los Ríos et al., 2002, 2003). The accumulation of EPS is also present in Antarctic lichenized fungi (De los Ríos et al., 2005a) and other endolithic microorganisms (De los Ríos et al., 2004), thus it could be an universal protector mechanism. Strains of the Antarctic genus *Cryomyces*, presumably thanks to their highly pigmented structures, showed a higher resistance to UV radiation doses compared with that of a strain of the Antarctic filamentous species *Arthrobotrys ferox* Onofri and Tosi, the latter having a UV resistance higher than an European strain of *Arthrobotrys oligospora* Fresen (Zucconi et al., 2002; Onofri et al., 2007a).

A reduction in the thickness of the niche in which terrestrial algae can survive may occur, because higher UV-levels may increase the depth of the zone of photoinhibition but not the depth to which algal photosynthesis can happen (Hughes, 2006); similar changes could also happen in the thickness of the cryptoendolithic communities.

Besides a large number of cold-tolerant mesophilic strains of filamentous fungi, cryptoendolithic rock black fungi show a more psychrophilic behavior, even if their minimum temperatures for growth are not known. A selection of black fungi tested for temperature preferences, were able to grow in the range between 0°C (minimum value tested) and 20–25°C, with optimal temperatures generally higher than those experienced *in vivo* (Selbmann et al., 2005). This situation has been also recorded for other members of the cryptoendolithic community, both autotrophs and heterotrophs, while the photosynthetic optimum for the community falls within the temperatures prevailing in nature. This apparent paradox has been recently clarified; in fact, it has been demonstrated that both lichens and lichen-dominated communities can adapt to a wide range of thermal regimes by regulating producers/consumers balance; therefore single microorganisms, not itself fully adapted, find their chance of surviving in the additional and emergent properties of the whole community (Friedmann and Sun, 2005; Sun and Friedmann, 2005).

In general under stress conditions fungi give up sexual reproduction as a simplification of their life cycles, which can be concluded in shorter time and without high metabolic costs. The Antarctic *Thelebolus* species reproduce sexually but it has been recently reported that the endemic species *T. globosus* Brumm. and de Hoog and *T. ellipsoideus* Brumm. and de Hoog, living in the biomat of some Antarctic lakes, show a strong reduction of the ascocarps compared with the species living in more permissive conditions and produce anamorphs as adaptations at the extreme conditions of sealed Antarctic lakes (de Hoog et al., 2005).

Cryptoendolithic black fungi show an extreme simplification of their life cycles: in fact, most of them are even unable to produce complex and well-differentiated anamorphic reproductive structures, and propagules are produced directly from disarticulation of toruloid preexisting hyphae. Some of them are mostly yeast-like organized and can formally conclude their life cycles with the production of a single cell being itself a resistant propagule. These high levels of simplification match very well with the prohibitive environment they colonize where the climatic conditions for active life happen just few days a year. Growth is restricted to the short time in which favorable conditions do exist and a metabolic inactivity probably characterizes fungal surviving during the long Antarctic winter.

Finally, black fungi are usually able to metabolize a wide selection of carbon sources, including D and L forms of sugars but, in some cases, also hydrocarbons. These abilities allow them to colonize oligotrophic environments where they can survive metabolizing dusts and pollutants blown by the winds. In the meantime they require lot of energy to synthesize high-costs molecules necessary for their survival such as melanins, or exopolysaccharides, which allow them to cope with many different stressors (Ruisi et al., 2007). As a consequence, black fungi show a very slow growth rate and do not produce secondary metabolites such as organic acids or antibiotics. Therefore, they are unable to compete with fast-growing cosmopolitan fungi which thrive in permissive conditions of rich-nutrients

environments and they remain confined in extreme environments, finding in extremotolerance the chance of surviving (Sterflinger, 2006).

Chertov et al. (2004) proposed a simulation growth model for a single fungal microcolony, considering it as the main and basic component of a complex system; a continued lack of available organic nutrition was reported as the dominating environmental factor limiting rock black fungi growth, making them good candidates to conquer environments hostile for any other growth form (Chertov et al., 2004).

4.2. PHYLOGENETIC PLACEMENT OF ROCK BLACK FUNGI

The massive increase in molecular studies in last decades has produced a wide number of data from microbial genomes, which represent an invaluable resource for comparative analyses. The increasing number of Antarctic environmental genomic projects will increase the amount of molecular information available in the near future, providing tools for studying natural selection as well as the link between organisms and environment (Clark et al., 2004; Peck et al., 2005).

Phylogenetic studies on cryptoendolithic Antarctic organisms are particularly intriguing; they colonize a strictly sealed environment that became isolated from the global gene pool over a timescale of evolutionary significance. Furthermore, the absence of interactions with higher organisms such as plants or animals makes them simple and fine models for general questions about evolution. It is known that isolation and environmental pressure deeply influence the degree of adaptive radiation and endemism formation (Vincent, 2000). In fact, phylogenetic analyses, based on both ITS and SSU ribosomal genes, carried out on a selection of Antarctic cryptoendolithic black meristematic fungi highlighted the presence of unique genotypes, clearly distinct from any other fungal lineages known at the moment. Some of these are apparently confined to the McMurdo Dry Valleys. On the basis of these results two new endemic genera (*Friedmanniomyces*, see Fig. 4, and *Cryomyces*) with four new endemic species (*F. endolithicus*, *F. simplex*, *C. antarcticus*, and *C. minteri*) have been published (Onofri et al., 1999; Selbmann et al., 2005), but many other unidentified new taxa have been noticed and are waiting to be described. These new genotypes could have evolved as results of the strong environmental pressure promoting speciation processes even more rapidly than those taking place in more permissive climates.

The clades of *Friedmanniomyces* and *Cryomyces* do not suggest a close phylogenetic relationship of these genera, whereas their grouping with clades of other extremotolerant fungi (e.g., from acidic or in salt environments, or rock-inhabiting fungi), rather indicates an independent origin. When Antarctica was located at higher latitudes and was characterized by milder climatic conditions, the mycodiversity on/within rocks was similar to that found in other parts of the world, including fungi predisposed to endure extreme temperatures. After the continent moved to the South Pole and became cold and dry as we know at present, selection

may have favored extremotolerant species among black fungi. This hypothesis suggests that the phylogenetic history and adaptation of these microorganisms could be a consequence of the geographic isolation and the cooling of the Antarctica in the South Pole, in a period ranging 60 and 30 million years ago.

It is known that the environmental conditions of the McMurdo Dry Valleys are similar to those on Mars during its early history, and this fact drives speculations about the possible pathway for Martian life, if it ever existed. In fact, it is possible that microbial life-forms experienced similar environmental constraints as the Antarctic cryptoendolithic associations, and found the last niches for surviving by withdrawing inside the rocks, before its ultimate extinction during the cooling of the Red Planet.

5. Conclusions

Environmental stresses concerning low water availability (low precipitations, extremely rare in some zones, low relative humidity, desiccating winds), low temperatures, and high spring UV irradiation characterize climates of the Antarctic and produce severe limitations to microbial growth. Despite such stresses, microbial communities colonize almost all terrestrial niches; even in the most prohibitive area of the Antarctic desert, some among the most extremotolerant microorganisms live together and find proper conditions for growth, coexistence, and potential interactions in rocks microhabitats. Black meristematic fungi are particularly suited to cope the harsh conditions by morphological and physiological adaptations. For instance, they show meristematic growth as stable character which ensure an optimal surface/volume ratio to the colony; the cells are characterized by the presence of thick and strongly melanized walls, allowing them to resist to dryness and high UV exposition; hyphae are surrounded by extracellular polymeric substances, probably polysaccharides, which may protect from desiccation and freeze damages. Another successful adaptation is the extreme simplification of the life cycles, which can be concluded within the very short time during which active life is possible, by producing even single cells being at least themselves resistant propagules. Presumably, they could also escape stresses by switching in a dormant state when conditions become too harsh, remaining metabolically inactive until conditions are once again suitable for growth and metabolic processes can be resumed promptly. The same is well known from lichen symbiosis. Lichenized fungi follow a different strategy to cope with the hostile conditions by forming symbiotic physiological units with algae. Both obligate partners in these symbioses contribute to a mutually beneficial antioxidant protective system (Kranner et al., 2005). Moreover, sugar alcohols, as photosynthetic products by the algae, can also act as osmolytes in lichens, and allows them to pursue a coordinated metabolism also under conditions that are too stressful for other most organisms. Thus the lichen “system” seems perfectly organized to endure extreme conditions for life.

The rather narrow suitable microhabitats in extreme environments allow only phenotypically preadapted organisms to co-occur. If these are still different enough to offer a potential benefit to unrelated neighbors, and if competition is low, this may result in more or less distinct, mutualistic interactions. While lichens represent clearly an obligate symbiosis, the observation that microcolonial rock-inhabiting fungi may also undergo interactions with lichen photobionts (Øvstedral and Lewis Smith, 2001; Gorbushina et al., 2005) could point on a facultative case. It still remains to be addressed to what extent bacteria or their products are involved in these microecosystems.

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Biodata of **Wladyslaw Altermann**, author of the chapter “*The Early Earth’s Record of Supposed Extremophilic Bacteria and Cyanobacteria, at 3.8 to 2.5 Ga*”

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THE EARLY EARTH'S RECORD OF SUPPOSED EXTREMOPHILIC BACTERIA AND CYANOBACTERIA, AT 3.8 TO 2.5 GA

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1. Introduction

The unambiguous evidence for the presence of life in the Archean is only limited by the preservation potential of sedimentary rocks. Throughout Earth's preserved sedimentary deposits, prokaryotic bodily fossils and geochemical fossils, for example, products of the Calvin-cycle dependent carbon isotopic fractionation, can be found. Nevertheless, irreproducible analyses in organic geochemistry, misinterpretations of artifacts from sample preparation and of organic contaminants, and uncertainties on the age and nature of the Archean rock formations are copious in evaluation of the earliest traces of life.

The understanding of geological processes strongly influence discussions of the ancient, supposed biological relicts from c. 3.8 billion years old (3.8 Ga) metasedimentary rocks. The evidence for prokaryotic bodily preserved microfossils of the Neoarchean, at 2.7 to 2.5 Ga is by orders of magnitude stronger, as rocks of this age are abundant and better preserved.

The enduring discussion of the significance of carbon isotopic measurements and microscopic carbon inclusions in rocks from Greenland, considered metasediments of 3.6 to 3.8 Ga (Schidlowski et al., 1979; Mojzsis et al., 1996; Lepland et al., 2002; van Zuilen et al., 2002; Rosing and Frei, 2004) became heated in recent years. The controversy was further ignited by the surprising claims of misinterpretation of what was until then *uni sono* recognized as the world's oldest bodily preserved microfossils, from the 3.46 Ga sedimentary rocks of Western Australia (Schopf, 1993; Brasier et al., 2002; Schopf et al., 2002). These claims have provoked a critical look at Archean paleobiology by the scientific community interested in the exploration of the oldest traces of life on Earth. The credibility of early Archean traces of life is being painstakingly restored by detailed, *in situ* microanalyses and elaborate field work (e.g., Schopf, 2004; Altermann, 2005; Allwood et al., 2006; Tice and Lowe, 2006a, b; Ueno et al., 2006a, b).

The aim of this contribution is to review and assess the nature of the microfossil evidence from the first c. 1.5 billion years of sedimentary record from the point of view of a “bio-geologist.” Processes ruling the preservation of ancient microbial cells and communities, their recognition in the rock matrix and the possibilities of

postdiagenetic contamination of rocks by younger bacterial communities and *postmortem* bacterial remains are discussed.

2. General Geological Constraints on Microfossil Preservation

Sediments formed by precipitation and fallout from suspension are buried by successively younger deposits and undergo diagenetic processes of dewatering, compaction, cementation and recrystallization. Organic remains in sedimentary environments are prone to *postmortem* degradation, before the onset of diagenesis, as they are subject to lysis and decomposition by heterotrophs. Therefore, they rarely reach the process of diagenesis in their original form, but mostly as indigestible remnants and leftovers buried in mud, sand or in carbonate and silica precipitates. With burial they become rescued from further organic decomposition. With increasing temperature and pressure however, sediments lithify (become "rocks") and successively enter metamorphic conditions between 100–200°C and 2–4 kbar. During this process the trapped organic matter undergoes significant changes, leading to maturation. Rearrangement of the bonding in C–H–O–N ring compounds and other elements of life is the consequence. These processes, crucial for the development of the hydrocarbon deposits on Earth are well investigated. Microbial catalysts under elevated temperature of up to above 80°C, can be responsible for hydrogenation processes and formation of steranes from algal sterols next to abiotic H₂S reaction with organic molecules, at the aerobic-anaerobic boundary in the sediment (Hayes, 2006; Hefting et al., 2006). Nevertheless, bodily fossils and macroscopic organo-sedimentary structures embedded in and penetrated (petrified) by minerals, may be well-preserved at low metamorphic P/T conditions. Thus, original depositional environments can be thoroughly reconstructed from the sedimentary record.

With progressively rising P/T conditions, mineralogical changes in sedimentary rocks become so severe that the prospects of preserving microstructures of organic origin diminish rapidly. Because of the growth force of new minerals (neomorphism), microscopic organic remains have no chance of being preserved in their original morphology. Organic matter is pushed along the new crystal boundaries and trapped in minerals and inclusions, and converted to highly indurated kerogenous material. The kerogen is microscopically amorphous, displaying typical peaks in Raman spectroscopy. The destruction of cellular morphology depends not only on the degree of metamorphism, but also on the mineralogy of the embedding material. Because of the tendency to build a very fine, sub-micron crystalline mosaic, cherts (SiO₂) preserve microfossils best, up to about greenschist facies metamorphism (c. 300°–450°C and 3–7 kbar), but carbonates are mostly destructive. It has been demonstrated on nano-scale, that even excellently conserved Precambrian microfossils in cherts do not preserve a continuous carbon film in their walls. The carbon is disrupted and arranged in few tens of nanometer spaced platelets between the SiO₂ crystallites (Kempe et al., 2005).

Carbon preserves its primary isotopic composition and can be diagnostic of Rubisco type enrichment of lighter ^{12}C and discrimination against ^{13}C , in greenschist facies metamorphism. From P/T conditions of approximately low amphibolite facies, at some 500°C and 5.5 kbar, metamorphism progressively and irreversibly destroys the remnant morphology of organic microstructures and the carbon isotopic signature. Sedimentary structures allowing for the reconstruction of the original depositional environment can survive metamorphism of almandine–muscovite facies, approximately corresponding to P/T conditions of up to 600°C and 6 kbar. Under favorable conditions (e.g., primarily distinct vertical change of material or grain sizes), this is also possible in folded, lustrous (micaceous), garnet bearing schists.

The oldest reported sedimentary rocks preserved on Earth, between 3.7 and 3.8 Ga, are located in the Isua and Akilia Provinces of Greenland and are metamorphosed to amphibolite facies and above it. The original nature (sedimentary or not) of the Isua and Akilia metamorphic rocks is highly doubtful. The stratigraphic subdivision and timing of sedimentation are heavily obliterated by subsequent processes of tectonic thrusting and dislocation, faulting and metamorphism and thus brusquely disputed (e.g., Fedo and Whitehouse, 2002; Friend et al., 2002; Mojzsis and Harrison, 2002). The metamorphism in the 3.7 to 3.8 Ga rocks is polyphase, implying that the rocks have been successively brought into varying P/T conditions during their history. Metamorphism is much younger than the rocks themselves. Metamorphic peaks postdate the origin of the rocks by hundreds of millions of years. Myers (2004) has argued that virtually all supposed Isua metasediments are actually extremely deformed mylonites, in which the sedimentary origin has been misinterpreted from metamorphic and recrystallization structures.

The record of relatively low metamorphosed rocks (lower greenschist facies) stretches back for 3.5 billion years. Such rocks are exposed within the greenstone belt terrains or ancient plate tectonic sutures forming the old continental cores (cratons) in South Africa (e.g., Barberton greenstone belt) and in Western Australia (Pilbara). Due to lower metamorphic degree and preservation of these rocks, largely in their stratigraphic context, the depositional environment can be reconstructed with a high degree of confidence (Buick et al., 1981; Buick and Dunlop, 1990; Tice and Lowe, 2004a; Allwood et al., 2006).

3. Rules for Microfossil Recognition

The morphological criteria for microfossil recognition in Archean rocks have been repeatedly cited by many authors, claiming to have meticulously obeyed all the rules (for a review comp. Altermann and Kazmierczak, 2003; Schopf, 2004) yet few researchers came up with undisputed new findings. Schopf (2004), has requested that the sample must be of established Archean age. The fossils must be indigenous to the rock and syngenetic with its deposition later contamination by

microbes must be excluded. Morphological recognition of biological origin must be assured by morphometric statistical data. Carbonaceous matter must constitute the structures and have the right isotopic composition. According to Buick (1991), to be considered “microfossils” structures must (i) occur in petrographic thin sections; (ii) they must be found in sedimentary or low-grade metasedimentary rocks; (iii) be larger than smallest organisms ($>0.01 \mu\text{m}^3$); (iv) be composed of kerogen; (v) occur with others of similar morphology; (vi) be hollow and (vii) be of cellular elaboration. “Pseudofossils” are structures not meeting the first 4 criteria. “Dubiofossils” are those meeting only the first 4 criteria. “Possible microfossils” are those meeting the first 5 criteria. “Probable microfossils” are those fulfilling the first 6 criteria. For paleontologists and geologists understanding rock formation, the first criterion of Buick (1991) is the crucial one for any meaningful microfossil discussion; only in petrographic thin sections and a petrographic microscope their mode of petrification and relationship to the rock, that is, the syngeneity, can be reliably ascertained. A very balanced evaluation of coccoid-like microstructures was recently given by Ueno et al. (2006a). The authors discuss the structures as possible 3.0 Ga microfossils and conclude on the base of petrographic analysis that the microspherules are most probably artefacts of geological processes.

With the advance of analytical techniques criteria for fossil recognition are being widened. They include *in situ* Raman spectroscopic investigations and Raman imagery, demonstrating the nature of the carbon (polycyclic aromatic kerogen) of the supposedly microfossiliferous structure, in comparison to the carbon disseminated in the sample (e.g., Schopf, 2004; Tice and Lowe, 2004a). Carbon isotopic investigations evidencing Calvin-cycle type ^{12}C enrichment were performed *in situ* on Archean microfossils by House et al. (2000), and by Ueno et al. (2001, 2004). Molecular fossils (steranes and related products of carbon maturation) were found in Archean shales by Brocks et al., 1999. Beaumont and Robert (1999) and Pinti et al. (2001) have investigated nitrogen isotopes in Archean kerogens, adding negative ^{15}N values and C/N ratios to the list of possible criteria. Much of the molecular and *in situ* isotopic evidence is extremely difficult to obtain and require complicated, very sensitive, high resolution technological effort. On not a single Archean microfossil occurrence all investigations were performed independently and led to positive results (review in Schopf, 2004).

3.1. POSSIBLE SOURCES OF ORGANIC CONTAMINATION OF ROCKS

Modern contamination of very old rocks is easiest to trace. In the case of Isua rocks some cracks and fissures may be colonized by modern bacteria (e.g., Westall and Folk, 2003). The comparison of the metamorphic degree (coalification, maturation) of the carbonaceous organic remains to the regional metamorphism of the rocks hosting the microfossiliferous objects under scrutiny, is one of the most important proofs. If the carbon maturation does not correspond to the

surrounding metamorphic facies, the objects are clearly contaminants introduced later into the environment. The logic in such considerations follows the reasoning that the objects and the rocks must have the same thermal and pressure history to be regarded as contemporary. However, because the metamorphic peak of the rock is usually tens or hundreds of millions of years younger than the rock itself, identification of contamination is difficult. Microbial communities can invade the rock long after its deposition but prior to metamorphism and subsequently undergo fossilization under progressively higher metamorphic conditions. Thus, it is possible that a rock (e.g., 3.8 or 3.5 Ga) barren of biogenic matter becomes colonized by microbes or contaminated with foreign organic matter through percolating fluids, in hundreds or thousand meters of depths of burial and few hundred million years subsequently to its deposition. In such a case the possibility of identifying the late introduction of the organic matter to the rock is almost zero. Polyphase metamorphism is the rule in ancient rocks. For example, the amphibolite to greenschist facies metasedimentary and metavolcanic rocks of the Barberton greenstone belt (3.55 Ga) experienced at least two episodes of deformation and metamorphism, the earlier one at 3.45 Ga and the later at 3.3 Ga (Diener et al., 2005 and references therein).

How probable are processes of late microbial invasion or of hydrocarbon bearing fluid migration into consolidated, relatively deeply buried rocks? Very little is known about the biomass living in subsurface rocks, but it has been estimated that the subsurface biomass can make up to 30% of the Earth total living biomass (D'Hondt et al., 2004). Actively metabolizing microbial communities were discovered in subsurface rocks, in relatively young submarine sediments and within cracks and cavities in oceanic basalts, or in oil fields, down to the depth of hundreds of meters. Contamination of older rocks by younger microbes and of the migration of biosphere within the lithosphere is thus inevitable. Cowen et al. (2003) described microbial communities living in c. 350 m subsurface depth, in c. 3.5 million years (Ma) old oceanic basalts, at 65°C. The boundaries of these findings are not limited by physical conditions but rather by the limitation to investigate deeper formations by the restricted reach of the drill.

Microbiota in deep subsurface sediments and basalts were also described also from depths of up to 800 m below the water interface, in rocks of 35 Ma (D'Hondt et al., 2004). Such living, mostly thermophilic bacteria are naturally easily discernible from the 35 million year old original organic content, acquired at times when the rock was still a sedimentary deposit exposed to the surface biosphere. Microbes spread universally between their ideal living conditions and do not need "corridors" of their favorable metabolic conditions to migrate from one place to another. When meeting a suitable environment they spread and colonize it, and, although true examples only rarely have been reported, can become fossilized during further lithification processes. It is reasonable to assume that when lithified, such late colonization of the lithosphere undergoes metamorphic changes and may become geochemically indiscernible from the older organic matter stored in the rock.

Fossilized microbial contamination of rocks more than 3 billion years old may generally be negligible if it occurs only few millions of years later than the rock's origin. It is however significant, when claims are raised for the oldest remnants of life and its evolutionary advance based on the dating of the host rock. This impediment is difficult to overcome as long as kerogenous microbial remains can not be dated directly. Therefore, a detailed study of minerals and the supposed microbial remnants must be performed using petrographic methods and, if possible, relative dating of minerals and fluid inclusions (compare the criteria for microfossil recognition above).

Of larger significance is the possibility that matured molecular organic matter, originating from postmortem decay of microorganisms can be transported in fluids through permeable rocks and fill their pore space and cavities. Fluid migration along aquifers or in hydrothermal vents in particular, but also along joints and fissures, can carry hydrocarbons over very long lateral distances and upward and downward in rock formations. Such migration is the result of pressure gradients and follows ways of permeability and less resistance. The pathways of fluid migration have been studied in great detail, as they are of crucial importance for economic geology (e.g., Toth, 1980) Tertiary to Holocene fluid movements on lateral scale of tens to hundreds of kilometers and down to more than 2 km depth from the surface, through Devonian, c. 360–400 million years old rocks of the Red Earth region of northern Alberta, Canada, are not exceptional. Oil fields are built this way but also mineral deposits like the Mississippi Valley Type lead–zinc deposits, where mineralizing fluids can migrate across giant basins for hundreds of kilometers and deposit their mineral wealth in rocks where P/T and Eh/pH conditions are favorable. It has been repeatedly shown that these processes were operational in the Precambrian as well. Because of elevated temperatures and the filtering effect of sedimentary rocks, most probably such fluids do not transport extant life or objects that could be misidentified as microfossils for very long distances, but they do influence the C, S and N-isotopic composition of the bulk rock and the possible biomarker content. Other fluids can be silica (SiO_2) rich and precipitate opaline silica, later transferred to chert, embedding and penetrating microbes and organic remains introduced to the rock in ways described above.

The existence of C-isotopic values of -15 to $-35\text{\textperthousand}$ (vs. PDB standard) is insufficient to prove Archean life. The chemical processes of Fischer–Tropsch synthesis at temperatures of 200 – 300°C and pressure of 500 bar can abiotically produce similarly fractionated carbon molecules. This fractionation mechanism was proposed by Lindsay et al. (2005), for the 3.5 Ga carbon in the Apex chert (compare below). Although particulate carbon has never been observed to be produced by processes of the Fischer–Tropsch synthesis, it may result from metamorphic processes acting over the rock and the carbon-rich products of hydrothermal degassing. Equally, UV-polymerization of simple organic compounds may have formed carbonaceous matter that was subsequently buried in Archean sediments and underwent thermal alteration during metamorphism. It could also be imagined that the more dense meteoritic rain of the early Archean

has supplied the reduced carbon. In meteorites, C displays strongly negative $\delta^{13}\text{C}$ -values of up to $-50\text{\textperthousand}$. These negative values can be shifted during metamorphism towards the typical Archean range of -15 to $-35\text{\textperthousand}$ $\delta^{13}\text{C}$. Structurally, the carbonaceous matter (kerogen) in Archean (meta) sediments is not clearly distinguishable from meteoritic or naturally synthesized nonbiological carbon. Based on Raman spectroscopy, Pasteris and Wopenka (2003), have described the insoluble carbonaceous matter from Archean metasediments as “graphite-like”, characterized by a predominance of sp^2 C–C bonds. The nonbiological “diamond-like” carbon is dominated by sp^3 C–C bonding. The “graphite-like” carbon in Archean metasediments displays structural order in correlation to the metamorphic overprint of the rock, from “disordered” (very low-grade metamorphism or only diagenetically altered) to “fully ordered graphite” (highly metamorphosed). With increasing metamorphic overprint the carbon becomes structurally closer to the “diamond-like” end member. The demonstration of a continuum in structural order of carbon in correlation to the age and metamorphic degree of a series of samples would be a strong indication for a primary biological origin of the carbonaceous matter. But such series of differently metamorphosed samples from the same formation are difficult to obtain.

4. Archean Microfossils, Molecular Fossils and Isotopic Signatures of Microbial Activity

The debate on the significance and authenticity of the Archean microfossils, especially those regarded as the world's oldest evidence for life, on the reports of the oldest cyanobacterial and eukaryotic biomarkers, and on the isotopic signatures from the oldest metasediments on Earth, has been frequently reviewed in detail since the discussion triggered by Brasier et al., 2002 (i.e., Altermann and Kazmierczak, 2003; Schopf, 2004; van Kranendonk, 2006; Tice and Lowe, 2006b). To avoid repetition, only the geological evidence will be briefly discussed in this review.

4.1. THE ISUA CASE OF >3.5 GA NEGATIVE $\delta^{13}\text{C}$ -VALUES

The possibly oldest signs of life are the carbon signatures from the Isua rocks of Greenland. Strongly negative bulk rock $\delta^{13}\text{C}$ -values (c. $-25\text{\textperthousand}$ $\delta^{13}\text{C}$) in the Isua rocks were reported originally by Schidlowski et al. (1979). Subsequently, similar $\delta^{13}\text{C}$ -values were measured directly on selected, microscopic carbon particles enclosed in thermally stable apatite minerals in the Isua rocks, but the reproducibility of these measurements could not be demonstrated, (Mojzsis et al., 1996; Lepland et al., 2002). Whether real or not, generally such negative $\delta^{13}\text{C}$ -values measured on singular kerogen or graphite grains, or on bulk rock, give little information on the possible metabolism of the supposed microbiota which could have

been involved in the carbon fractionation. The Calvin-cycle C-isotopic fractionation overlaps widely between -15 and $-40\text{\textperthousand}$ $\delta^{13}\text{C}$ in oxygenic photosynthesis, methanogenic and S-reducing bacteria. Thus, the different metabolic pathways can not be separated with confidence in rocks of this age and complicated history. Finally, as discussed above, such negative carbon values could have originated from other sources than biology, although as rightly argued by many authors, this would be highly improbable within the given geological setting.

The most compelling attempt to interpret the $-25\text{\textperthousand}$ $\delta^{13}\text{C}$ -values in some of the Isua rocks is by Rosing and Frei (2004). Although opposed by Myers (2004), these authors argue based on structural and petrographic arguments that their samples are indeed taken from metasedimentary rock, where sedimentary bedding is still recognizable, despite amphibolite facies metamorphism and tectonic overprint. They claim that carbon in these metamorphosed pelagic muds occurs as $2\text{--}5\text{ }\mu\text{m}$ globular grains of graphite, and its distribution within the bedding is controlled by sedimentary processes. Their arguments build on comparison of these graphitic carbon globules to graphite inclusions in metamorphic minerals within the bedding and exclude the possibility of postmetamorphic contamination. However, considering that peak metamorphism occurred at 2.8 Ga, there is about 1 billion years of time in which the carbon could have entered these rocks after their deposition.

Rosing and Frei (2004) demonstrate that the U/Th ratio and Pb isotopic composition of the rocks do not correspond to the crustal average values, but are typical of pelagic sediments. They argue that the original sediments must have had a high U/Pb ratio acquired during sedimentation or diagenesis, while Th was introduced to the system during the 2.8 Ga metamorphism. From the high U/Th ratio they conclude that U was transported in solution and reacted with buried organic components in the sediment. A redox gradient between the point of U fixation in the sediment and the more oxidized environment of solution and transportation can be deduced, implying that some compartments of the Archean (>3.7 Ga) ocean must have been oxidized to a higher degree. Such relatively more oxidized environments are envisaged to have hosted planktonic prokaryotes that released oxygen in the photic zone. However, Tice and Lowe (2004b), proposed that the Archean ocean, 300 million years later, at 3.5 Ga, was not oxidized at all, despite of the presence of photosynthetic organisms and that the U-rich water derived from shallow to deep realms with turbidity currents. Ohmoto (2004) has argued that oxidized compartments in Early Archean oceans could have existed through nonbiogenic processes. Moreover, the entire Archean ocean might have been significantly higher oxidized than assumed. In such a case uranium would mainly be in solution, except in very deep, stagnant or otherwise restricted basins.

It becomes clear from the above summary that the geological uncertainties do not allow for an unequivocal interpretation of the environment in which the carbon analyzed by Rosing and Frei (2004) accumulated. Independently of the uncertainties as to the oxygenation state of the early Archean ocean and to the possible transport and deposition mechanisms for the uranium, the elegant

reasoning by Rosing and Frei (2004) fails to fully exclude Archean contamination. The only possible life signature in Isua rocks is the C-isotopy of negative $\delta^{13}\text{C}$ -values. No other hints for existing life, like stromatolitic structures or microbial bodily remains are preserved. As long as the geological questions are not answered (comp. above; Lepland et al., 2002; Myers, 2004), all possible organic remains in these rocks can be considered as evidence but not as definite prove of pre 3.6 Ga life. Nevertheless, the reasoning by Rosing and Frei (2004) is geologically logical and stands within the context of much clearer and diverse arguments for microbial life, in less metamorphosed, 3.5 Ga rocks from Australia and South Africa, as discussed below. It is indisputable that life must have evolved well before 3.5 Ga.

4.2. MICROFOSSILS AND $\delta^{13}\text{C}$ -VALUES IN C. 3.5 GA SEDIMENTARY ROCKS FROM WESTERN AUSTRALIA AND SOUTH AFRICA

In contrast to those in Isua, the rocks of the Pilbara craton and of the Barberton greenstone belt exhibit ample evidence for their origin in manifold sedimentary environments at c. 3.5 Ga,. In the eighties and nineties of the last century these rocks were mainly interpreted as formed in shallow marine, restricted and evaporitic environments (Buick and Dunlop, 1990). They contain not only the typical “Archean” carbon but also abundant stromatolitic structures and few occurrences of bodily preserved microbial fossils (Walsh, 1992; Schopf, 1993; Ueno et al., 2001). Subsequently, the depositional environments have been re-investigated and found to be have had a significant hydrothermal influence (Brasier et al., 2004; Nijman and de Vries, 2004; van Kranendonk, 2006.). Not all investigators agree with this reinterpretation and many make a good case for “normal” shallow marine conditions (Tice and Lowe, 2006a, b; Allwood et al., 2006), with hydrothermal conditions arguably postdating the deposition.

Judging from Si and O- isotopy of cherts however, the “normal” Archean seawater temperature might have been on average at temperatures of around 70°C (Knauth and Lowe, 2003; Robert and Chaussidon, 2006). Consequently, the prokaryotic life recorded in these rocks must have been thermophilic or alternatively, thriving in low temperature shelters. For theoretical reasons however, such high temperatures, at around 70°C seem untenable because they would probably lead to an overheated atmosphere and eventually to an atmospheric “run away” effect and to a different mineralogy than found in Archean sedimentary rocks (Sleep and Hessler, 2006). Evolutionary microbiologists, still do not agree whether thermophily or extremophily in general are original, ancient characteristics of prokaryotes or were acquired relatively late in the evolutionary pathway (Islas et al., 2003).

The 11 Archean microfossil taxa described from the 3.46 Ga Apex chert (Schopf, 1993) are of outstanding importance and represent one of the oldest and the best investigated cellularly preserved evidence of microbial life. They testify

for an advanced early evolution of probable oscillatoriacean prokaryotes. New examination of some of the original sections used by Schopf (1993) and Brasier et al. (2002, 2004) allows investigators to establish the validity of the microfossils, based on petrographic assessment of the thin sections and on hundreds of photomicrographs taken at varying depth of focus (Altermann, 2005; Altermann et al., 2006).

The Apex chert sections contain cellularly preserved kerogenous microfossil remains, revealing advanced biostratonomic to metamorphic, taphonomic changes. The microfossils are preserved in chert clasts embedded in hydrothermal chert dike matrix. The clasts contain some stromatolitic laminae and relict carbonate minerals and therefore must have been silicified during early diagenesis at their source of origin, possibly in a carbonate environment or by direct silification of microbial mats. The younger, hydrothermal chert is distinctly different from the original clasts, in which the fossils are preserved. In places hydrothermal recrystallization strongly affects the clasts and they become almost nondiscernible from the hydrothermal chert matrix. Therefore, the clasts clearly are older than the hydrothermal dike in which they were found. The clasts could have been fallen into the hydrothermal fissure from above or have been brought up by the hydrothermal activity from layers below and thus be somewhat younger or somewhat older than their stratigraphic position between the c. 3.5 Ga cherts and lavas of the Apex formation. As there is no possibility to directly date the dike intrusion itself, besides by the unconformity eroding the dike (2.75 Ga, Buick and Dunlop, 1990), the precise stratigraphic position of these fossils can not be pointed out. No genetic connection to the hydrothermal activity and thus no evidence for thermophily of the preserved microorganisms can be constructed.

The Warrawoona Group, 3.5 Ga cherts of Pilbara Craton, were investigated by Ueno et al. (2001, 2004), who found spiral, thread-like and branched filaments in black vein cherts. Raman spectroscopic analysis of the filaments and clothed kerogen showed that the graphitization has reached the lower greenschist facies. $\delta^{13}\text{C}$ measurements on these filaments yielded strongly negative values of -42 to $-32\text{\textperthousand}$. The morphology of these filaments is rather simple and not directly comparable to the filaments described by Schopf (1993). They do not show septation or hollow, cellular shapes. They are nevertheless discussed as possibly genuine microfossils (comp. Schopf, 2004). If real, they could be good candidates for remains of thermophilic microbes, as they are found in hydrothermal vein cherts. However, their occurrence can be disputed. Some authors (e.g., Ueno et al., 2001; Nijman and de Vries, 2004), contend that the chert veins were formed immediately after or during the deposition of the hosting layered chert-baryte unit of the Warrawoona Group and are thus older than the overlying units of 3.458 Ga. Earlier workers (Buick et al., 1981; Buick and Dunlop, 1990) argued, however, that the chert veins extruded upward in the chert-baryte beds as sills, and can only be estimated as of 3.45 to 2.75 Ga, on regional grounds. Whatever the exact age of the veins, the carbon and the filaments are also confined to clasts of c. 1 cm diameter, embedded in vein silica, leaving the problem unresolved (compare discussion earlier).

The volcano-sedimentary succession of the Barberton greenstone belt of South Africa encompasses cherts similar to those of the Apex Formation. From cherts of the Hooggenoeg and Kromberg Formations of the Onverwacht Group, spheroids and filaments were described and interpreted as bacterial microfossils (Walsh and Lowe, 1985; Walsh, 1992). The microfossils are embedded in laminated black and black and white, banded to massive chert, partly recrystallized but well-preserved and exhibiting original sedimentary texture. Samples were examined from all major cherts in the Hooggenoeg, Kromberg and Mendon Formations, where most chert beds exhibit some influx of tuffaceous material. Detrital carbon flakes and finely disseminated carbon particles, of a few μm in diameter, down to sub-micrometric size, are present in trace amounts in the primary chert precipitate. In silicified detrital sediments, carbon content is generally lower, but the particles are of larger size, above 10 μm . Pervasive silicification of sedimentary structures like laminae and crossbeds and a "blocky" chert appearance are widespread (e.g., Lowe and Byerly, 1999; Hofmann, 2005). In general, several generations of silicification can be recognized. Silica replacement of carbonates and evaporites and botryoidal quartz replacing chert and filling cavities are common (Lowe and Byerly, 1999). Redeposited carbon rich laminae, contorted during resedimentation and interpreted as cryptic microbial mats, show little compaction before thorough silification. Such cryptic microbial mats offer a rich source for the carbon disseminated in all cherty sedimentary rocks of the Barberton greenstone belt. From the description above, no extreme environments can be recognized for the microfossil findings in these sediments.

The only direct claim for the existence of lithophilic microbial life on the 3.5 Ga Earth was made by Furnes et al. (2004). These authors have recognized microtubes in glassy rims of metamorphosed, pillowed, submarine basaltic lavas of the Barberton greenstone belt. The lavas are unequivocally 3.5 Ga in age and the alteration is due to hydrothermal reactions between the chilling lava and the ocean water and thus has occurred shortly after the extrusion. No doubt exists, that the microtubes are contemporary to this alteration because they have the typical mineralization that must have taken place prior or simultaneously with tube formation and reaction with sea water. The microtubes are interpreted as products of microbial corrosion in order to dissolve nutrients from rock. But the arguments that the microtubes were formed by the activity of lithophilic microbes are unconvincing upon close examination. Microbial fossils were not found and the arguments used to prove microbial origin of the tubes in pillow lava rims are circumstantial and can be refuted. The question is, were the microtubes indeed produced by lithophilic microbes or do they represent degassing structures subsequently colonized by microbes? Microbial pits on lava and other rocks are well known from many occurrences and were also produced in laboratory conditions (comp. Einen et al., 2004). Lithophilic bacteria dissolving rock surfaces produce typical pitting of μm size, usually of shallow, irregular, saucer-shaped morphology. Tubes of 1–9 μm diameter and up to 200 μm deep in to the rock are not very effective energetically, as bacterial colonies tend to spread and act upon relatively wide surface and less into the depth of the rock (availability of resources from

water and ambience). In the Barberton case, the occurrence of degassing pipes in the rims of almost all pillows of the relevant outcrops at the Komati River is conspicuous. The pillows exhibit a radial arrangement of pipes of few mm in diameter up to microscopic dimensions of few μm , partly filled with carbonate spar and other minerals and partly hollow, penetrating the pillow rim from the inside towards the outside. Moreover, the microtubes are often associated with cracks filled with secondary minerals and are extremely reminiscent of ambient pyrite grains as described from many hydrothermal silicate environments and from experimental work on silica crystallization (Iller, 1979). The main argument used by Furnes et al. (2004) is the presence of carbon in the microtubes that correlates negatively with carbonate. Disseminated carbonate from bulk rock samples of the pillow rims has $\delta^{13}\text{C}$ values of +3.9 to -16.4‰, while the crystalline interiors of individual pillows have values of 0.7 to -6.9‰. Secondary carbonate-rich amygdalites in these rocks have $\delta^{13}\text{C}$ values clustering around zero. These values (C from carbonates of pillow rims and from the pillow interior) widely overlap and the claimed organic signature is not reliable, especially as hydrothermal degassing is known to produce comparably negative $\delta^{13}\text{C}$ values. Signs of hydrothermal alteration and degassing are plentiful in the pillow rims. Last but not least, the bacteria could have occupied the tubules at any stage after their formation, as long as exchange with the hydrosphere and biosphere was possible and thus, be responsible for the carbon isotope values in bulk pillow rim samples. Thus, bacterial activity, but not necessarily lithophilic or thermophilic processes could be indicated.

4.3. PHOTOSYNTHESIS AND METABOLIC PATHWAYS IN THE EARLY ARCHEAN

The origin of kerogen in 3.5 Ga rocks is controversial, although C and N isotopes suggest widely that the kerogens were produced by chemo-autotrophic microorganisms (e.g., Pinti et al., 2001; Ueno et al., 2004). Possible metabolic pathways of the microbes have been the subject of wide discussion triggered by different geochemical investigations and chemotrophy, phototrophy, sulfate reduction, and methanogenesis have been proposed by various groups of authors.

In his review on Archean photosynthesis, Olson (2006) implies that early photosynthesis involved H_2 and H_2S as reductants. Only when the source of H_2 was reduced by the production of methane by methanogens was sulfur-driven photosynthesis developed at around 3.5 Ga. Another reductant for photosynthesis might have been ferrous iron. The ability to use ferrous Fe was probably developed between 3.5 and 3.0 Ga and might have been responsible for the deposition of banded iron formations (BIF; Kappler et al., 2005). Oxygenic photosynthesis was perhaps only developed after 3.0 Ga. Nevertheless the interpretation by Olson (2006) is based on the data discussed above and thus prone to the same fallacies.

Evidence for early Archean microbial methanogenesis in the Warrawoona Group vein chert rocks has been found by Ueno et al. (2006b). Fluid inclusions in the chert veins contain methane with carbon isotopic composition of $-58\text{\textperthousand}$, next to H₂O and CO₂. The authors discuss the possibility of hyperthermophilic methanogens thriving above 80°C as being responsible for this large isotopic fractionation and compare the preserved methane to that produced by CO₂ reduction or acetate fermentation in modern methanogens. Pyrite (FeS) with significant ³⁴S depletion was found in the same rocks by Shen et al. (2001) and interpreted as a product of sulfate-reducing bacteria. Such findings are common in Archean rocks (for a recent overview see Altermann and Kazmierczak, 2003; Altermann et al., 2006). Tice and Lowe (2006a) argue, based on evidence from the Buck Reef Chert of the Barberton greenstone belt, that H₂ was the primary electron donor for carbon fixation in the early Archean, instead of H₂O, Fe²⁺ and H₂S. The same type of photosynthesis has been generally proposed for the time at 3.8 to 3.5 Ga, on the ground that there would be no evolutionary pressure for oxygenic photosynthesis in the presence of H₂ (Olson, 2006).

4.4. EVIDENCE FOR MICROBIAL ACTIVITY IN ROCKS OF 2.9 TO 2.5 GA

The evidence for microbial life improves dramatically with the recuperating rock record. From c. 2.9 Ga on, carbonate rocks with complex stromatolitic reefs become so abundant and that their biogenic origin is beyond all doubts. Excellently preserved microbial fossils of Neoarchean age were found in many formations (Lanier, 1986; Klein et al., 1987; Altermann and Schopf, 1995). In general, only filamentous, but not colonial coccoid cyanobacteria have yet been discovered in rocks older than about 2.6 Ga, although their very early origin is suggested by molecular phylogenetic analyses (Turner et al., 1997). Ueno et al. (2006a) give a detailed description of spherical microfossil like objects found in 3.0 Ga cherts of Australia, but come to the conclusion that these coccoid-like structures are most probably not microfossils.

Most reports of Neoarchean microfossils are from diagenetically silicified carbonates or primary cherts. Carbonate rocks, however, are conventionally excluded as a potential source of microfossils because of the destruction of prokaryotic cell-sized structures by the growth force of carbonate minerals. Exceptions hereof are the filaments described as *Siphonophycus transvaalensis*, which are partly preserved in late diagenetic, euhedral dolomite crystals and partly in chert (Klein et al., 1987). These filaments were interpreted as possible remains of cyanobacterial calcification. Wright and Altermann (2000) reported on possible microbial mediation in calcification and dolomitization processes in microbial laminites and oolites from the Campbellrand Subgroup of South Africa (2.6–2.5 Ga).

Direct morphological evidence for biomineralization by benthic coccoid Neoarchean cyanobacteria was presented also from the Campbellrand Subgroup

(Kazmierczak and Altermann, 2002). The biostructures obtainable in the SEM images are essentially similar to capsules and common mucilage sheaths of modern benthic cyanobacteria classified within the orders Chroococcales (particularly the family Entophysalidaceae) and Pleurocapsales. The process of early *postmortem* mineralization in these Neoarchean cyanobacteria was interpreted as reflecting the action of heterotrophic bacteria upon the dead cyanobacterial biomass.

In all these findings, the sedimentary relationships always point to shallow, “normal,” nonextremophilic origin. The environmental setting for all Neoarchean fossiliferous units ranges from shallow marine intertidal to subtidal and although hydrothermal influence on the rocks is often clearly visible, it always arguably postdates the origin of the sedimentary rocks.

5. Conclusions

Many of the above described traces of Archean life may be equivocal if viewed in isolation. The geological and evolutionary context however, links all of them into a well-fitting and robustly based theory, that is, that the prokaryotic life on Earth thrived in sedimentary environments well before 3.5 billion years ago. Doubts as to the interpretation of the exact paleoecological and sedimentary settings of these habitats are justified in some cases. However, these uncertainties only concern detailed interpretations, while the general picture is rather well-reconstructed.

From all described microfossil occurrences in the Archean it is difficult to construct the case for extremophilic prokaryotes. Perhaps the most likely are thermophiles as suggested by paleotemperatures of Archean oceans measured by Si and O isotopes of cherts. The question, whether the elevated temperatures of up to 70°C are indeed primary ocean water signature or perhaps secondary is not unequivocally answered yet. No biological hint for thermophily can be found in the fossils themselves and the environments where they are found imply restricted marine areas, where temperatures, judging from the deposited sediment, must have been comparable to those of equivalent recent environments.

Even if we assume that the early Archean oceans were acidic (MacLeod et al., 1994), the abundant relicts of carbonate minerals in fossiliferous and stromatolitic Archean cherts clearly indicate that the microbes thrived in neutral to rather alkaline conditions, and thus in niches. In case of a soda-dominated, alkaline oceans, as discussed by Kempe and Kazmierczak (1994) and by Kazmierczak et al. (2004), however, early Archean prokaryotic life must have been well adapted to such conditions, as it is today in many alkaline lakes world wide.

The desire of finding traces of Archean extremophilic life was born out of astrobiology and the search for life on Mars. Compared to the Earth, the only place in the universe where life is known to exist, all the other celestial bodies have particularly “extreme” conditions. Extremophiles, tolerating or depending on extremely high or low or extremely fluctuating temperatures, acidic environments, high and low pressure conditions, high salinity, extremely low or high gravity,

radiation, darkness, or other extreme conditions would have a larger chance of existence on these planets. The question, however, should be, would they also have had sufficient time and suitable conditions to evolve? In research, model-driven thinking is common. Microbiologists were until now not in the position to decide whether extremophily in prokaryotes is an evolutionarily ancient or rather young development. It seems therefore hopeless to expect such a definitive information from geologists and palaeontologists, who can only judge from heavily altered, poorly preserved and extremely incomplete rock record.

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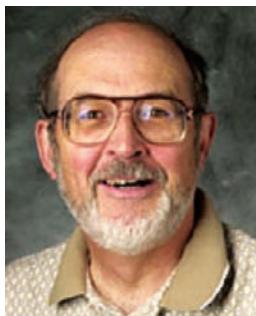
PART 9: OUTLOOK - SUMMARY

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ALGAE AND CYANOBACTERIA UNDER ENVIRONMENTAL EXTREMES:

Final Comments

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The more than 40 chapters in this book provide an updated overview of our current understanding of the life of oxygenic phototrophs – from simple prokaryotic cyanobacteria to multicellular eukaryotic macroalgae – in a wide variety of extreme biotopes in which they are exposed to diverse forms of stress: high and low pH, high and low temperatures, including below zero temperatures, excessively high and extremely low light intensities, salt concentrations up to saturation, and xeric environments. We should not forget situations, e.g., the intertidal where conditions, especially related to water availability, fluctuate on a daily basis. In addition, many contributions deal with “polyextremophilic” phototrophs, which are simultaneously adapted to multiple forms of environmental stress. The unicellular red alga *Cyanidium caldarium* and its relatives *Galdieria sulphuraria* and *Cyanidioschyzon merolae* are among the best known examples of such polyextremophiles, being adapted to life in hot thermal pools at temperatures in the range of 45–56°C and pH values as low as <2–4.

Cyanidium is one of the more extensively studied extremophilic oxygenic phototrophs. Other examples of such well-studied extremophiles are the thermophilic cyanobacterium *Thermosynechococcus* and the halophilic or extremely halotolerant members of the eukaryotic green algal genus *Dunaliella*, one representative of which, *Dunaliella acidophila*, is acidophilic rather than halophilic. Most of our insights on the mechanisms of the extremophilic tolerance and function of phototrophs are based on the study of such model organisms. Many other, probably no less interesting organisms remain virtually unexplored. A prominent case is the alkaliphilic and halophilic (and therefore polyextremophilic) chlorophyte *Picocystis* which is the main primary producer in Mono Lake, California. This is a lake at pH 9.8 and salinity ca. 90 parts per thousand. Algae that not only survive, but actually

are able to grow at near-zero light intensities, as “highlighted” in several chapters in this volume, are another example.

The definition of the organisms described in the chapters of this book as extremophiles and their environments in which they live as extreme is primarily based on the fact that they by far exceed the limits of what our own species, or those species that typically inhabit human landscapes, i.e., the multicellular plants and animals that make up most of the eukaryotic domain, can tolerate. We must not forget that for many of those organisms that live in unusual habitats with their temperature extremes, acidic pH values, high concentrations of heavy metals etc., the “extreme” conditions under which they live may represent a normal state of affairs, and this is the habitat to which they are adapted. We therefore have to ask the question whether these habitats are really extreme, or are they only so from a human perspective?

The term “extreme” is usually defined in a mathematical sense as belonging to the outer parts of a Gaussian distribution, i.e., as “extraordinary.” However, among biologists it should be clear that this definition has to be handled carefully. As soon as there are organisms able to settle habitats characterized by parameters that are unusual in comparison to the mainstream of ecosystems, those habitats are no longer extreme in a biological sense. They now are within the range in which organisms can settle in principle. Organisms may show traits of adaptation to such special and seldom found habitats, but the conditions are not “extreme” or “extraordinary” for those species.

Some extremophilic phototrophs are strictly dependent on their specialized environment and will not survive even a short exposure to “normal” conditions. Alkaliphilic cyanobacteria of the genus *Spirulina* present an example: they lyse when exposed to neutral pH. In many other cases, however, the requirement for the environmental extremes is much less strict. The already mentioned red alga *Cyanidium* not only survives, but even is able to grow (though slowly) at ambient temperatures and in the neutral pH range. However, in nature we only find it in hot acidic environments, as only there it is able to successfully compete with other types of microorganisms.

A resolution to the question as to what really represents extreme may be approached not from the environmental conditions per se, but rather from the biodiversity that is present in the environment. Thus, species richness and species abundance may be the critical parameters to resolving the questions posed above. One would expect low species richness and low structural complexity of the truly “extreme” environments.

Another prominent feature that characterizes habitats of extremophiles and ecology “on the edge” is the lack of continuity between the “normal” and the “extreme” environments. In most terrestrial and aquatic habitats one proceeds from one suitable habitat space with a particular community to another habitat space where there is a different community. Whether the transitions between communities are sharp or diffuse is not significant, but the fact that there is a continuous community of living things. In these transitions it is typically competitive

interactions between species that regulate their abundance and not their physiological tolerances per se. This has been demonstrated experimentally in the intertidal zone of rocky shores where species easily colonize lower on the shore when space becomes available (hence competitive interactions), but typically have difficulty moving upward on the shore because of physiological limitations. So we may consider defining truly extreme habitats by the lack of a clear zone of transition in which conditions change gradually and a continuum of adaptation is observed in the organisms present. Many of the ecosystems mentioned in the chapters in this book are characterized not only by low species diversity, but also by transitions from conditions that barely allow life to be maintained to conditions where life becomes virtually untenable (at least for macroscopic cyanobacteria and eukaryotic algae). These transitions are often accompanied by the presence of biological deserts, with subsequent sudden changes to completely different life forms.

The key word in trying to evaluate the potentials of life on Earth is not "extreme" but "adaptation." It is a most interesting observation that uni- or oligocellular organisms usually show a greater ability to adapt to various and changing environmental conditions than multicellular ones that have to pay the price for specialization by loss of adaptive abilities. Those observations are well illustrated by algae: most species are aquatic and thus the aquatic habitat often is biased as being typical for algae. However, the biological concept "alga," i.e., a uni- or oligocellular eukaryote performing oxygenic photosynthesis, has a much broader adaptive spectrum, and this is well illustrated by soil algae. Those algae show that, in principle, the concept "alga" is fit for all types of light-exposed habitats conceivable on our planet.

Biologically, the organisms that inhabit extreme environments are important not only because of the unique species they represent, but also because of their unusual physiological and biochemical properties. A question, which is extremely relevant but has hardly been addressed thus far, is therefore whether the specific adaptations to allow for tolerances that have evolved in different lineages of phototrophs are convergences, or whether they may result from re-expression of ancient genes. For example, the properties of the Cyanidiophyceae, a group of red algae evolutionarily divergent from the other classes, raise interesting questions about the evolution of the red algae and the paleo-environment in which they originated and evolved. However, we must not forget that the paleo record rarely leaves an indication of the metabolic capabilities of the organisms present at the time and we need to be cautious about extrapolating back in time in this regard.

This brings us to the question when the extremophilic properties of the different types of cyanobacteria and eukaryotic phototrophs have evolved: are these ancient traits, or can they be relatively recent additions to the genetic potential of these organisms? We should also bear in mind that features of most and therefore in statistical terms "normal" ecosystems have not been constant during the long history of life on Earth. What is today "normal" (e.g., current ambient temperatures, concentrations of carbon dioxide, etc.) has been very different in earlier times. For example, the polyextremophile *Oscillatoria limnetica* (*Phormidium*

(*hypolimneticum*) is found in Solar Lake, Sinai, growing in salt concentrations around 15% and temperatures as high as 55°C. This species facultatively undergoes anoxygenic photosynthesis. The ability of *O. limnetica* to use sulfide as an electron donor instead of water may represent an ancient type of metabolism, preserved until now in a specialized "extreme" environment.

A thorough understanding of the particular genetic adaptations of extremophilic phototrophs on Earth may provide the basis for the genetic engineering of organisms to be used in the exploration and the future exploitation of other planets. By combining specific genes that bestow tolerance to a range of environmental extremes, the possibility that organisms may be produced with increased capacity not only survive, but also be active on currently sterile landscapes of, for example, Mars, may well become a reality in the future. We should also consider that our genetic understanding might lead to genetic engineering of crop plants for establishment of viable crop plants in "extreme" environments, e.g., saline, alkaline soils which are frequently inhospitable environments.

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