

Handbook of Microalgal Culture

Handbook of Microalgal Culture: Biotechnology and Applied Phycology

Edited by

Amos Richmond

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Preface

An introduction into the state of the art

Over 15 years have elapsed since the previous Handbook addressing mass cultivation of microalgae (CRC Press, 1986) was published. At that time, it was already evident that the original concept viewing microalgae as a future agricultural commodity for solving world nutrition needs has no basis in reality. Photosynthetic efficiency in strong sunlight falls far short of the theoretical potential resulting in low yields which are the major culprits for the forbiddingly high production cost of algal cell mass. Economically, therefore, outdoor cultivation of photoautotrophic cell mass is inferior to conventional production of commodities such as grains or soybeans. At this stage of our experience with mass production of photoautotrophic microalgae, it is indeed evident that certain very ambitious roles that have been suggested for large-scale microalgaeculture – e.g. reduction of global carbon dioxide using large areas of unlined, minimally mixed open raceways – are unrealistic, being based on unfounded assumptions concerning, in particular, maintenance costs and the expected long-term productivity. Notwithstanding, schemes for local reduction of carbon and nitrogen emissions from, e.g. power plants, using intensive microalgal cultures in efficient photobioreactors, may have economic prospects based on winning valuable environmental credits for the polluting industry and provided such environmental treatments are, in effect, subsidised by State laws in which strict demands for reducing combustion gases within a definite period are imposed.

Similarly, the grand idea of using algal systems for the sole purpose of industrial energy production, such as hydrogen or methane (unlike the bacterial–algal systems meant to produce these chemical energies coupled to processes of waste clearance), is simply unrealistic: Technologies by which to harness solar energy, e.g. wind machines, photovoltaic systems or a whole array of solar collectors, are much closer to becoming an ongoing economic reality than microalgal cultures bent on producing, with dismal efficiency, bio-hydrogen.

One unique grand scheme, however, sea nourishment to augment phytoplankton growth, is worthy of critical examination. Several land and ocean areas on our planet are exhibiting low productivity due to lack of factors required for plant growth and large ocean expanses are essentially barren due to an acute shortage of some mineral element, e.g. nitrogen or iron. The productivity of such desert oceans could be readily improved by a small, judicious addition of the growth-limiting factor and, there are experimental indications showing this idea to be feasible. The growing world population in certain areas of this planet mandates urgent efforts to achieve a substantial increase in local food production, and barren oceans may be regarded as an

extension of land in which rather extreme manipulations of the natural environment for the purpose of food production have been acceptable for years. Such schemes naturally arouse intense criticism based on fears of evoking unknown deleterious environmental consequences. Nevertheless, adding small amounts of a growth-limiting nutrient to desert oceans carries the prospects of benefitting from both carbon dioxide sequestration and fish productivity. A reassuring aspect of this scheme rests on the fact that ocean nourishment may be quickly modified or altogether stopped if the results are judged to bring about negative environmental consequences.

A development which may soon lead to massive production scale of microalgae stems from the fact that production of heterotrophic microalgae has significant economic advantages over photoautotrophic production. The recent successful attempts to convert the trophic level of strictly autotrophic species (e.g. *Porphyridium cruentum*) into that of heterotrophic producers represent, therefore, a landmark in microalgal biotechnology. It is conceivable that once efficient trophic conversions become readily available for practical use, several photoautotrophic microalgae will be grown commercially in very much the same simple and effective mode by which bacteria, yeast or fungi are commercially produced. Indeed, if the requirement for light is eliminated, microalgae could be grown in accurately controlled, very large-culture vessels of a few hundred thousands liters, holding cell densities higher by about two orders of magnitude above the optimal for an open raceway. A cut of perhaps one order of magnitude in the cost of production, compared with that of photoautotrophic microalgae, has thus been envisioned.

Presently, the most important endeavor unfolding in commercial microalgaculture is the use of heterotrophic microalgae for a whole line of new products to supplement animal and aquacultural feed, as well as human nutrition. The first production lines so far developed by MARTEK, USA, concerns long chained polyunsaturated fatty acids (PUFAs), mainly docosahexaenoic acid (DHA). Soon to follow will probably be production facilities of microalgal feed for animal husbandry, particularly for aquaculture. It is significant that the first truly large-scale industrial production of microalgae in a photobioreactor, the 700 000 l tubular reactor (divided into some 20 subunits), constructed and run by IGV Ltd in Germany which is producing *Chlorella* as a food additive for poultry, is based on a mixotrophic mode of nutrition.

Is then the strictly photoautotrophic production mode in commercial microalgaculture on the verge of phasing out? Despite the imminent onslaught of trophic conversions of several microalgal species, which would to some extent undermine phototrophic production, photoautotrophic microalgae do have a rather safe future for several specific purposes, most prominent of which are in aquaculture, bioactive compounds, water clearance for a sustainable environment as well as fresh water supplies, nutraceuticals regarded as healthfood and finally, as a basic human food.

Since most artificial substitutes are inferior to live microalgae as feed for the critical stages in the life cycles of several aquacultural species, a growing demand for microalgae will go hand in hand with the expected growth of aquaculture throughout the world. Presently, most aquacultural enterprises

produce (albeit with only limited success in many cases) their own supply of microalgae. Since the algal cultures can be often fed directly to the feeding animals, eliminating thereby the necessity for harvesting and processing, such rather small scale on-site production makes economic sense. Centralized microalgal facilities which sell (for a high price) frozen pastes or highly concentrated refrigerated stock cultures cover at present only a small part of the aquacultural demand for live microalgae. Once heterotrophic production is established and inexpensive microalgal feed becomes widely available, it seems certain that centralized production of microalgae for aquaculture will receive a strong impetus. Nevertheless, costs of local, in situ production of microalgae could be greatly reduced through improved implementation of practical know-how in mass cultivation giving cause to expect that on-site production of photoautotrophic microalgae carried out presently in many hatcheries, will at least to some extent, maintain its ground.

Wastewater clearance represents another important niche in which photoautotrophic microalgae are prominent. Using photosynthetic microalgae to take up the oxidized minerals released by bacterial action and, in turn, enrich the water with oxygen to promote an aerobic environment and reduce pathogens, makes good practical sense and could be well used in suitable locations the worldover. An interesting and promising variation on this general theme may be seen in land-based integrated systems, in which microalgae together with bacteria play a role in clearing aquacultural wastes, becoming in turn feed for herbivores and filter-feeders. These systems well integrate with the environment and will probably become widespread in favorable locations the world over.

Ever since the inception of commercial mass cultivation of microalgae in the early 1950s, the mainstream of product development has been diverted to the nutraceutical and health food, markets. There are good reasons to believe this trend will continue, considering the growing economic affluence the world over as well as the growing interest in the western world in vegetarian eating modes. The collection of pills and powders made from *Chlorella*, *Spirulina* (or *Arthrospira*) and *Dunaliella* is being enriched by a promising newcomer, *Haematococcus pluvialis*. Originally meant to produce the carotenoid Astaxanthine for fish and shrimp pigmentation, astaxanthine was discovered to be an outstanding antioxidant with antiaging potential, so the present primary production target is focused on the usual nutraceutical venue.

Concerning this trend, it is my opinion that a gross mistake has been made by the microalgal industry in focusing all marketing efforts on health foods and the like. It is a lucrative market, but is naturally rather small and cannot stir a large demand for microalgae. This marketing focus may be as culpable in impeding progress of industrial-scale microalgal culture, as high production costs, by curbing potential demand. It is an erroneous approach in that it overlooks the fact that several microalgae (such as *Spirulina*, *Chlorella*, *Dunaliella*, as well as other species such as *Scenedesmus*) when correctly processed have an attractive or piquant taste and could be thus well incorporated into many types of human foods, greatly expanding demand for microalgae. I thus believe the microalgal industry would much benefit from

a closer interaction with the food industry, employing food technology methods to create a myriad of possible new food products. Incorporating suitably processed microalgae into nearly all food categories would add not only nutritional value, but also new, unique and attractive tastes to such food items as pasta, pretzels, potato and corn chips, soup mix and seasonings, an assortment of dairy products, and even an assortment of candies, and ice-creams, to mention but a few obvious possibilities.

Much effort has been expended on the search for new compounds of therapeutic potential, demonstrated in microalgae of all classes, possessing antibacterial, antifungal and anticancer activities. Indeed, there are many promising prospects for new chemicals reported in recent years, the most prominent of which are carotenoids of nutritional and medical values, new polysaccharides and radical scavengers, as well as a whole array of unique chemicals in cyanobacteria, and in the vast diversity of marine microalgae. Considering the untapped resources with which it may be possible to enrich the pharmaceutical arsenal, it seems safe to predict that the search for photoautotrophic microalgal *gold mines* will continue for years to come. The prospects for generating bioactive products using photoautotrophic cultures, however, would unfold only if alternative sources, i.e. an inexpensive heterotrophic production mode or chemical synthesis of the active substances, will not present a more economically attractive venue.

Photobioreactor design was the subject of much research in recent years, yet little real progress was accomplished. Meaningful improvements in this field would no doubt strengthen the economic basis of commercial photoautotrophy by reducing production costs. The tubular design seems to have gained popularity at present, yet it is questionable whether it represents the optimal design for strictly photoautotrophic production. Small tube diameters do not go hand in hand with very high cell densities, for which fast, turbulent flows are strictly mandatory. Flat plate reactors (without alveoli), which facilitate cultures of very high cell densities devoid of oxygen accumulation in greatly reduced optical paths together with the required turbulent streaming, may be readily scaled-up. Well suited for utilizing strong light, plate reactors offer hope for obtaining a significant increase in productivity of cell mass, once the growth-physiology of very high cell concentrations (mandatory for efficient use of strong light) will be sufficiently understood, so as to prevent or control the growth-inhibition effects, which unfold in cultures of ultra-high cell densities, barring at present industrial use of such cultures.

It is well to note that the type of reactor used has a profound effect on the cost of production of cell mass and cell products, considering the investment, as well as the running costs. Much of the future of the photoautotrophic mode of production depends on success in greatly reducing these costs. The rather simple, less expensive techniques involved in mass production in open tanks and raceways have, under certain circumstances, advantages in this respect, well seen in many hatcheries as well as commercial plants. Most algal species, however, cannot be long maintained as continuous, monoalgal cultures in open systems, which in addition may not be suitable for general use as human food.

Some 50 years of experience, the world over, with microalgal mass cultures have witnessed an exciting canvass of successes as well as some failures reflected in this Handbook to which leading authorities in their respective fields have contributed. The accomplishments, during this period, in addressing the various aspects of mass microalgal production seem somewhat overshadowed by the outstanding achievements the pioneers of this biotechnology who were active in the '50 and '60, had attained in laying out, with great intuition, the basic physiological principles involved in mass cultivation of photoautotrophic microalgae outdoors.

It is, therefore, somewhat surprising that an output rate of some 70 g dry cell mass m^2 (ground) day $^{-1}$ was envisioned at that time as a practical goal for open systems which could be well reached and surpassed. This daily output rate of protein-rich cell mass represents an annual yield of some 250 t ha $^{-1}$, i.e. several times that of any agricultural commodity. Such expectations were, in effect, translated into a firmly held premise, enthusiastically perceiving outdoor mass cultivation of microalgae as a means by which to avert hunger in a fast growing humanity. Today, this prospect is justifiably regarded as nothing but a dream.

Were the early pioneers, then, completely wrong? This is not as easy to answer as it may seem, for the future will unfold possibilities that presently border on sheer fantasy. The methodology of genetic engineering which already facilitates such feats as effective trophic conversions and combating Malaria by use of microalgae incorporated with bacterial toxins lethal to the mosquito larvae, are but the harbingers of vast future opportunities in microalgal culture. The future could well see greatly improved, fast-growing microalgal species with significantly improved capabilities to carry out effective photosynthesis utilizing strong sunlight, and photoautotrophic microalgal culture may yet become an economic alternative for provision of food and feed in the sunny, more arid, parts of our planet.

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Acknowledgments

Work on this Handbook extended for two years. Whatever merit this volume deserves, would be primarily due to the high-level professional efforts exerted by its many contributors, to whom I wish to extend my sincere appreciation and thanks.

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The major trust of editing and writing this Handbook took place during my sabbatical leave as a guest of the Marine Bioproducts Engineering Center of the University of Hawaii at Manoa. I wish to acknowledge the University of Hawaii for this generosity and thank Dr Charles Kinoshita, Director of MarBEC at the time, who was a kind host, as were the friendly administrative personnel of the Center whose assistance and good will are much appreciated. The final phase of preparing the book for publication took place during my visit at the University of Wageningen, with the group of Dr Rene Wijffels, to whom I wish to thank.

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Part I

The Microalgae: With Reference to Mass-Cultivation

1 The Microalgal Cell

Luisa Tomaselli

1.1 What is the meaning of *microalgae* in applied algology?

Phycologists regard any organisms with chlorophyll *a* and a thallus not differentiated into roots, stem and leaves to be an alga (Lee, 1989). Cyanobacteria are included in this definition, even though they are prokaryotic organisms. Therefore, in applied phycology the term microalgae refers to the microscopic algae *sensu stricto*, and the oxygenic photosynthetic bacteria, i.e. the cyanobacteria, formerly known as Cyanophyceae.

The interest for these two groups of phototrophic organisms lies in their potential utilization, in a similar way to heterotrophic microorganisms, to produce biomass for food, feed and fine chemicals, using solar energy. The origins of applied phycology most probably date back to the establishment of a culture of *Chlorella* by Beijerinck (1890). Even today *Chlorella* takes up the first place in the commercial use of these microorganisms.

Microalgae are found all over the world. They are mainly distributed in the waters, but are also found on the surface of all type of soils. Although they are generally free-living, a certain number of microalgae live in symbiotic association with a variety of other organisms.

1.2 Structural and morphological features of microalgae

1.2.1 Microscopy: examining fresh material; making permanent slides

Examination of fresh material can be directly performed on a drop of liquid sample, or after the solid sample has been mixed with distilled water or saline solution. In presence of motile cells the sample should be mixed with a weak acid, such as acetic acid. Settling, centrifugation or filtration can be used to concentrate the living or preserved material. To minimize changes in the composition of the samples after collection, fixation using formaldehyde, Lugol's solution and glutaraldehyde should be carried out quickly, or the sample should be cooled and stored in total darkness to ensure a low activity rate.

Permanent slides can be simply prepared, placing the cell suspension on a coverslip and drying over gentle heat. The sample is then inverted onto a slide with a mounting medium of suitable refractive index. Canada Balsam is commonly used (Reid, 1978). Sometimes the removal of free water from the cells requires dehydration procedures, which are carried out using gradually increasing concentrations of an alcohol series. Staining techniques are

used to distinguishing some special features, such as sheath and specific organelles (Clark, 1973). Finally, the coverslip with the sample in the mounting medium is sealed to a glass slide usually using clear nail polish.

1.2.2 Types of cell organization: unicellular flagellate, unicellular non-flagellate (motile, nonmotile); colonial flagellate, colonial non-flagellate; filamentous (unbranched, branched)

Microalgae may have different types of cell organization: unicellular, colonial and filamentous. Most of the unicellular cyanobacteria are nonmotile, but gliding and swimming motility may occur. Baeocytes, cells arising from multiple fission of a parental cell, may have a gliding motility. Swimming motility occurs in a *Synechococcus* sp., even if flagella are not known. Unicellular microalgae may or may not be motile. In motile forms, motility is essentially due to the presence of flagella. The movement by the secretion of mucilage is more unusual. Gametes and zoospores are generally flagellate and motile. Some pennate diatoms have a type of gliding motility, as well as the red alga *Porphyridium* and a few green algae.

Cyanobacteria with colonial cell organization have nonmotile colonies (e.g. *Gloeocapsa*). In microalgae motile flagellate cells may aggregate to form motile (e.g. *Volvox*) or nonmotile colonies (e.g. *Gloeocystis*). Nonmotile cells may be organized into coenocytic forms with a fixed number of cells in the colony (e.g. *Scenedesmus*), or into non-coenocytic forms with a variable number of cells (e.g. *Pediastrum*). Many filamentous cyanobacteria may have gliding motility often accompanied by rotation and by creeping (e.g. *Oscillatoria*), but others may be motile at the stage of hormogonia (e.g. *Nostoc*). Microalgae, with unbranched or branched filamentous cell organization are nonmotile, zoospores and gametes excepted. Siphonaceous and parenchymatous cell organization occur mostly in macroalgae.

1.2.3 Cellular organization: prokaryotic; eukaryotic: uninucleate, multinucleate (coenocytic)

The DNA of prokaryotic Cyanobacteria and Prochlorophytes is not organized in chromosomes, lies free in the cytoplasm together with the photosynthetic membranes, and is not surrounded by a membrane. Moreover, the prokaryotes have no membrane-bounded organelles (Fig. 1.1). The eukaryotic microalgae possess a true membrane-bounded nucleus, which contains the major part of the genome distributed on a set of chromosomes, and the nucleolus. They have cytoplasm divided into compartments and membrane-bounded organelles (Golgi body, mitochondria, endoplasmic reticulum, vacuoles, centrioles and plastids) devoted to specific functions (Fig. 1.2). Many microalgae are uninucleate, those with multinucleate cellular organization (coenocytic) usually have a peripheric cytoplasm containing nuclei and chloroplasts, which are the most important plastids.



Fig. 1.1. Electron micrograph of a dividing cell of *Synechococcus* sp. in longitudinal section. Abbreviations: **cw** – cell wall, **t** – thylakoids, **cs** – carboxysomes, **n** – nucleoplasm with DNA fibrils. Scale = 0.5 µm (Courtesy of M.R. Palandri).

1.2.4 Colony features: orderly (e.g. netted) or random; shape and investments

Different shapes of colonial organization occur: flat, spherical, cubic, palmelloid, dendroid, flagellate, and non-flagellate. The cells are held together by an amorphous (e.g. *Microcystis*) or microfibrillar polysaccharide envelope (e.g. *Gloeothecace*). Inside the colony the cells may be orderly or irregularly arranged in the mucilage (e.g. *Microcystis*). Both colonies with orderly (e.g. *Pediastrum*) and irregularly arranged cells (e.g. *Palmella*) occur in microalgae. Moreover, nonmotile (e.g. *Coelastrum*) and motile colonies formed of flagellate cells, embedded in a mucilage, are common (e.g. *Gonium*). The polysaccharide investment may be amorphous or laminated with a microfibrillar structure; depending on its consistency, it may be called sheath, glycocalyx, capsule, or slime. Cyanobacteria sheaths may contain pigments functioning as sun-screen compounds (Garcia-Pichel *et al.*, 1992), or UV-A/B-absorbing mycosporine-like amino acids (Ehling-Schulz *et al.*, 1997). Capsule and slime envelopes are particularly abundant in many species (*Cyanospira capsulata*).



Fig. 1.2. Electron micrograph of a cell of *Chlorella vulgaris* in longitudinal section. Abbreviations: **cw** – cell wall, **ch** – cup shaped chloroplast, **t** – thylakoids, **st** – starch grains (leucoplasts), **n** – nucleus, **nl** – nucleolus, **m** – mitochondria. Scale = 1 µm (Courtesy of M.A. Favali).

1.2.5 Morphological adaptation: specialized cells (spores, heterocysts, hormogonia), pili, flagella, light shielding and flotation structures

Specialized cells as akinetes, heterocysts, hormogonia, and pili or fimbriae occur in many cyanobacteria. Akinetes, or spores, are cells with thick walls and granular content, which originate from vegetative cells under unfavourable conditions and germinate when favourable conditions for growth are restored. Heterocysts are unique cells where nitrogen fixation takes place. They have thick wall and rarefied cytoplasm, characterized by two polar nodules of cyanophycin. Hormogonia are short trichome pieces or development stage of filamentous cyanobacteria. They usually have gliding motility, smaller cell size, and/or gasvacuolation. Gas vacuoles are specific subcellular inclusions that appear highly refractile in the light microscope. They are composed of elongated gas vesicles with pointed ends, which may function in light shielding and/or buoyancy. Pili or fimbriae are non-flagellar proteinaceous appendages protruding from the cell wall.

Spores and flagella may occur in microalgae. The spores, or resting cells, have thick walls and like akinetes are formed under unfavourable conditions. Resting cells of *Botryococcus braunii* may accumulate in the cell wall, a hydrocarbon up

to 70% of its dry weight (Knights *et al.*, 1970). Flagella are locomotory organs with a complex structure consisting of an axoneme of nine peripheral double microtubules surrounding two central microtubules; the whole structure is enclosed by the plasma membrane. The flagella may be smooth or hairy, and are inserted in the outer layer of the cytoplasm *via* a basal body.

1.3 Ultrastructure and cell division

1.3.1 Prokaryotes

1.3.1.1 Cell wall

Cyanobacteria and Prochlorophytes have a four layered cell wall which is of the Gram-negative type; the structural part consists of a murein (peptidoglycan) layer, outside which there is a lipopolysaccharide layer. The high digestibility of cyanobacteria cells, due to the lack of cellulose, unlike the majority of algae, facilitates their use for human consumption (e.g. *Spirulina* – health food). Mucilaginous envelopes may surround the cell wall (sheaths, glycocalix, capsule or slime). The cell wall may be perforated by small pores and may also have appendages such as fimbriae and pili.

1.3.1.2 Plasma membrane

Beneath the cell wall there is the plasma membrane, or plasmalemma. It is a thin unit membrane of about 8 nm thickness.

1.3.1.3 Thylakoid arrangement

Thylakoids are the most evident membrane system occurring in the cyanobacterial cell; they lie free in the cytoplasm and contain the photosynthetic apparatus. Thylakoids appear as flattened sacs showing phycobilisomes attached to the protoplasmic surface in regularly spaced rows. The phycobilisomes contain the phycobiliproteins that are widely used as fluorescent tags (Glazer, 1999); phycocyanin from *Arthrospira* is commercialized as natural pigment (linablue). Thylakoids may be arranged in concentric rings, in parallel bundles, dispersed, etc. They are not present in *Gloeobacter*, which possesses only a peripheral row of phycobilisomes. Phycobilisomes are absent in the prochlorophytes, which possess an extensive membrane system with stacked thylakoids.

1.3.1.4 Cell inclusions

The most common cell inclusions of cyanobacteria are the glycogen granules, cyanophycin granules, carboxysomes, polyphosphate granules, lipid droplets, gas vacuoles, and ribosomes. The glycogen granules (α -1,4-linked glucan) lie between the thylakoids and represent a reserve material, such as the

cyanophycin granules, polymer of arginine and aspartic acid. Carboxysomes, containing the enzyme ribulose 1,5-bisphosphate carboxylase-oxygenase, lie in the central cytoplasm. Poly-hydroxybutyrate granules, appearing as empty holes, represent unusual inclusions and a potential source of natural biodegradable thermoplastic polymers (Suzuki *et al.*, 1996). Ribosomes are distributed throughout the cytoplasm. In the planktonic forms there are gas vacuoles.

1.3.1.5 Cell division

Cell division may occur through binary fission, with constriction of all the wall layers that grow inward, or invagination of the plasma membrane and peptidoglycan layer without involvement of the outer membrane. Cell division may also occur by multiple fission leading to the formation of baecocytes. A very particular type of cell division, similar to budding, occurs in *Chamaesiphon*. Cyanobacteria also reproduce by fragmentation (hormogonia). Moreover, some filamentous genera produce akinetes. Although the cyanobacteria have no evident sexual reproduction, genetic recombination by transformation or conjugation may occur.

1.3.2 Eukaryotes

1.3.2.1 Cell wall, outer investments

A microfibrillar layer of cellulose, which may be surrounded by an amorphous layer, generally composes the microalgal cell wall. The cell wall is secreted by the Golgi apparatus. It may be silicified or calcified, and it may be strengthened with plates and scales. Some species are naked, lacking the cell wall. Outside the outer amorphous layer there may occur a laminated polysaccharide investment. The nature of the outer cell wall layers supports polysaccharide production (alginates, agar and carrageenans) from various macro algae as well as from the microalga *Porphyridium* (Arad, 1999).

1.3.2.2 Plasma membrane, periplast, pellicle

The plasma membrane is a thin unit membrane that bounds the cytoplasm. The Chryptophyta do not possess a cell wall but there is an outer cell wall covering the cytoplasm, called periplast. In the Euglenophyta the proteinaceous outer covering is called pellicle.

1.3.2.3 Cytoplasm, nucleus, organelles

The cytoplasm contains the nucleus and different kinds of organelles and compartments formed by invagination of the plasma membrane and endoplasmic reticulum. Among the organelles there are: chloroplast, Golgi apparatus, endoplasmic reticulum, ribosomes, mitochondria, vacuoles, contractile vacuoles, plastids, lipid globules, flagella, and microtubules. Chloroplast and cytoplasmic lipids represent an important source of polyunsaturated fatty

acids, such as eicosapentaenoic, docosahexaenoic and arachidonic acids (Pohl, 1982). The nucleus is bounded by a double nuclear membrane; it contains the nucleolus and several DNA molecules distributed among the chromosomes, and undergoes division by mitosis.

1.3.2.4 Chloroplast

The chloroplast contains a series of flattened vesicles, or thylakoids, containing the chlorophylls, and a surrounding matrix, or stroma. Thylakoids also contain phycobiliproteins in phycobilisomes in the Rhodophyta, whereas in the Cryptophyta the phycobiliproteins are dispersed within the thylakoids. Thylakoids can be free or grouped in bands. Pyrenoids can occur within chloroplasts. In many motile forms there is an orange-red eyespot, or stigma, made of lipid globules. A double membrane envelops the chloroplast; in some algal division besides this double membrane one or two membranes of endoplasmic reticulum are present.

1.3.2.5 Cell division and reproduction

Vegetative reproduction by cell division is widespread in the algae and related, in many species, to an increase in cell or colony size. Other types of asexual reproduction occur by fragmentation and by production of spores, named zoospores if flagellate and aplanospores or hypnospores if non-flagellate. Autospores are also produced by various algae and are like aplanospores lacking the ontogenetic capacity for motility.

Although sexual reproduction occurs in the life-history of most of the species, it is not a universal feature in algae. It involves the combination of gametes, often having different morphology and dimension, from two organisms of the same species (isogamy, anisogamy or oogamy). Five schematic types of life-histories are recognizable: (i) predominantly diploid life history with meiosis occurring before the formation of gametes (haploid part of life cycle); (ii) predominantly haploid life history with meiosis occurring when the zygote germinates (zygote only diploid part of life cycle); (iii) isomorphic alternation of generation (alternation of haploid *gametophytic* plants bearing gametes with diploid *sporophytic* plants bearing spores); (iv) heteromorphic alternation of generations (alternation of small haploid plants bearing gametes with large diploid plants bearing spores, or large haploid plants alternating with smaller diploid plants); (v) triphasic life cycle, in red algae, consisting of haploid gametophyte, diploid carposporophyte and diploid tetrasporophyte.

1.4 Cell growth and development

1.4.1 Cell growth

Growth is defined as an increase in living substance, usually the number of cells for unicellular microorganisms or total mass of cells for multicellular organisms. The most used parameter to measure change in cell number or cell

mass per unit time is the growth rate. Cell growth is treated in detail elsewhere in this volume (Chapter 4).

1.4.2 Cell cycle

In unicellular microalgae the cell size generally doubles and then the cell divides into two daughter cells which will then increase in size. The cell cycle in eukaryotic algae involves two phases: mitosis and interphase. During the interphase the cell grows and all cellular constituents increase in number so that each daughter cell will receive a complete set of the replicated DNA molecule and sufficient copies of all other constituents and organelles. During the mitosis the nuclear division occurs.

1.4.3 Cell decline

Microbial growth is influenced by several chemical and physical conditions. As the substrate concentration or other factors become limiting, or toxic metabolites accumulate, the growth rate decreases. In this growth phase, the production of secondary metabolites often takes place. As long as there is consumption of storage material the organism remains viable. When energy is no longer produced for cell maintenance, the cell declines and finally dies. In some cases this process is accompanied by the formation of a few spores or similar structures, which may survive and overcome adverse conditions giving rise to new individuals when favourable conditions are resumed.

1.5 Microalgal systematics

1.5.1 Principles of classification

Traditionally algae have been classified according to their colour and this characteristic continues to be of a certain importance. The current systems of classification of algae are based on the following main criteria: kinds of pigments, chemical nature of storage products and cell wall constituents. Additional criteria take into consideration the following cytological and morphological characters: occurrence of flagellate cells, structure of the flagella, scheme and path of nuclear and cell division, presence of an envelope of endoplasmic reticulum around the chloroplast, and possible connection between the endoplasmic reticulum and the nuclear membrane.

Lee (1989) was one of the first scientists to stress the phylogenetic importance of the additional membranes around the chloroplast envelope. He separated the algal divisions into four groups. The first group includes the prokaryotic algae: Cyanobacteria and Prochlorophyta. The other groups are classified with respect to the evolution of the chloroplast, and include the eukaryotic algae, which probably acquired the chloroplast along different evolutionary events. The second group, which includes Glaucophyta, Rhodophyta and Chlorophyta, has the chloroplast surrounded only by two chloroplast membranes. The third and fourth group have the chloroplast surrounded respectively by one (Dinophyta and Euglenophyta) or two additional

membranes of the endoplasmic reticulum (Cryptophyta, Chrysophyta, Prymnesiophyta, Bacillariophyta, Xanthophyta, Eustigmatophyta, Raphidophyta and Phaeophyta). The phylum Prochlorophyta contains chlorophylls *a* and *b* and, according to Castenholz (2001), the described genera (*Prochloron*, *Prochlorothrix* and *Prochlorococcus*) are included in the phylum Cyanobacteria.

The systematic position of the various algal group has changed many times over the years. The system of classification proposed by Lee (1989) has been largely adopted here to give a brief description of some of the divisions that this author considers.

1.5.2 General description of major Divisions and Classes

1.5.2.1 Prokaryotes

Cyanobacteria (*Cyanophyta* and *Prochlorophyta*)

The Cyanophyta and the Prochlorophyta are prokaryotic algae that contain chlorophyll *a*. The traditional name of blue-green algae for the Cyanophyceae is due to the presence of phycocyanin and phycoerythrin, which usually mask the chlorophyll pigmentation. The main storage product is glycogen (α -1,4-linked glucan). Cyanophycin is stored in large structured granules. Cell wall is composed of peptidoglycan with an external lipopolysaccharide layer, and it may also have a mucilaginous sheath. The cells may occur singly or in filaments, unbranched or branched, with uniserial or multiseriate arrangement. The cells may aggregate to form colonies, which are surrounded by a firm or amorphous mucilage. Filaments may have cells differentiated into heterocysts and/or akinetes. Some planktonic forms can float owing to the presence of gas vacuoles, and most of the filamentous forms have gliding motility. The Cyanophyceae have a cosmopolitan distribution and inhabit marine and freshwater environments, moist soils and rocks, either as free-living or as symbiotic organisms.

The Prochlorophyta synthesize chlorophyll *b* in addition to chlorophyll *a*, and lack phycobiliproteins and phycobilisomes. Other major differences concern the morphology of thylakoids, which are paired rather than single, the synthesis of a starch-like polysaccharide as main storage compound, the absence of both cyanophycin and gas vacuoles, and the diffuse localization of DNA through the cytoplasm. They have unicellular or filamentous cell organization. They occur as free-living organisms in freshwater and marine habitats, and as symbionts in colonial ascidians.

Microcystis Kützing. *Microcystis* is a planktonic unicellular cyanobacterium that grows in freshwaters. Cells are spherical, 3–6 µm in diameter, and irregularly arranged in mucilaginous, spherical or irregularly lobed, colonies. The cells generally have a light refractile appearance due to the presence of gas vesicles. Cell division occurs by binary fission and reproduction by disintegration of colonies into single cells or group of cells. At the ultrastructural level, cells are characterized by thylakoids arranged in short bent or sinuous bundles running around the cell wall and occupying a large portion of the cytoplasm (Fig. 1.3). Gas vacuolated *Microcystis* species may give rise

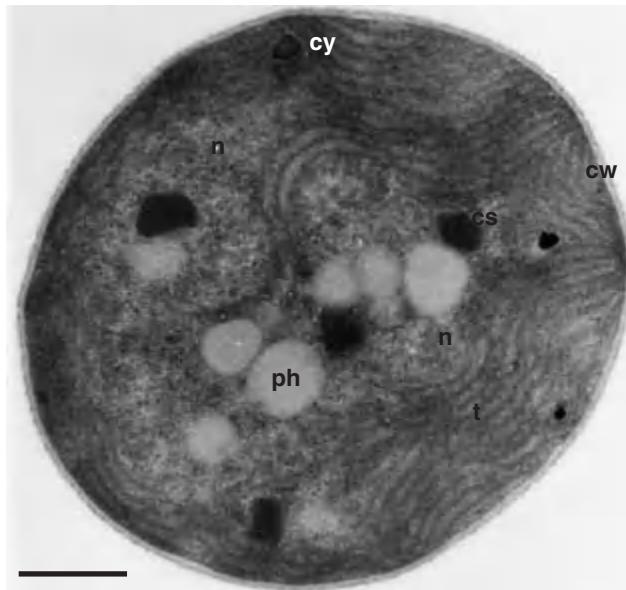


Fig. 1.3. Electron micrograph of a cell of *Microcystis* sp. in cross-section. Abbreviations: **cw** – cell wall, **t** – thylakoids, **n** – nucleoplasm, **cs** – carboxysomes, **cy** – cyanophycin granule and **ph** – poly-hydroxyalkanoate granules. Scale = 0.5 µm (Courtesy of M.R. Palandri).

to toxic blooms (Watanabe & Oishi, 1982). Like other cyanobacteria, they may produce toxins, such as microcystins (cyclic peptides) (Carmichael, 1996), which have become a concern for human health.

Arthrospira Stizenberger. *Arthrospira* is a planktonic filamentous cyanobacterium. It has multicellular left-handed helical trichomes, composed of shorter than broad cells (mean cell diameter 8 µm), and shows gliding motility by rotation along its axis (Fig. 1.4). Cells have visible cross walls, often masked by numerous gas vesicles (Fig. 1.5) (Tomaselli, 1997). Multiplication occurs by cell division in one plane and reproduction by hormogonia formation through trichome breakage at the sites of a lysing cell (necridium) (Tomaselli *et al.*, 1981). *Arthrospira* grows profusely in alkaline lakes of subtropical regions (Vonshak & Tomaselli, 2000). *Arthrospira*, the most cultivated phototrophic prokaryote as food supplement, is also used as a source of feed and fine chemicals (Richmond, 1986), and exploited as a therapeutic agent (Belay *et al.*, 1993). This genus is currently known under the name *Spirulina*, although *Arthrospira* and *Spirulina* have been recognized as separate genera (Castenholz & Waterbury, 1989; Tomaselli *et al.*, 1996).

1.5.2.2 Eukaryotes

Rhodophyta

The class Rhodophyceae, or red algae, includes multicellular and filamentous forms, whereas unicellular species are less represented. These algae have chlorophyll *a* and *d*, phycobiliproteins (phycoerythrin and phycocyanin), and floridean starch (α -1,4-linked glucan) as storage products accumulated

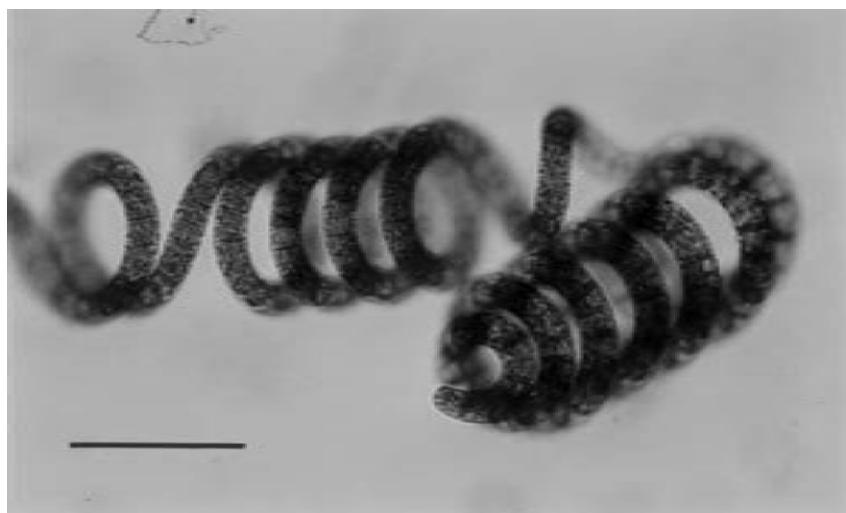


Fig. 1.4. Light micrograph of *Arthrospira maxima*. Scale = 50 µm (Courtesy of C. Sili).

in the cytoplasm outside the chloroplast. Rhodophyceae lack flagellate cells. Cell wall is composed of a microfibrillar layer of cellulose or xylan and amorphous polysaccharidic mucilages (agar or carrageenans). Chloroplasts contain the thylakoids with phycobilisomes, and pyrenoids.

Red algae represent the majority of seaweeds distributed mostly in temperate and tropical regions. Commercial utilization of red algae concerns the polysaccharidic mucilages of the cell wall, agar and carrageenan. The red microalga *Porphyridium* is an important source of sulphated polysaccharides (Arad *et al.*, 1985), and of polyunsaturated fatty acids, such as arachidonic acid (Ahern *et al.*, 1983).

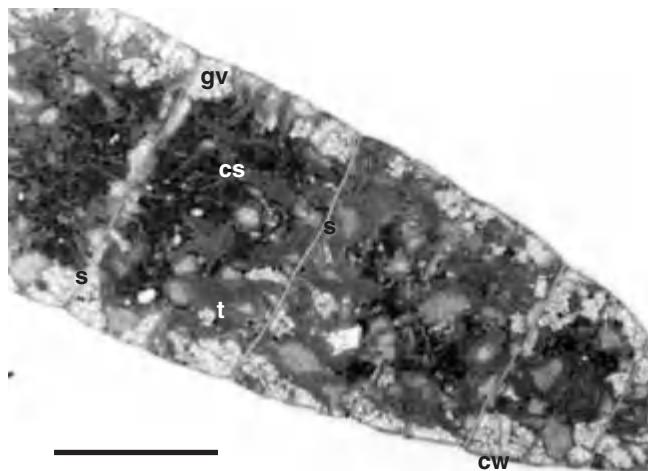


Fig. 1.5. Electron micrograph of a trichome of *Arthrospira maxima* in longitudinal section. Abbreviations: **cw** – cell wall, **s** – septa or cross walls, **t** – thylakoids, **gv** – gas vesicles, **cs** – carboxysome. Scale = 5 µm (Courtesy of M.R. Palandri).

Chlorophyta

The Chlorophyta, or green algae, embrace a large group of organisms with a great morphological variability, ranging from microscopic to macroscopic forms. They comprise four classes: Micromonadophyceae, Charophyceae, Ulvophyceae and Chlorophyceae. They have chlorophyll *a* and *b* and several carotenoids, that may be synthesized and accumulated outside the chloroplast under conditions of nitrogen deficiency and/or other stress, colouring the alga orange or red. The storage product is starch (α -1,4-linked glucan), composed of amylose and amylopectin. Unlike the other algae, it is formed within the chloroplast. Cell walls generally contain cellulose. Some species are naked. Chloroplasts may have an eyespot and pyrenoids. The group includes coccoid, unicellular or colonial flagellates, multicellular or multinucleate filaments. Most species have flagellate stages with the flagella apically inserted into the cell, the flagellar root system anchored with four sets of crucially arranged microtubules. The green algae are cosmopolitan. They are primarily freshwater, but a great number grow in marine, terrestrial and subaerial habitats. Some species occur in symbiotic associations, mostly with lichens.

Commercial exploitation of microscopic green algae comprises relatively few chlorophycean genera among which there are *Chlorella*, *Dunaliella* and *Haematococcus*. Moreover, *Botryococcus braunii* was proposed and cultivated as a renewable source of liquid fuel (Wolf *et al.*, 1985), owing to the high hydrocarbon content (Knights *et al.*, 1970). *Dunaliella* spp. from hypersaline environments have been extensively studied, cultivated and commercialized as a source of natural β -carotene (Ben-Amotz & Avron, 1983; Borowitzka *et al.*, 1984; Richmond, 1990; Ben-Amotz, 1999).

Haematococcus Agardh. *Haematococcus* is a freshwater unicellular alga with ovoid cells actively motile by two smooth apical flagella. During its growth stages nonmotile cells, or cysts, also occur. The cell peculiarity is that the protoplast is separated from the cell wall, to which it is connected by radiating protoplasmic threads. Chloroplast is usually cup-like, parietal, and it contains pyrenoids and the eyespot. The cell content is usually green with a small part of the cell orange-red pigmented owing to the accumulation of the ketocarotenoid astaxanthin in globules outside the chloroplast (Lee & Ding, 1994). As the conditions become unfavourable, green and motile cells round off, then produce thicker walls, loose the flagella and finally cease to swim. In these cysts the protoplast fills the whole cell and a great increase in cell dimension occurs together with a change of pigmentation from green to orange-red, due to the increase of astaxanthin deposition. When the conditions become favourable for growth, the cysts germinate releasing a large number of new motile cells. The astaxanthin represents a high valuable product not only as a colouring agent for fishes and crustaceans in aquaculture but, since it is a potent natural antioxidant, it may also find important applications in medicine (Palozza & Krinsky, 1992). The process of astaxanthin production has been successfully developed in Israel (Boussiba & Vonshak, 1991).

Chlorella Beijerinck. *Chlorella* is a cosmopolitan genus with small, unicellular, ovoidal nonmotile cells; it does not produce zoospores. Cells have a thin cell wall, and cup-shaped chloroplast. Pyrenoid may be present. The accumulation of starch occurs within the chloroplast. *Chlorella* reproduces by forming daughter cells or autospores (4–8–16) of the same shape as the parent cell. It grows in autotrophic, heterotrophic and mixotrophic conditions. Besides autotrophic strains, heterotrophic strains are also cultivated. *Chlorella* is the most important species in the microalgal industry; it is cultivated and sold essentially as health food (Richmond, 1990).

Dinophyta

The class of Dinophyceae, formerly named Pyrrrophyceae, includes a diverse assemblage of unicellular biflagellate planktonic algae of marine and freshwater habitats. They have chlorophylls *a* and *c₂* and carotenoids. Starch is accumulated in the cytoplasm outside the chloroplast. The cell is composed of two parts, an epicone and a hypocone, and divided by the transverse girdle. Perpendicular to the latter there is a longitudinal groove. A layer of flat vesicles usually containing the cellulose plates surrounds the cell wall. In many species a polysaccharidic glycocalix surrounds the cell. Chloroplasts may have pyrenoids and a complex eyespot. The nucleus has the particularity of chromosomes always condensed. The Dinophyceae can produce large blooms, red or brown tides that colour the water and are highly toxic (Shilo, 1967). They are the main contributors to marine bioluminescence. The dinoflagellates are a potential source of ω-3 unsaturated fatty acids, eicosapentaenoic and docosaeanoic acids (Pohl, 1982).

Chrysophyta

The Chrysophyta or golden-brown algae include two classes: the Chrysophyceae and the Synurophyceae. They mainly occur in freshwaters, especially in oligotrophic waters low in calcium. The chloroplasts contain chlorophylls *a* and *c₁*, *c₂*, fucoxanthin and β-carotene, which are responsible of the golden-brown colour. The storage product is chrysolaminarin (β-1,3-linked glucan), which is accumulated in a cytoplasmic vesicle. Most of the species are unicellular or colonial. Cells usually have two different apical flagella, one smooth, the other hairy, and contain two parietal chloroplasts with an eyespot. The cell wall is often lacking, or composed of cellulose. Silicified scales, polysaccharidic envelopes, or loricas and various cytoplasmic processes may occur external to the cell wall. Characteristic of the chrysophyta is the formation of special resting spores, statospores, enclosed in a silicified wall. Some species require vitamins and growth substances. *Ochromonas malhamensis*, which requires vitamin B12 to grow, has been used as an assay organism for this vitamin.

Prymnesiophyta

The Prymnesiophyta, also named Haptophyta for the presence of a thin filamentous appendage between two smooth flagella, called haptonema, include only the class Prymnesiophyceae. The cells are flagellate and have chlorophyll *a*, *c₁* and *c₂* and fucoxanthin as the major carotenoid. The storage

product is chrysolaminarin (β -1,3-linked glucan). Cells are usually covered with scales embedded in a mucilage and sometimes are calcified (coccoliths). These microalgae are widespread in marine environment, forming a major part of marine phytoplankton. Since many years, *Prymnesium parvum* is known for producing a potent exotoxin lethal for fish and molluscs (Shilo, 1967). Recently, toxic blooms of *Chrysochromulina polylepsis* have been reported (Nielsen *et al.*, 1990). Strains of *Isochrysis* and *Pavlova* are investigated as a source of polyunsaturated fatty acids (PUFAs) (Lopez-Alonso *et al.*, 1992).

Bacillariophyta

The class of Bacillariophyceae, or diatoms, includes a very conspicuous number of golden brown unicellular organisms. The diatoms live mostly singly or attached to one another in chains of cells or in colonial aggregations, in aquatic and terrestrial habitats. Their colour is due to the masking of the green colour of chlorophylls *a*, *c₁* and *c₂* by the brown and yellow pigmentation of the fucoxanthin and β -carotene. The storage product is chrysolaminarin (β -1,3-linked glucan). Lipids are also present. The cytoplasm is enclosed in a siliceous cell wall, the frustule, showing different structures and ornamentation, which are used as key features for diatom classification. The frustule consists of two overlapping halves joined by a girdle. The upper longer and wider half (epitheca) fits on the lower half (hypotheca) as the cover of a box. Cells contain two parietal chloroplasts; the nucleus is laterally or centrally placed and suspended by protoplasmic threads. Pyrenoids are sometimes present. Some diatoms may have a central raphe, or fissure. Diatoms with a raphe possess gliding motility. There are two major groups of diatoms: the pennate diatoms with bilateral symmetry and the central diatoms with radial symmetry. The latter are mostly widespread in marine environments, where they have a key role in food chains. Diatoms include photoautotrophic, auxotrophic and colourless heterotrophic species.

Deposits of fossil diatoms, known as diatomaceous earth, have many industrial uses (filtration and absorption processes), while commercial uses of living cells are mainly related to the aquaculture, since some diatoms contain significative amounts of PUFAs, especially eicosapentaenoic acid (Pohl, 1982).

Xanthophyta

The class of Xanthophyceae, also known as Tribophyceae, comprises freshwater and terrestrial species. Only few members are marine. The yellow-green colour is due to the presence of both chlorophyll *a* and carotenoids. Chlorophyll *c* may also be present, whereas fucoxanthin is absent. Most species are unicellular or colonial. Species with amoeboid, multicellular, filamentous or siphonaceous cell organization may also occur. A few unicellular species are motile and flagellate with two unequal flagella: a long hairy and a shorter smooth flagellum. Cells usually contain several parietal discoid chloroplasts, usually with pyrenoids and an eyespot. The cell wall, mainly formed by cellulose, is composed of two overlapping halves: a cap of constant size and a tubular basal portion, which elongates as the cell grows.

The storage product is chrysolaminarin (β -1,3-linked glucan). Mannitol and glucose are also accumulated in plastids. Some species, like *Monodus subterraneus*, contain appreciable amounts of arachidonic and eicosapentaenoic acids (Pohl, 1982).

Eustigmatophyta

The class of Eustigmatophyceae includes unicellular and coccoid organisms producing a small number of zoospores and living in freshwaters and soil. Previously these organisms were classified in the Xanthophyceae. The name was chosen because of the large size of the eyespot in the zoospore. The chloroplast have chlorophyll *a*; violaxanthin is the major light-harvesting carotenoid pigment. Vegetative cells usually have a polygonal pyrenoid, which is absent in the zoospores. The zoospores have one or two apical flagella. The cell wall is polysaccharidic. Unlike the Chrysophyta, the eyespot is not enclosed in the chloroplast. Cytoplasm and photosynthetic lamella lipids of many species are promising sources of eicosapentaenoic acid, particularly in *Monodus subterraneus* (Cohen, 1999).

Nannochloropsis. *Nannochloropsis* is a picoplanktonic genus of marine environment. The cells are small (2–4 μm in diameter), spherical to slightly ovoid, non-flagellate. They have one single chloroplast without pyrenoid and containing several bands of photosynthetic lamellae, each with three thylakoids per band. The chloroplast endoplasmic reticulum is continuous with the nuclear envelope. *Nannochloropsis* has polysaccharide cell walls. The cells do not accumulate starch. This alga has an important applied interest as a source of polyunsaturated fatty acids, since it accumulates significant amounts of eicosapentaenoic acid (Boussiba *et al.*, 1987).

Rhaphidophyta

The class of Rhaphidophyceae, or chloromodas, includes a small group of unicellular flagellate containing chlorophyll *a*, *c₁* and *c₂*, and some carotenoids, often fucoxanthin in marine species and heteroxanthin in freshwater species. The cells are naked and have two different apical or subapical flagella; there is no eyespot. The cytoplasm is subdivided into a peripheral part occupied by a layer of many discoid chloroplasts and vacuoles, and a central part containing the nucleus and the mitochondria. Species of marine genera (e.g. *Fibrocapsa japonica*) may give rise to massive blooms, red tides, that cause serious damages to fish-farming (van den Hoek *et al.*, 1995).

Phaeophyta

The class of Phaeophyceae, or brown algae, includes a few microscopic filamentous forms and many giant forms, mostly seaweed. The colour results from the dominance of the fucoxanthin over the chlorophylls *a*, *c₁* and *c₂*. The principal storage product is laminarin (β -1,3-linked glucan). The Phaeophyta occur almost exclusively in marine habitat, where they form rich underwater forests. Cell wall is composed of an inner cellulose microfibrillar layer and an outer amorphous slimy layer made up of alginates. Commercial uses of brown algae mainly concern the algaite industry.

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2 Photosynthesis in Microalgae

Jiří Masojídek, Michal Koblížek and Giuseppe Torzillo

It's not love or money that makes the world go round, it's photosynthesis.

2.1 The process of photosynthesis

Photosynthesis represents a unique process of sunlight energy conversion. In this process, inorganic compounds and light energy are converted to organic matter by photoautotrophs. Virtually, all forms of life on Earth depend directly or indirectly on photosynthesis as a source of energy for their metabolism and growth.

The earliest photoautotrophic organisms, anoxygenic photosynthetic bacteria, evolved 3.5 billion years ago. They use light energy to extract protons and electrons from a variety of donor molecules, such as H₂S, to reduce CO₂ to form organic molecules. In this treatise, we focus on oxygen-producing photosynthetic microorganisms – prokaryotic cyanobacteria and eukaryotic algae – which emerged later and created our oxygenous atmosphere on Earth.

Cyanobacteria (blue-green algae) are frequently unicellular, some species forming filaments or aggregates. The internal organisation of a cyanobacterial cell is prokaryotic, where a central region (nucleoplasm) is rich in DNA and a peripheral region (chromoplast) contains photosynthetic membranes. The sheets of the photosynthetic membranes are usually arranged in parallel, close to the cell surface.

Eukaryotic autotrophic microorganisms are usually divided according to their light-harvesting photosynthetic pigments: Rhodophyta (red algae), Chrysophyceae (golden algae), Phaeophyceae (brown algae) and Chlorophyta (green algae). Their photosynthetic apparatus is organised in special organelles, the chloroplasts, which contain alternating layers of lipoprotein membranes (thylakoids) and aqueous phases, the stroma (Staehelin, 1986).

Oxygenic photosynthesis can be expressed as a redox reaction driven by light energy (harvested by chlorophyll molecules), in which carbon dioxide and water are converted to carbohydrates and oxygen. The conversion is traditionally divided into two stages, the so-called *light reactions* and *dark reactions* (Fig. 2.1). In the light reactions, which are bound on photosynthetic membranes, the light energy is converted to chemical energy providing a *biochemical reductant* NADPH₂ and a *high energy* compound ATP. In the

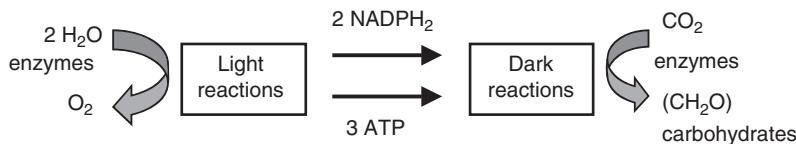


Fig. 2.1. Major products of the light and dark reactions of photosynthesis (adapted from Hall & Rao, 1999). The process of oxygenic photosynthesis is divided into two stages, the so-called *light reactions* and *dark reactions*. The light reactions include light absorption, transfer of excitons and electron and proton translocation resulting in the production of NADPH₂, ATP and O₂. The other phase, the dark reactions, which occur in the stroma, represent the reduction of carbon dioxide and the synthesis of carbohydrates using the NADPH₂ and ATP produced in the light reactions.

dark reactions, which take place in the stroma, NADPH₂ and ATP are utilised in the sequential biochemical reduction of carbon dioxide to carbohydrates.

The classical description of photosynthetic activity is based on measurements of oxygen evolution in proportion to light intensity, the so-called light-response (P/I) curve (Fig. 2.2). The initial slope $\alpha = P_{\max}/I_k$, where I_k represents the saturation irradiance and P_{\max} is the maximum rate of photosynthesis. In the dark, there is a net consumption of oxygen as a consequence of respiration (the negative part of the curve in Fig. 2.2). Thus, gross photosynthesis is considered as the sum of net photosynthesis (O₂ evolution) and respiration (O₂ uptake). At low irradiance (light-limited region), the rate of photosynthesis depends linearly on light intensity. With increasing light intensity, photosynthesis becomes less and less efficient. Finally, it reaches a plateau – the light-saturated value – where enzymatic reactions utilising fixed energy become rate limiting. Under prolonged supra-optimal irradiance, photosynthetic rates

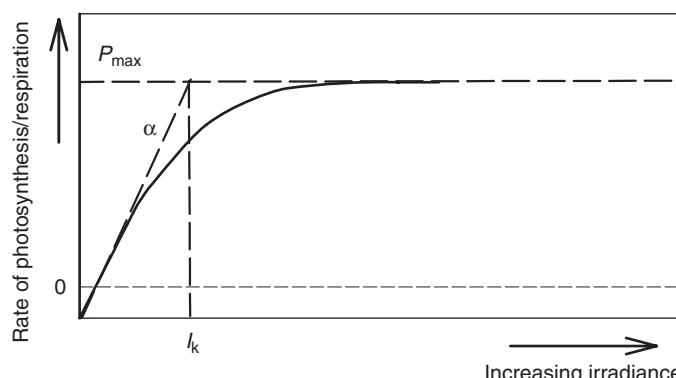


Fig. 2.2. A schematic representation of photosynthetic light-response curves, i.e. the dependency of photosynthesis vs irradiance. The initial slope of the curve (α) is the maximum light utilisation efficiency. The intersection between the maximum rate of photosynthesis P_{\max} and α is the light saturation (optimum) irradiance. At supra-optimum irradiance, photosynthesis declines, which is commonly called down-regulation or photoinhibition.

usually decline from the light-saturated value. This phenomenon is commonly referred to as *photoinhibition* of photosynthesis.

2.2 The nature of light

The energy for photosynthesis is delivered in the form of light. Light is electromagnetic radiation and travels at the speed $c \sim 3 \times 10^8 \text{ m s}^{-1}$. Based on the wavelength, electromagnetic radiation can be divided into several components (Fig. 2.3). As light is usually denoted radiation with wavelengths between 10^{-3} and 10^{-8} m. Gamma and X-rays have shorter wavelengths, while radio waves are above 10^{-3} m. The visible part of the spectrum ranges from the violet of about 380 nm to the far red at 750 nm. This range is usually expressed in nanometers ($1 \text{ nm} = 10^{-9} \text{ m}$). The wavelengths of visible light also correspond to photosynthetically active radiation (PAR), i.e. radiation utilisable in photosynthesis.

According to the quantum theory, light energy is delivered in the form of separated packages called *photons*. The energy of a single light quantum, or *photon*, is the product of its frequency and Planck's constant, i.e. $h\nu$ ($h = 6.626 \times 10^{-34} \text{ J s}$). Since the energy is inversely related to wavelength, a photon of blue light (about 400 nm) is more energetic than that of red light (around 700 nm). Photosynthetic pigments absorb the energy of photons, and transfer it to the reaction centres where it is utilised for photochemistry. The photon should possess a critical energy sufficient to excite a single electron from one pigment molecule and initiate charge separation. According to Einstein's law, one mole of a compound must absorb the energy of N photons

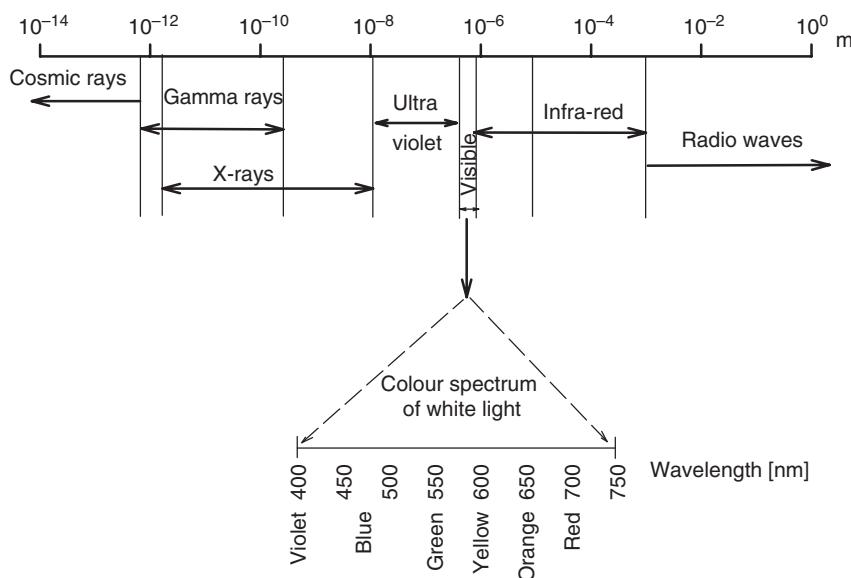


Fig. 2.3. Spectra of electromagnetic radiation and spectral pattern of visible light (adapted from Hall & Rao, 1999). Photosynthetically active radiation (PAR) ranges from 400 to 750 nm.

($N = 6.023 \times 10^{23}$, the Avogadro number) to start a reaction, i.e. $N h\nu$. This unit is called an Einstein ($E = 6.023 \times 10^{23}$ quanta).

Light flux is measured in *lumens* (lm); intensity of illumination is expressed in *lux* (lm m^{-2}) or historically in footcandles (1 lm ft^{-2} , i.e. 1 ft candle equals 10.76 lux).

Photobiologists prefer to measure light energy incident on a surface, i.e. radiant flux energy or *irradiance*, in units of power per area (W m^{-2} or $\text{J m}^{-2} \text{ s}^{-1}$). Since photochemical reactions in photosynthesis depend on the number of photons incident on a surface, it is logical to express irradiance as number of quanta (photons) reaching unit surface in unit time, i.e. as the photosynthetic photon flux density measured in $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ or $\mu\text{E m}^{-2} \text{ s}^{-1}$.

On a sunny day, average direct solar irradiance reaching the earth's surface is about 1000 W m^{-2} ($100\,000$ lux), of which photosynthetically active radiation (PAR, between 400 and 750 nm) represents about 40%, i.e. 400 W m^{-2} or $1800 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$. Thus, the approximate conversion factor for sunlight is 1 W m^{-2} equals about $4.5 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$. There exist different types of instruments to measure irradiance; most of them measure PAR in $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ or in W m^{-2} .

2.3 Photosynthetic pigments

All photosynthetic organisms contain organic pigments for harvesting light energy. There are three major classes of pigments: chlorophylls, carotenoids and phycobilins. The chlorophylls (green pigments) and carotenoids (yellow or orange pigments) are lipophilic and associated in Chl–protein complexes, while phycobilins are hydrophilic.

Chlorophyll (Chl) molecules consist of a tetrapyrrole ring (polar *head*, chromophore) containing a central magnesium atom, and a long-chain terpenoid alcohol (except for Chl *c*) (Fig. 2.4A). These molecules are non-covalently bound to apoproteins. Structurally, the various types of Chl molecules designated *a*, *b*, *c* and *d* differ in their side-group substituents on the tetrapyrrole ring. All Chl have two major absorption bands: blue or blue-green (450–475 nm) and red (630–675 nm). Chl *a* is present in all oxygenic photoautotrophs as a part of the core and reaction centre pigment–protein complexes, and in light-harvesting antennae it is accompanied by Chl *b* or Chl *c*. The so-called accessory (antennae) pigments Chl *b*, *c* and *d* extend the range of light absorption.

Carotenoids represent a large group of biological chromophores with an absorption range between 400 and 550 nm. The basic structural elements of carotenoids are two hexacarbon rings joined by an 18-carbon, conjugated double-bond chain. They are usually either hydrocarbons (carotenes, e.g. α -carotene, β -carotene) or oxygenated hydrocarbons (xanthophylls, e.g. lutein, violaxanthin, zeaxanthin, fucoxanthin, peridinin) (Fig. 2.4C,D). Carotenoids have several roles in the photosynthetic apparatus, functioning as (i) accessory light-harvesting pigments transferring excitation to Chl *a*,

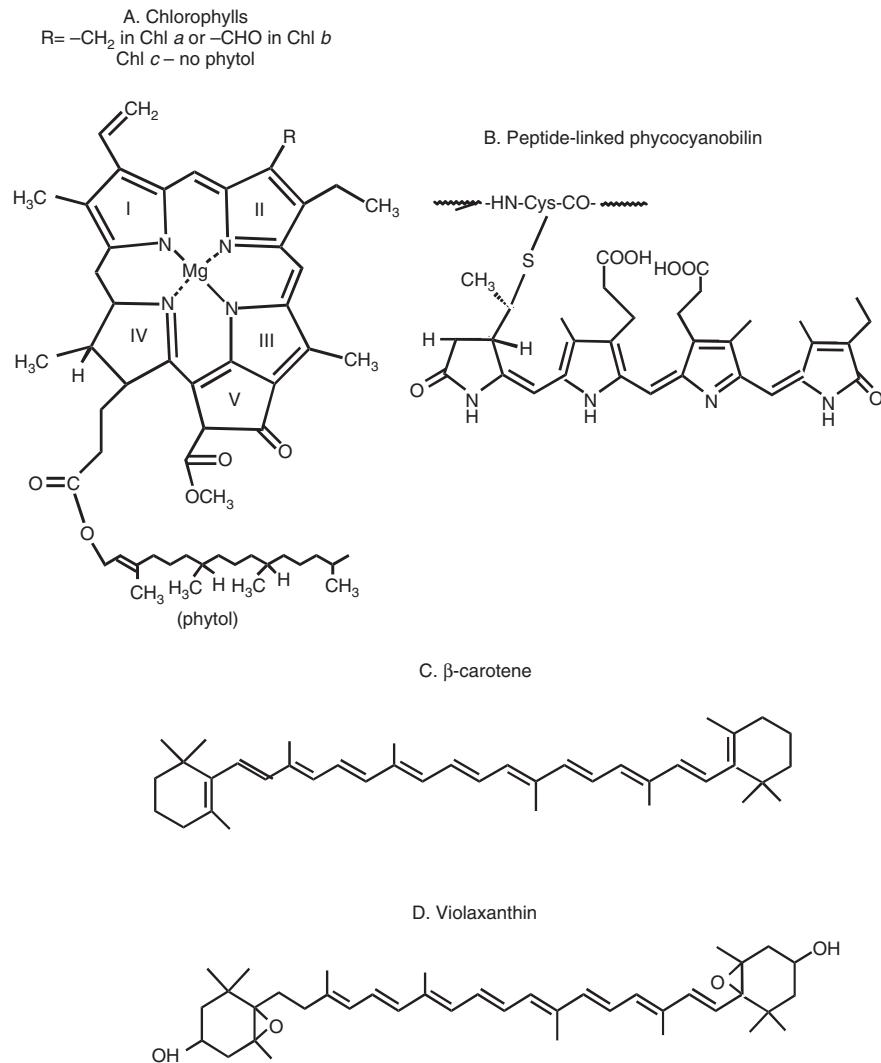


Fig. 2.4. The structures of the three principal groups of pigments in algae and cyanobacteria – chlorophylls (Chls), phycocyanobilin and carotenoids (β -carotene and violaxanthin). All Chls are tetrapyrroles, where nitrogen atoms are co-ordinated around a Mg atom. Chl *a* and *b* differ in the R group while Chl *c* does not contain a side chain of phytol. Phycobiliproteins are open tetrapyrroles, which are covalently linked to a protein. Carotenoids are conjugated isoprenes with cyclic 6-carbon side groups, whereas compared to carotenes, xanthophylls such as violaxanthin, are oxygenated.

(ii) structural entities within the light-harvesting and reaction centre pigment–protein complexes; and (iii) molecules required in the protection against excess irradiance, chlorophyll triplets and reactive oxygen species.

In cyanobacteria and red algae, the major antennae contain phycobilins (phycerythrobilin, phycocyanobilin and phycourobilin), which are linear tetrapyrroles, nor are they associated with a magnesium atom. These accessory

pigments absorb blue-green, green, yellow, or orange light (500–650 nm). In contrast to the Chl-proteins and carotenoid-proteins, phycobiliproteins are water-soluble and the pigments are covalently bound to apoprotein (Fig. 2.4B).

Some pigments in algae do not transfer excitation energy. One group called secondary carotenoids, e.g. orange-red coloured xanthophylls, astaxanthin and canthaxanthin, are overproduced in some algal species (e.g. *Haematococcus pluvialis*) when grown under unfavourable conditions (i.e. combinations of nutrient deficiency, temperature extremes and high irradiance). These pigments are found in the cytoplasm and their metabolic role is unknown.

For quantification of Chls and carotenoids the pigments are extracted in organic solvents (methanol, ethanol, acetone, etc.). The absorbance of the extract is determined spectrophotometrically and the pigment content is calculated using mathematical formulae (e.g. Lichtenthaler & Wellburn, 1983). The separation and quantification of individual carotenoids can be achieved using high-performance liquid chromatography equipped with an absorption or fluorescence detector (e.g. by the method of Gilmore & Yamamoto, 1991).

2.4 The light reactions of photosynthesis

2.4.1 The photosynthetic membranes

The photosynthetic light reactions are located in the thylakoid membranes. These are composed of two major lipid components mono- and digalactosyldiglycerol arranged in a bilayer, in which proteins are embedded forming a liquid mosaic (Singer & Nicholson, 1972). They form closed, flat vesicles around the intrathylakoidal space, the lumen. Some protein–protein or pigment–protein complexes span the thylakoid membrane, whereas others only partially protrude with some functional groups facing the lumen or stroma.

In cyanobacteria (and also eukaryotic red algae), the photosynthetic lamellae occur singly, most likely as a result of the presence of hydrophilic phycobilisomes serving as outer (major) light-harvesting complexes. In the chloroplasts of higher plants, highly appressed regions of stacked thylakoids called *grana* are connected by single thylakoids called stromal lamellae. By contrast, in most algal strains, the thylakoids are organised in pairs or stacks of three.

The thylakoid membrane contains five major complexes: light-harvesting antennae, photosystem II (PS II) and photosystem I (PS I) (both containing a reaction centre), cytochrome b_6/f and ATP synthase, which maintain photosynthetic electron transport and photophosphorylation (Fig. 2.5).

2.4.2 Photosynthetic electron transport and phosphorylation

The main role of the light reactions is to provide the biochemical reductant (NADPH_2) and the chemical energy (ATP) for the assimilation of inorganic carbon. The light energy is trapped in two photoreactions carried out by two

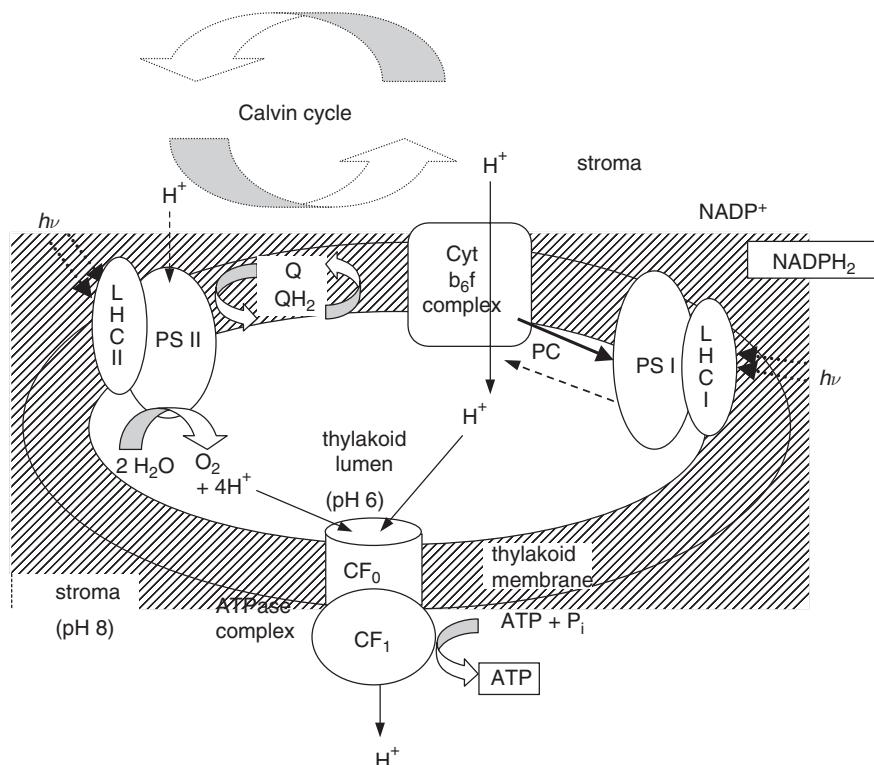
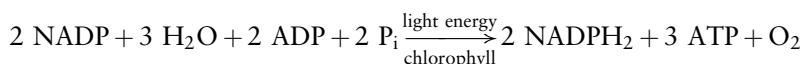


Fig. 2.5. Vectorial arrangement of photosystem I and II, the cytochrome b_6/f complex, and the ATP synthase within the thylakoid (adapted from Stryer, 1988). Electrons are removed from molecules of H_2O resulting in the evolution of O_2 as a by-product transported outside the thylakoid. Protons are translocated from an external space (stroma) into the intrathylakoid space during the light-induced electron transport. The flow of protons through the ATP synthase to the stroma leads to the generation of ATP from ADP and P_i in the stroma where the Calvin–Benson cycle reactions are carried out. $NADPH_2$ is also formed on the stromal side of the thylakoid.

pigment–protein complexes, PS I and PS II. The photosystems operate in series connected by a chain of electron carriers usually visualised in a so-called ‘Z’ scheme (Hill & Bendall, 1960). In this scheme, redox components are characterised by their equilibrium mid-point potentials and the electron transport reactions proceed energetically downhill, from a lower (more negative) to a higher (more positive) redox potential (Fig. 2.6).

Upon illumination, two electrons are extracted from water (O_2 is evolved) and transferred through a chain of electron carriers to produce one molecule of $NADPH_2$. Simultaneously, protons are transported from an external space (stroma) into the intrathylakoid space (lumen) forming a pH gradient. According to Mitchell’s chemiosmotic hypothesis, the gradient drives ATP synthesis, which is catalysed by the protein complex called ATPase or ATP synthase (Fig. 2.5). This reaction is called photophosphorylation and can be expressed as:



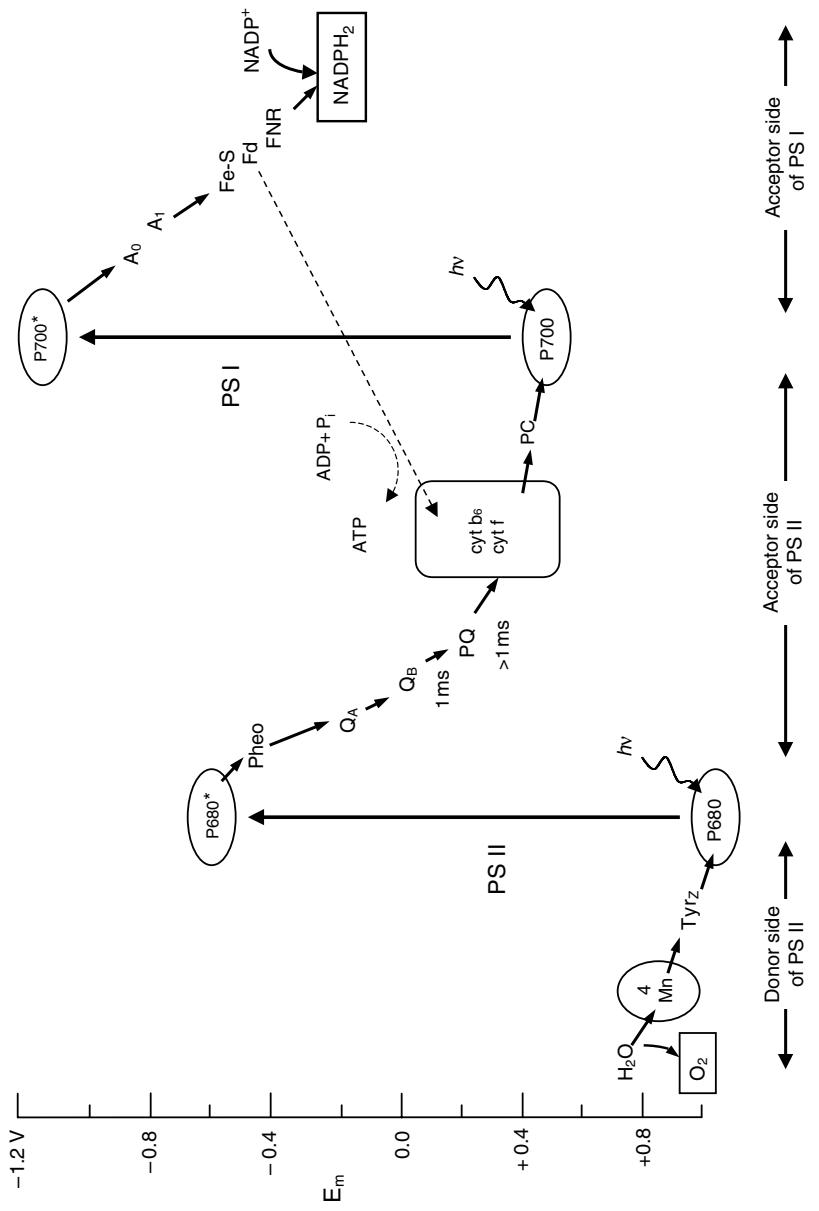


Fig. 2.6. The Z scheme for photosynthetic electron flow from water to NADPH₂ (Hill & Bendall, 1960). The electron transport carriers are placed in series on a scale of mid-point potentials. The oxidation of the primary electron donor P680 leads to a charge separation of about 1.2 V. The electron hole in P680⁺ is filled by an electron from tyrosine Tyr Z, which obtains an electron from water via the four Mn ions. On the acceptor side of PS II, the pheophytin (Pheo) reduces the primary acceptor, Q_A, which is a plastquinone molecule bound to a protein. Two electrons are sequentially transferred from Q_A to the secondary acceptor Q_B, the time constant of which is dependent on the level of reduction of Q_B. The reduced plastquinol PQH₂ is the slowest reaction in the photosynthetic electron transport pathway. Plastocyanin (PC) carries one electron to the reaction centre of PS I, P700. On the acceptor side of PS I, the electron is passed through a series of carriers to ferredoxin, resulting finally in the reduction of NADP. The dotted straight arrow shows the pathway of cyclic photophosphorylation, where the electrons cycle in a closed system around PS I (from ferredoxin to the cyt b₆/f complex) and ATP is the only product.

2.4.3 The outer light-harvesting antennae

The primary function of the antenna systems is light-harvesting and energy transfer to the photosynthetic reaction centres (Fig. 2.7). The energy is funnelled to the reaction centres placed energetically *downhill*; some amount of heat is released during the transfer. All photosynthetically active pigments (chlorophylls, carotenoids and phycobilins) are associated with proteins, which are responsible for conferring a variety of specific functions in light-harvesting and electron transfer.

Two major classes of light-harvesting pigment–protein complexes can be identified: (i) hydrophilic phycobiliproteins, which are found in cyanobacteria and red algae, and (ii) hydrophobic pigment–protein complexes, such as LHC II and LHC I that contain Chl *a*, Chl *b* and carotenoids.

In cyanobacteria and red algae, the phycobiliproteins are assembled into multimeric particles called phycobilisomes, which are attached to the protoplasmic side of the thylakoid membrane. Phycobilisomes are assembled around an allophycocyanobilin-containing cores, which are coupled to the cores of PS II. The disks adjacent to the core of phycobiliproteins contain phycocyanobilin. The more distal disks consist of phycoerythrobilin or phycochlorobilin depending on the species. A special subdivision of cyanobacteria is Prochlorophyceae; unlike other cyanobacteria, they contain Chl *b* but no phycobiliproteins (Bryant, 1994).

In green algae (and higher plants), outer light-harvesting Chl *a/b*-protein complexes (called LHC II and LHC I) bind Chl *a* and *b* as well as xanthophylls (oxygenated carotenoids). A group of complexes LHC II serves PS II, and a genetically and biochemically different group called LHC I is associated with PS I.

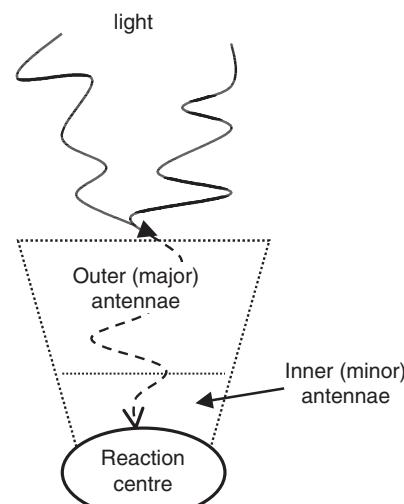


Fig. 2.7. The funnelling of excitation energy through the antenna array to the reaction centre (adapted from Ort, 1994). The light is captured by outer light-harvesting antennae, which are usually mobile. The excitation energy is transferred to the reaction centre via the inner antennae; it is the inner antennae and reaction centre that form the core of the photosystem.

In diatoms, the outer light-harvesting complexes contain Chl *a* and *c*, and fucoxanthin as the major carotenoid.

2.4.4 Photosystem II

Photosystem II represents a multimeric complex located in the thylakoid membrane, with more than 20 subunits and a relative molecular mass of about 300 kDa, composed of the reaction centre, the oxygen-evolving complex and the inner light-harvesting antennae. The PS II reaction centre contains the D1 and D2 proteins and the α and β subunits of cyt *b*₅₅₉. D1 and D2 proteins carry all essential prosthetic groups necessary for the charge separation and its stabilisation, tyrosine Z, the primary electron donor, P680, pheophytin and the primary and secondary quinone acceptors, Q_A and Q_B (Fig. 2.8). The inner core antennae are formed by the intrinsic Chl *a*-proteins CP43 and CP47, transferring excitation energy from the outer antennae to reaction centre (Fig. 2.7). As shown in Fig. 2.8, CP43 and CP47 are located on opposite sides of the D1-D2 reaction centre (Hankamer *et al.*, 2001). Recently, the X-ray crystal structure of PS II isolated from *Synechococcus elongatus* was resolved at 3.8 Å resolution (Zouni *et al.*, 2001).

2.4.5 Plastoquinone, the cytochrome b_{6/f} complex and plastocyanin

Electron transport between PS II and PS I is linked via the cytochrome b_{6/f} complex and assisted by two kinds of mobile carriers (Fig. 2.5). Plastoquinones (lipophilic benzoquinones with an isoprenoid chain) serve as

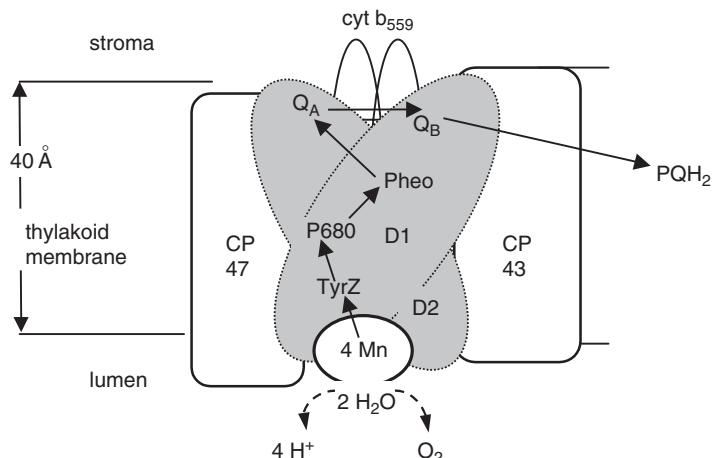


Fig. 2.8. Schematic diagram of molecular organisation of the PS II core. The major protein subunits CP43, CP47, cyt *b*₅₅₉, and the D1 and D2 proteins are labelled with bold letters. The two shaded protein subunits D1 and D2 are known to bind most of the electron carriers (a manganese cluster – 4 Mn, a tyrosine molecule Tyr Z, the special pair of chlorophyll *a* molecules P680, pheophytin Pheo, the plastoquinones Q_A and Q_B, and the plastoquinone pool PQH₂). The water-splitting complex represented by four manganese atoms is located in the thylakoid lumen. Arrows indicate principal electron transport pathways.

two-electron carriers between PS II and cytochrome b₆/f complexes. In parallel, the plastoquinone molecule translocates two protons from the stroma into the lumen. Plastocyanin (Cu-binding protein) operates in the thylakoid lumen, transferring electrons between the cytochrome b₆/f complex and PS I (for review, see Gross, 1996).

2.4.6 Photosystem I

Photosystem I is a multi-subunit intermembrane complex composed of about ten proteins, 100 chlorophylls and a molecular mass of about 360 kDa. PS I performs the photochemical reactions that generate the low redox potential (about -1 V) necessary for reducing ferredoxin and subsequently producing NADPH₂. The two large *PsaA* and *PsaB* proteins are located at the centre of the monomer which bears the major prosthetic cofactors of the reaction centre. Embedded within the complex are the Chl dimer P700 (where primary charge separation is initiated) and electron carriers A₀ (Chl *a*), A₁ (phylloquinone) and F_X (4Fe–4S). Generated electrons are further transported to the 4Fe–4S electron acceptors F_A and F_B of the *PsaC* subunit and to the terminal mobile electron acceptor, which is ferredoxin (Fig. 2.6). Recently, the 2.5 Å X-ray structure of cyanobacterial PS I was resolved (Jordan *et al.*, 2001).

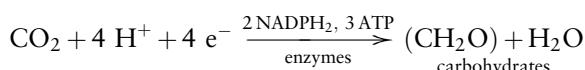
2.4.7 ATP synthase/ATPase

ATP synthase is a membrane-bound enzyme that is composed of two oligomeric subunits, CF₀ and CF₁ (with relative molecular masses of 110–160 kDa and about 400 kDa, respectively). The complex powered by the pH gradient catalyses the synthesis of ATP from ADP and P_i (Fig. 2.5). The hydrophobic CF₀ spans the thylakoid membrane, whereas the hydrophilic CF₁ is attached to CF₀ on the stromal side of the membrane. The subunits CF₀ act as a proton channel and the flux of protons drives the subunits CF₁, which form a ring structure with catalytic sites for ATP synthesis. A passage of about four protons is required for the synthesis of one ATP molecule (Kramer *et al.*, 1999).

2.5 The dark reactions of photosynthesis

2.5.1 Carbon assimilation

The fixation of carbon dioxide happens in the dark reaction using the NADPH₂ and ATP produced in the light reaction of photosynthesis. The reaction can be expressed as:



In order to fix one molecule of CO₂, two molecules of NADPH₂ and three molecules of ATP are required (representing an energy of $5.2 \times 10^4\text{ J}$, about

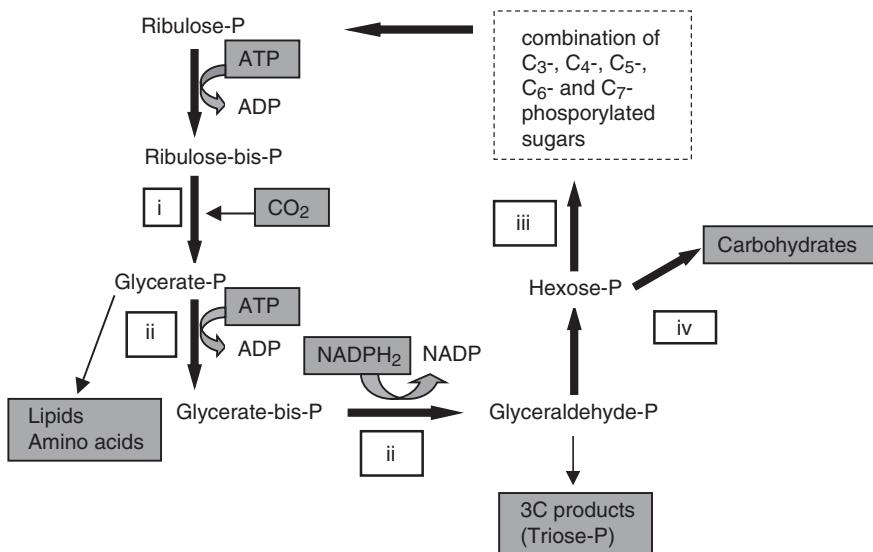


Fig. 2.9. The photosynthetic carbon fixation pathways – the Calvin–Benson cycle. The fixation of CO_2 to the level of sugar can be considered to occur in four distinct phases: (i) *Carboxylation phase* – a reaction whereby CO_2 is added to the 5-carbon sugar, ribulose bisphosphate (Ribulose-bis-P), to form two molecules of phosphoglycerate (Glycerate-P). This reaction is catalysed by the enzyme ribulose bisphosphate carboxylase/oxygenase (Rubisco); (ii) *Reduction phase* – to convert Glycerate-P to 3-carbon products (Triose-P), the energy must be added in the form of ATP and NADPH₂ in two steps, the phosphorylation of Glycerate-P to form diphosphoglycerate (Glycerate-bis-P), and the reduction of Glycerate-bis-P to phosphoglyceraldehyde (Glyceraldehyde-P) by NADPH₂; (iii) *Regeneration phase* – Ribulose-P is regenerated for further CO_2 fixation in a complex series of reactions combining 3-, 4-, 5-, 6- and 7-carbon sugar phosphates, which are not explicitly shown in the diagram; (iv) *Production phase* – primary end-products of photosynthesis are considered to be carbohydrates, but fatty acids, amino acids and organic acids are also synthesised in photosynthetic CO_2 fixation.

13 kcal). As concern the quantum efficiency of CO_2 fixation, it was found that at minimum ten quanta of absorbed light are required for each molecule of CO_2 fixed or O_2 evolved.

The reaction mechanism of carbon fixation was worked out by Calvin and Benson in the 1940s and early 1950s using ¹⁴C radiolabelling technique (Nobel Prize, 1961). The conversion of CO_2 to sugar (or other compounds) occurs in four distinct phases (Fig. 2.9) forming the so-called Calvin–Benson cycle:

1. *Carboxylation phase* The reaction whereby CO_2 is added to the 5-carbon sugar, ribulose bisphosphate (Ribulose-bis-P), to form two molecules of phosphoglycerate (Glycerate-P). This reaction is catalysed by the enzyme ribulose bisphosphate carboxylase/oxygenase (Rubisco).
2. *Reduction phase* In order to convert phosphoglycerate to 3-carbon products (Triose-P) the energy must be added in the form of ATP and NADPH₂ in two steps: phosphorylation of phosphoglycerate to form diphosphoglycerate and ADP, and secondly, reduction of diphosphoglycerate (Glycerate-bis-P) to phosphoglyceraldehyde (Glyceraldehyde-P) by NADPH₂.

3. *Regeneration phase* Ribulose phosphate (Ribulose-P) is regenerated for further CO₂ fixation in a complex series of reactions combining 3-, 4-, 5-, 6- and 7-carbon sugar phosphates. The task of generating 5-carbon sugars from 6-carbon and 3-carbon sugars is accomplished by the action of the transketolase and aldolase enzymes.
4. *Production phase* Primary end-products of photosynthesis are considered to be carbohydrates, but fatty acids, amino acids and organic acids are also synthesised in photosynthetic CO₂ fixation. Various end-products can be formed under different conditions of light intensity, CO₂ and O₂ concentrations, and nutrition.

2.5.2 Photorespiration

Photorespiration represents a competing process to carboxylation, where the organic carbon is converted into CO₂ without any metabolic gain. In this process, Rubisco functions as an oxygenase, catalysing the reaction of O₂ with ribulose bisphosphate to form phosphoglycolate. After dephosphorylation, glycolate is converted, in several steps, to serine, ammonia and CO₂.

Photorespiration depends on the relative concentrations of oxygen and CO₂ where a high O₂/CO₂ ratio (i.e. high concentration of O₂ and low concentration of CO₂) stimulates this process, whereas a low O₂/CO₂ ratio favours carboxylation. Rubisco has low affinity to CO₂, its K_m (half-saturation) being roughly equal to the level of CO₂ in air. Thus, under high irradiance, high oxygen level and reduced CO₂, the reaction equilibrium is shifted towards photorespiration. Photosynthetic organisms differ significantly in their rates of photorespiration: in some species it may be as high as 50% of net photosynthesis.

For optimal yields in microalgal mass cultures, it is necessary to minimise the effects of photorespiration. This might be achieved by an effective stripping of oxygen and by CO₂ enrichment. For this reason, microalgal mass cultures are typically grown at a much higher CO₂/O₂ ratio than that found in air.

2.6 Light adaptation (Falkowski & Raven, 1997)

In the natural environment, photosynthetic organisms can face frequent changes in irradiance – in the range of one to two orders of magnitude. To cope with such changes plants have developed several acclimation mechanisms. The aim of acclimation processes is to balance the light and dark photosynthetic reactions. Since the levels of Rubisco seem to be relatively constant (Sukernik *et al.*, 1987), the major regulation occurs on the light reactions' side, mainly in PS II. The regulation of the PS II output can be performed in two ways – by modulation of its light-harvesting capacity, or by changes in the number of PS II reaction centres.

In light-limiting conditions, the organism increases pigmentation, i.e. increases the number of photosynthetic units, the size of light-harvesting complexes. Under supra-optimal irradiance the pigmentation is reduced. The changes of pigmentation occur at a timescale of days; so, to respond

to fast changes in irradiance, other mechanisms have to be employed. In many species, the build-up of the pH gradient results in enhanced thermal dissipation (quenching) of harvested quanta, reducing the amount of energy utilised in photochemistry (Briantais *et al.*, 1979). Though, in cyanobacteria, the ΔpH -regulated dissipation does not seem to exist. In higher plants and green algae, the pH gradient build-up is accompanied by a reversible conversion of violaxanthin into zeaxanthin. In higher plants, it was demonstrated that zeaxanthin content correlates well with the extent of thermal dissipation (Demmig *et al.*, 1987). However, in green algae, the zeaxanthin-dependent dissipation seems to play only a minor role (Casper-Lindley & Björkman, 1998; Masojídek *et al.*, 1999). An analogous cycle (monoepoxide diadinoxanthin \leftrightarrow diatoxanthin) has been found in Chrysophyceae and Phaeophyceae. As in the case of zeaxanthin in green plants, the presence of diatoxanthin results in enhanced thermal dissipation of light energy (Arsalane *et al.*, 1994).

The light inactivation of the PS II function (PS II photoinactivation) can be viewed as an emergency acclimation process reducing the number of redundant PS II units. As it happens, light energy causes an inevitable modification of the PS II reaction centres, which, if not repaired by continuous D1 replacement, leads to the inactivation of the PS II function (Prasil *et al.*, 1992). The photoinactivation is manifested as an exponential (single-order) decline of variable fluorescence F_v (F_0 remains constant), paralleled by a decline of the Hill reaction (Šetlik *et al.*, 1990). The rate of this decline is directly proportional to light intensity (Šetlik *et al.*, 1987; Tyystjärvi & Aro, 1996) suggesting that the damage represents a single-photon process with a very low quantum yield. The inactivation of a part of the units caused by excess irradiance does not necessarily reduce the overall rates of electron transfer. At saturating light intensities, the rate of photosynthesis usually depends on the CO₂ fixation rate (Sukenik *et al.*, 1987), and a moderate reduction in the number of active PS II units might not have any effect (Behrenfeld *et al.*, 1998).

2.7 Selected monitoring techniques used in microalgal biotechnology

2.7.1 Measurement of photosynthetic oxygen evolution (Walker, 1993)

Routine measurements of photosynthetic oxygen production in algal cultures are usually carried out with an oxygen electrode. It is a special form of electrochemical cell, in which the generated current is proportional to the activity of oxygen present in a solution, capable of detecting changes of the order of 10 µM.

A Clark-type oxygen electrode, which is the most widely used, consists of a platinum cathode (but gold or other metals can also be used) and a silver/silver chloride anode. When the voltage (-0.71 V) is applied across the electrodes, the oxygen undergoes electrolytic reduction (O₂ + 2e⁻ + 2H⁺ → H₂O₂ + 2e⁻ + 2H⁺ → 2H₂O). The electrodes are placed in an electrolyte (saturated KCl) separated from the suspension by a thin,

gas-permeable membrane (Teflon, polypropylene). The electrode consumes oxygen, and therefore the suspension has to be mixed. Oxygen production is usually expressed in μmol or mg O_2 per mg Chl h^{-1} , or per cell h^{-1} .

Recently, optical oxygen sensors have been developed that are based on the fluorescence and phosphorescence quenching of certain luminophores in the presence of oxygen (e.g. PreSens, Precision Sensing GmbH, Germany; Optod Ltd, Moscow, Russia). Although not widely used, these sensors have sensitivity comparable to Clark-type electrodes, and yet show a few advantages, namely: no consumption of oxygen, stability against electrical and thermal disturbances, and high storage and mechanical stability.

2.7.2 Measurement of photosynthetic carbon fixation

Since photosynthetic carbon fixation in cell suspension cannot be easily followed by infrared gas analysis, special electrodes are used to measure the partial pressure of carbon dioxide (p_{CO_2}) in solutions. The principle is based on the relationship between pH, and the concentration of CO_2 and bicarbonate in the solution ($K_s = [\text{HCO}_3^-] \times [\text{H}^+]/[\text{CO}_2]$). The p_{CO_2} electrode is constructed as a combined glass and Ag/AgCl electrode.

The method of ^{14}C radiolabelling has been widely used to study photosynthetic carbon metabolism, but it also provides a measure of the photosynthetic assimilation rate. The population (or culture) of microalgae is exposed to ^{14}C for a fixed period of time. The reaction is then stopped by the addition of concentrated HCl and the amount of ^{14}C incorporated is determined by a scintillation counter. This technique is widely employed in phytoplankton studies, but can also be exceptionally used in mass cultures in photobioreactors.

Biomass production might be roughly estimated as optical density (OD) at 750 nm, or measured as dry weight per volume of sample. Exact determination of carbon (and nitrogen) content in the biomass can be done by a CHN analyser.

2.7.3 Chlorophyll fluorescence

Chlorophyll fluorescence has become one of the most common and useful techniques in photosynthesis research. Its non-invasiveness, sensitivity, as well as the wide availability of reliable commercial instruments, also make it a convenient and suitable technique in algal biotechnology.

Chlorophyll fluorescence directly reflects the performance of photochemical processes in PS II; the contribution of PS I emission in the total signal at ambient temperature is rather small and for practical purposes is often neglected. However, in cyanobacteria, the fluorescence of numerous PS I complexes and phycobilisomes contributes significantly to the total signal, which affects the correct determination of certain parameters (e.g. F_v/F_M). Upon illumination, the PS II chlorophyll molecules are excited to a singlet excited state (Chl α^*). The energy of the excited state is transferred to the reaction centre to be used for photochemical charge separation. Alter-

natively, the excitation energy can be dissipated as heat, or re-emitted as fluorescence (Fig. 2.10). The sum of energy entering these three competing processes is equal to the absorbed light energy. Any change of photochemistry or dissipation results in a change of fluorescence, providing a direct insight into the energetics of PS II.

In the dark, all the reaction centres are in the so-called *open* state and photochemistry is at a maximum. The fluorescence yield in this state is low, designated as F_0 . When PS II is exposed to a strong pulse of light, the reaction centres undergo charge separation and the electron is moved to the first electron acceptor Q_A . When Q_A is reduced, the reaction centres are in the *closed* state and photochemistry is transiently blocked. Since the yield of photochemistry is zero, the dissipation and fluorescence yields rise proportionally. The high fluorescence yield of the closed centres is described as F_M . Since the fluorescence yield rises proportionally to the level of the PS II closure, the open reaction centre acts as a fluorescence quencher. This phenomenon is called photochemical quenching qP and can be calculated as $(F_M - F')/(F_M - F_0)$, where F' is a steady-state yield of fluorescence. The values of qP range from 0 to 1 reflecting the relative level of Q_A oxidation (Fig. 2.11).

The difference between the maximum fluorescence F_M (all Q_A reduced) and minimum fluorescence F_0 (all Q_A oxidised) is denoted as the variable fluorescence F_V . The ratio between the variable fluorescence and maximum fluorescence (F_V/F_M) ranges from 0.65 to 0.80 in dark-adapted green algae. This ratio is frequently used as a convenient estimate of the photochemical yield of PS II. The yield varies significantly, depending on the irradiance regime and physiological treatment.

When the photosynthetic apparatus is exposed to light, a decrease in F_M is usually observed. The lowered fluorescence yield is described as F'_M . This phenomenon is called non-photochemical quenching and indicates an

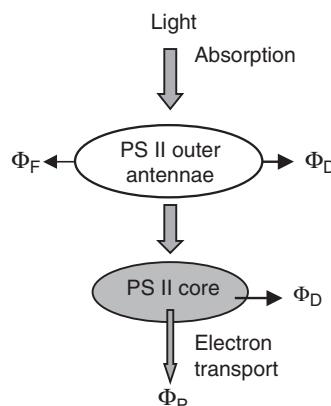


Fig. 2.10. A schematic representation of absorbed light energy distribution in the PSII complex between photochemistry Φ_P , fluorescence Φ_F and non-radiative dissipation Φ_D ; the latter (Φ_D) can occur in the antennae as well as in the reaction centre. Φ_P , Φ_F and Φ_D represent the yield of photochemistry, fluorescence and non-radiative dissipation, respectively.

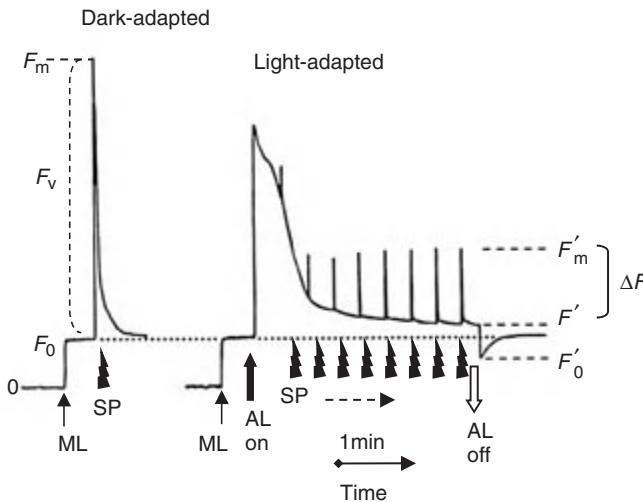


Fig. 2.11. A schematic representation of the saturation pulse method (adapted from Schreiber et al., 2000). The minimum and maximum fluorescence levels F_0 and F_m are measured in the dark-adapted sample, using modulated measuring light (ML) and a saturating light pulse (SP). Next, the sample is illuminated with actinic light (AL) and a series of saturating pulses in order to reach the steady state F' and F'_m level. Finally, the actinic light and saturating pulses are switched off to measure the F'_0 level.

increased heat dissipation of excitation (Fig. 2.10). In principle, non-photochemical quenching is inversely related to photochemistry, and is considered a safety valve protecting PS II reaction centres from damage by excess irradiance. Selected parameters calculated from chlorophyll fluorescence measurements are listed in Table 2.1.

Table 2.1. Selected parameters calculated from chlorophyll fluorescence measurements (Fig. 2.11). F_0 , F_v , F_m – minimum, variable and maximum fluorescence in dark-adapted state; F'_0 , F' , F'_v , F'_m – minimum, steady-state, variable and maximum fluorescence in light-adapted state; α_{PSII} – optical absorption cross-section of PS II; PPFD – photosynthetic photon flux density.

Parameter	Symbol	Formula
Maximum photochemical yield of PS II	F_v/F_m	$F_v/F_m = (F_m - F_0)/F_m$
Effective PS II photochemical yield	Φ_{PSII} or $\Delta F/F'_m$	$\Phi_{PSII} = (F'_m - F')/F'_m$
Relative electron transport rate through PS II (rate of photochemistry)	rETR	$rETR = \Phi_{PSII} \times PPFD$
Actual electron transport rate through PS II (correlated with primary productivity)	ETR	$ETR = \Phi_{PSII} \times PPFD \times \alpha_{PSII}$
Non-photochemical quenching	qN	$qN = 1 - (F'_m - F'_0)/(F_m - F_0)$
Photochemical quenching qP	qP	$qP = (F'_m - F')/(F'_m - F'_0)$
Stern–Volmer coefficient of non-photochemical quenching	NPQ	$NPQ = (F_m - F'_m)/F'_m$

2.8 Theoretical limits of algal productivity

An understanding of photosynthesis is fundamental for microalgal biotechnology. Mass cultures of unicellular microalgae (cyanobacteria and algae) grown in the laboratory and outdoors represent a special environment, where rather dense suspensions of cells, colonies, coenobia or filaments are usually cultivated under conditions of low irradiance per cell, high concentration of dissolved oxygen and limited supplies of inorganic carbon (carbon dioxide or bicarbonate). Therefore, the growth critically depends on the interplay of several parameters: average irradiance per cell, mixing, gas exchange and temperature.

Ideally, the theoretical maximum rate of growth of an algal culture should be equal to the maximum rate of photosynthesis. In a fast-growing culture adapted to high irradiance, the turnover of electron transport can reach 2 ms, which probably corresponds to the turnover of the PS II complex. At this rate, up to 50 atoms of carbon can be fixed per individual RC per second, if we consider that ten electrons are transferred per C atom or per molecule of O₂ fixed. Assuming 300 chlorophyll molecules per PS II unit, then the rate of photosynthesis can be about $660 \mu\text{mol C (or O}_2\text{)} \text{ mg}^{-1} (\text{Chl}) \text{ h}^{-1} = 7.9 \text{ g C (or O}_2\text{)} \text{ g}^{-1} (\text{Chl}) \text{ h}^{-1}$. This rate, considering a carbon per Chl ratio of 30 (w/w), results in a growth rate of $\mu \sim 0.2 \text{ h}^{-1}$. (See, however, the distinction made between productivity and growth rate in Chapter 7.)

In all cultivation facilities, the growth of algae is spatially confined by the dimensions of the cultivation vessel. This confinement, together with a given solar input, leads to a finite amount of light energy that can be delivered to such a system. Therefore, cultivation facilities have to be designed such that the light conversion efficiency is maximised, which means the use of dense cultures fully absorbing the delivered light. Unfortunately, the steep light gradient formed in these cultures results in an overexposure of the upper layers of the suspension and leads to a low efficiency of light conversion. To avoid this situation, the cultures have to be rapidly mixed to prevent prolonged light saturation (Nedbal *et al.*, 1996). Special designs of photobioreactors might improve the light distribution in a suspension, but proposed solutions are frequently difficult to scale up to the industrial level (Carlozzi & Torzillo, 1996; Tredici & Chini Zittelli, 1997). The third approach to the problem is to modify the optical properties of the cells in order to assure better light utilisation in the suspension (Melis *et al.*, 1999). The genetic modification of reducing the antenna size could reduce the excitation pressure of the photosynthetic units under high irradiance and maintain a high efficiency of light conversion.

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3 Basic Culturing Techniques

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Basic principles of microbial cultivation in general are applicable to microalgae. The unique ability of microalgae to utilize light energy, however, sets them apart from most microorganisms. The supply of light needs to be satisfied in isolation, cultivation and maintenance of photoautotrophic and mixotrophic microalgae.

3.1 Isolation of microalgae

A culture is a genetically homogenous clone propagated from one individual cell or filament, isolation of which involves the following steps:

3.1.1 Selection of sources of microalgae

Water and soil samples collected from diverse habitats, such as thermal springs, glacier ice and industrial-waste treatment sites are likely to yield very different algal isolates. These environments provide ongoing enrichment and selection of organisms adapted to specific conditions. Aquatic algae often appear in planktonic forms. They may be sampled by collecting a representative volume of water and observed directly, or after concentration by centrifugation, filtration, or sedimentation. Algae can attach to other algae, vascular plants, animals, and rocks, where they can be collected by scraping.

3.1.2 Enrichment of a culture

Enrichment is the process of providing a suitable environment for the growth and reproduction of a special group of microalgae while being inhibitory or lethal for nontarget organisms. An example of enriching photosynthetic algae in a soil sample is to add 2.5–3.0 ml of suitable nutrient medium to 5–10 mg soil and incubate in the wells of a 24-well tissue culture plate. After three to four weeks of incubation at 25°C in an atmosphere of 5% (v/v) CO₂ in air with continuous illumination, *blooms* of algae may be observed. The material from a bloom is plated onto mineral agar medium for the isolation of photosynthetic microalgae and onto organic carbon-substrate containing agar medium for the isolation of mixotrophic and heterotrophic microalgae. Following an incubation protocol as above, morphologically distinct isolates are picked up and transferred to a fresh agar medium until identical colonies are obtained on a plate.

For microalgae obtained from water sources, algal cells can be concentrated and then plated on agar medium. An example is to concentrate an aquatic sample on a filter membrane. The membrane is then washed with distilled water, and the cell suspension transferred to an agar medium with the inoculated side up. After two to three weeks of incubation in this manner, morphologically distinct colonies are picked up under a dissecting microscope and transferred to another agar plate or a sterile liquid medium. Cell suspension can also be mixed with soft agar and plated on solidified agar.

3.1.3 Direct isolation

Single cells or filaments can be picked up under a dissecting microscope, using micropipettes. The individual cells are transferred to agar medium or fresh sterile medium for isolation.

An atomizer has been used for separating cells in a liquid suspension for isolation (Pfau *et al.*, 1971). This method involves compressed air blown across a micropipette tip, drawing up the liquid sample, finally atomizing the cell suspension. The small droplets formed are evenly sprayed onto the surface of an agar plate. After incubation, single clones may be picked up and transferred to another agar or a sterile liquid medium.

3.1.4 Producing axenic cultures

Axenic cultures are pure, i.e. unicellular as well as free of other organisms (bacteria, protozoa). Some of the basic purification techniques are described below.

3.1.4.1 Cell washing

Under a dissecting microscope, an individual algal cell is picked up using a micropipette and placed in a sterile liquid medium in a spot plate. The organism is then transferred through a series of sterile media. Associated microorganisms adhering to the algal cells are separated through the action of pipetting and washing off during transfer.

3.1.4.2 Density gradient centrifugation

Microalgae are separated from bacteria using density gradient centrifugation. The algae at a particular position within the gradient are collected by fractionation of the gradient. Silica sol percoll has been used to produce density gradient for the separation of microalgae (Whitelam *et al.*, 1983).

3.1.4.3 UV irradiation

Most algae are slightly more resistant to ultraviolet light than bacterial cells. Thus, following UV irradiation, washing and diluting a sample, then spraying or streaking it on selective agar medium may produce pure algal culture free of bacteria.

3.1.4.4 Filtration

Filamentous algae can be separated from bacteria using membrane filters. Sonication is often employed to break up the algae into small length filaments (3–5 cells). The diluted sample is then vacuum filtered.

3.1.4.5 Antibiotics

Various antibiotics have been effectively used in removing bacteria from algae. For example, agar medium containing the antibiotic imipenem ($110\text{ }\mu\text{g ml}^{-1}$) was used to purify unicellular eukaryotic microalgae. The antibiotics nystatin ($100\text{ }\mu\text{g ml}^{-1}$) and cycloheximide ($100\text{ }\mu\text{g ml}^{-1}$) were used to eliminate fungal contaminants from cyanobacteria. Alternatively, low concentrations of organic nutrients and imipenem were added to a cyanobacterial culture. After incubation in the dark, the cyanobacteria were plated onto agar medium and incubated in light (Schwartz *et al.*, 1990).

To confirm the purity of an algal culture, the culture is inoculated into an organic nutrient medium and incubated in dark for two to three days. There should be no indications of any microbial growth.

3.2 Screening of microalgae for bioactive molecules

Direct and indirect methods can be used for the screening of useful biomolecules. Direct assays aim at detection of a specific target product while indirect assays measure the biological activity of the desired product.

3.2.1 Direct assays

Rapid advances in the development of analytical methods and micro-instrumentation allow rapid, selective and highly sensitive analytical procedures for the detection of metabolic products of microalgae. Instruments such as high-performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS) and nuclear magnetic resonance spectrometry (NMR) are widely employed in the screening. Algal products, detected by these methods, that have attracted attention in recent years include pigments, fatty acids, polysaccharides, pharmaceutical and agrochemicals (Cohen, 1999).

3.2.2 Indirect assays

Bio-assays are continuously being developed for the selection of organisms exhibiting antibiotic and antitumor activity. Screening for antitumor activity is carried out with cultures of tumor cells. Toxins and cytostatic agents of antitumor activities have been found in algae (Kerby & Stewart, 1988).

Screening for antibiotic activities is performed with pathogenic micro-organisms (Harmala *et al.*, 1992). Antibiotic compounds against other microalgae, bacteria, fungi, viruses and protozoans are produced by a diverse range of microalgae (Schwartz *et al.*, 1990).

3.3 Maintenance and preservation of microalgal strains

Algal strains can be maintained in liquid or on solid agar media. To maintain an algal strain, the culture can be kept at low irradiance, at room temperature (15–20°C) and transferred once in every six months. For preservation, most algae have to be kept at room temperature. Some species of algae can be kept in liquid nitrogen for long-term storage.

3.4 Measurement of growth parameters

3.4.1 Cell count

Determination of the cell number concentration (cell counting) requires that cells are suspended singly. In some cases, sonication or trypsinization is needed to disintegrate the aggregates of algae. A dilution step is normally required to achieve the necessary resolution of single cells in direct microscopic, Coulter Counter and flow cytometer enumeration. In some cases, biomass is estimated as colony forming units.

3.4.2 Dry and wet mass

Dry mass is measured with total biomass dry weight or ash free dry weight. The determination of dry weight requires cell separation, washing steps and drying to constant weight. Representative aliquots of algal cultures are taken and the cells are separated by membrane filtration or centrifugation. The filter membrane or centrifuge tubes should be pre-weighed. The cells are normally washed with diluted medium or buffer several times, followed by rinsing with distilled water. Marine algae may be washed by isotonic solution (0.5 M) of ammonium formate or ammonium bicarbonate (Zhu & Lee, 1997). Of the two, ammonium bicarbonate is readily available, less expensive and evaporates at a lower temperature (60°C), and is thus a satisfactory washing agent for marine algal dry weight determination. The lowest feasible drying temperature (60°C–100°C) should be used to prevent loss of volatile components. Lower drying temperature could be employed under reduced pressure (e.g. in vacuum oven).

It is usually assumed that determination of ash free dry weight of marine algal samples does not require washing of the sample, since the weight of the nonorganic matter (ash) will be subtracted from the total dry weight. It should be noted that a measurable amount of the seawater salts could evaporate at 540°C but not at 95°C, and contributes to the ash free dry weight (Zhu & Lee, 1997).

After the centrifuge tube or filter membrane has been cooled in a desiccator at room temperature for about 15–30 min, the dried sample should be weighed immediately once taken out of the desiccator to avoid air-moisture absorption.

Wet weight is measured using the same procedures described above, but without drying. Although faster, this determination method is by far less accurate since a defined water content is seldom obtained.

3.4.3 Protein concentration determination

A representative aliquot of an algal culture is taken and the cells are collected by centrifugation. The cells are homogenized in a buffer (e.g. 50 mM Tris-HCl, pH 7.8, 0.25 M sucrose, 25 mM KCl, 10 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM EDTA, 0.1 mM β-mercaptoethanol and 0.5% v/v Triton-100) and the proteins are precipitated by adding 20% (v/v) trichloroacetic acid (TCA). The proteins are collected by centrifugation, then washed with 90% ethanol in 20 mM Tris (pH 7.4). Protein concentration is determined by the Bradford method (Bradford, 1976) or using a Bio-rad protein assay kit. In the Bradford method, 0.1 ml of protein sample is mixed with 5 ml of the reagent (100 mg Coomassie Brilliant Blue G-250 dissolved in 50 ml 95% v/v ethanol). The solution is then mixed with 100 ml 85% w/v phosphoric acid and topped up with water to a final volume of 1000 ml. The absorbance of the mixture at 595 nm is measured within 2–60 min. Protein content is estimated from a calibration curve.

3.4.4 Chlorophyll determination

Cells are collected by centrifugation or membrane filtration. The pigments can be extracted with diverse solvents, such as 100% acetone, 90% methanol and 100% diethyl ether until the biomass appears colorless. After extraction, the cell debris is removed by centrifugation. The absorbance of the solvent extract is measured at the given wavelengths against a solvent blank. The chlorophyll content is estimated using the following equations:

For acetone (mg/l):

$$\begin{aligned}\text{Chlorophyll } a &= (12.7 \times A_{663}) - (2.69 \times A_{645}) \\ \text{Chlorophyll } b &= (22.9 \times A_{645}) - (4.64 \times A_{663}) \\ \text{Chlorophyll } a + b &= (98.02 \times A_{663}) + (20.2 \times A_{645})\end{aligned}$$

For 90% methanol (mg/l):

$$\begin{aligned}\text{Chlorophyll } a &= (16.5 \times A_{665}) - (8.3 \times A_{650}) \\ \text{Chlorophyll } b &= (33.8 \times A_{650}) - (12.5 \times A_{665}) \\ \text{Chlorophyll } a + b &= (4.0 \times A_{665}) + (25.5 \times A_{650})\end{aligned}$$

For diethyl ether (mg/l):

$$\begin{aligned}\text{Chlorophyll } a &= (9.92 \times A_{660}) - (0.77 \times A_{642.5}) \\ \text{Chlorophyll } b &= (17.6 \times A_{642.5}) - (2.18 \times A_{660}) \\ \text{Chlorophyll } a + b &= (97.12 \times A_{660}) + (16.8 \times A_{642.5})\end{aligned}$$

3.4.5 Total organic carbon (TOC) measurement

Cells in one ml of an algal sample (about 1×10^8 cells per ml) are harvested by centrifugation. The cell pellet is suspended in the test reagent (containing

0.2 g HgSO₄, 1 ml distilled water, 1 ml H₂SO₄, 0.5 ml Ag₂SO₄ and 1 ml 0.25 N K₂Cr₂O₇) with additional 1 ml H₂SO₄ added and incubated at 150°C for 1 h. The sample is then titrated with 0.1 N Fe(NH₄)₂(SO₄)₂ · 6H₂O, with three drops of ferroin solution (1/40 mol l) as the redox indicator. The TOC content is estimated from a calibration curve.

3.4.6 Growth rate and output rate

When the culture environment is favorable and all nutrients required for cell growth are present in a non-growth limiting quantity, i.e. at sufficiently high concentrations so that minor changes do not significantly affect the reaction rate, most unicellular algae reproduce asexually. The size and biomass of individual cells increase with time, resulting in *biomass growth*. Eventually, the DNA content is doubled in quantity and cell division ensues upon complete division of the cell into two progenies of equal genome and of more or less identical size. Population number is thereby increased, and *population growth* is therefore referred to as increase in population of the number of cells in a culture.

3.4.6.1 Doubling time, specific growth rate and output rate

The time required to achieve a doubling of the number of viable cells is termed *doubling time* (*t_d*). It is also termed *generation time*, as it is the time taken to grow and produce a generation of cells. The number of cells in an exponentially growing microbial culture could be mathematically described as follows:

$$2^0 N_0 \rightarrow 2^1 N_0 \rightarrow 2^2 N_0 \rightarrow 2^3 N_0 \rightarrow 2^n N_0$$

N₀ = Initial number of cells

n = Number of doublings (generations)

Number of doublings (*n*) at a time interval *t*, is determined by the relation *t/t_d*. Thus, the number of cells (*N_t*) in an exponentially growing culture after being incubated for some time, *t*, can be estimated:

$$\begin{aligned} N_t &= N_0 2^n \\ &= N_0 2^{t/t_d} \\ N_t/N_0 &= 2^{t/t_d} \\ \ln(N_t/N_0) &= (\ln 2)t/t_d \end{aligned} \tag{4.1}$$

During the exponential growth phase, the growth rate of the cells is proportional to the biomass of cells. Since biomass generally can be measured more accurately than the number of cells, the basic microbial growth equations are often expressed in terms of mass. A biomass component such as protein may

be used as an alternative to direct weighing of biomass. Hence, equation (4.1) can be modified, by assuming the biomass concentration at time 0 (initial) and time t as X_0 and X_t , respectively:

$$\begin{aligned}\ln(X_t/X_0)/t &= 0.693/t_d \\ d(\ln X)/dt &= 0.693/t_d \\ d(\ln X)/dX \cdot dX/dt &= 0.693/t_d \\ 1/X \cdot dX/dt &= 0.693/t_d \\ \mu &= 0.693/t_d\end{aligned}$$

where μ represents the *specific growth rate* (h^{-1}) of the culture. It defines the fraction of increase in biomass over a unit time, i.e. an increase of certain g-biomass from every g of existing biomass per hour. Specific growth rate represents the average growth rate of all cells present in a culture, but not necessarily the maximum specific growth rate of the individual cells, as most microbial cultures divide asynchronously.

The expression of the rate of microbial growth as a specific growth rate avoids the effect of cell concentration, i.e. the output rate of a culture at a concentration of 1 g l^{-1} is $1 \text{ g-biomass l}^{-1} \text{ h}^{-1}$ with a doubling time of 1 h, whereas the same culture with the same doubling time produces $10 \text{ g-biomass l}^{-1} \text{ h}^{-1}$ at a biomass concentration of 10 g l^{-1} .

3.4.7 Growth yield

Growth yield ($Y_{x/s}$, g-cells/g substrate) is defined as the amount of biomass produced (dX) through the consumption of a unit quantity of a substrate (ds), i.e.

$$Y_{x/s} = dX/ds$$

It is an expression of the conversion efficiency of the substrate to biomass.

3.4.8 Maintenance energy requirement

Microbes and cells require energy for both growth and other maintenance purposes, e.g. turnover of cellular materials or maintaining concentration gradient across cell membrane and motility.

Under a particular set of environmental conditions, it is assumed that energy is consumed at a constant rate for maintenance ($ds/dt)_m = mX$, where m is a constant called the *maintenance coefficient* (e.g. g-glucose/g-biomass/h), X is the biomass concentration (e.g. g-biomass l^{-1}). Thus, the energy balance is given by:

$$\text{Total rate of consumption} = \begin{aligned}&\text{Rate of consumption for growth} \\ &+ \text{Rate of consumption for maintenance}\end{aligned}$$

$$\mu X/Y_E = \mu X/Y_G + mX$$

where,

Y_E = Overall growth yield, that is the yield value determined experimentally,
 Y_G = True growth yield, where $m = 0$.

Hence

$$1/Y_E = 1/Y_G + m/\mu \quad (4.2)$$

Thus, if m is a constant, the graph of $1/Y_E$ against $1/\mu$ will be a straight line, with slope m , and intercept $1/Y_G$ on the ordinate.

Alternatively

$$q_E X = \mu X / Y_G + mX$$

where, q_E = Specific rate of energy uptake = μ/Y_E

$$q_E = \mu / Y_G + m \quad (4.3)$$

The maintenance coefficient of a *Chlorella* culture was reported to be $20 \text{ J/g-biomass h}^{-1}$ (Pirt *et al.*, 1980).

The requirement of cells for maintenance energy should affect the relation between growth rate and supply of energy substrate, i.e.

$$\mu = \mu_m s / (s + K_s) - m Y_G \quad (4.4)$$

Thus, if the maintenance energy requirement makes up a significant component in the energy balance equation, the plot of μ vs [s] would not pass through the origin.

3.5 Modes of culture

3.5.1 Batch culture

This is the most common method for cultivation of microalgal cells. In a simple batch culture system, a limited amount of complete culture medium and algal inoculum are placed in a culture vessel and incubated in a favorable environment for growth. Some form of agitation, such as shaking or impeller mixing, is necessary to ensure nutrient and gaseous exchange at the cell–water interface. The culture vessel can be a simple conical flask or an environment controlled fermentor. In a photosynthetic or mixotrophic culture, CO_2 is supplied by either purging the conical flask with CO_2 enriched air (e.g. 5% v/v CO_2 in air) and capped, or by gassing the culture continuously with CO_2 enriched air. The culture can be illuminated externally by either natural or artificial light sources (Pohl *et al.*, 1988; Javanmardian & Palsson, 1991; Muller-Feuga *et al.*, 1998), or sunlight through optical fiber (Mori, 1985; Matsunaga *et al.*, 1991; Ogbonna *et al.*, 1999), placed in the culture vessels.

Batch culture is widely used for commercial cultivation of algae for its ease of operation and simple culture system. Since the process is batch wise, there

is low requirement for complete sterilization. For mass algal culture production, a portion of the culture could be retained as inoculum for the next culture batch.

The different phases, which may occur in a batch culture, reflect changes in the biomass and in its environment.

3.5.1.1 Lag phase

An initial lag phase where the specific growth rate is at the sub-maximum level may often be observed. The growth lag could be due to the presence of non-viable cells or spores in the inoculum. The growth lag could also be the period of physiological adjustment due to changes in nutrient or culture conditions. For example, growth lag may be observed when shade-adapted cells are exposed to a higher irradiance. Lag phase may be abolished when cells at a later exponential growth phase are used as inoculum.

3.5.1.2 Exponential phase

At the late lag phase, the cells have adjusted to the new environment and begin to grow and multiply (*accelerating growth phase*), and eventually enter the *exponential* (or *logarithmic*) *growth phase*. At the latter phase, cells grow and divide as an exponential function of time, as long as mineral substrates and light energy are saturated.

3.5.1.3 Linear growth phase

The quantity of light energy absorbed by a photosynthetic culture is mostly determined by cell concentration and not the photon flux density. That is, most photons of low flux density could pass through a culture of low cell concentration, but all photons of high flux density could be captured by a culture of high cell concentration. Thus, cell concentration of a photosynthetic culture will continue to increase exponentially until all photosynthetically available photons impinging on the culture surface are absorbed. For example, a *Chlorella* cell suspension, the light-harvesting system of which has an optical absorption cross-section of $60 \text{ cm}^2 \text{ mg}^{-1}$ chlorophyll *a*, and a cellular chlorophyll *a* content of $30 \text{ mg Chl } a/\text{g-cell}$, will require $5.6 \text{ g-cell m}^{-2}$ or $0.56 \text{ g-cells l}^{-1}$ to take up all available photons impinging on a culture vessel $1 \text{ m wide, } 1 \text{ m long and } 0.01 \text{ m deep}$, irrespective of the photon flux density. Once this cell concentration is reached, biomass accumulates at a constant rate (linear growth phase) until some substrate in the culture medium or inhibitors becomes the limiting factor.

In the light limited linear growth phase, the relationship between the biomass output rate and the light energy absorbed by the culture can be expressed as follows (Pirt *et al.*, 1980).

$$IA = \mu X \cdot V/Y$$

where,

- I = Photon flux density in the photosynthetically available range ($\text{Jm}^{-2}\text{h}^{-1}$),
- A = Illuminated surface area (m^2),
- μ = Specific growth rate (1h^{-1}),
- X = Biomass concentration (g l^{-1}),
- V = Culture volume (m^3),
- Y = Growth yield (g J^{-1}).

The above equation suggests that, if the value of Y for a particular microalga is a constant, the specific growth rate (μ) changes with changing cell concentration (X). The only specific growth rate that could be maintained at a constant value over a period of time is the maximum specific growth rate at light saturation. Thus, it is only meaningful to compare the biomass output rates ($\mu X, \text{ g l}^{-1} \text{ h}^{-1}$) of light-limited photosynthetic cultures.

At the cell level, the growth rate (μ) of a light-limited photosynthetic cell is determined by the photon flux density. The relationship between the photon flux density (I) and the specific growth rate has the form of the Monod relation (Gobel, 1978).

$$\mu = \mu_m I / (I + K_I)$$

where,

- μ_m = Maximum specific growth rate,
- K_I = Light saturation constant, the photon flux density required to achieve half of the maximum specific growth rate.

In a study on the photosynthetic bacterium *Rhodopseudomonas capsulata*, the K_I for monochromatic light at 860 nm, in which photons are mainly absorbed by bacteriochlorophyll, was $25 \mu\text{mol m}^{-2} \text{ s}^{-1}$. The K_I value of carotenoid pigment (absorbing light at 522 nm) was $103 \mu\text{mol m}^{-2} \text{ s}^{-1}$. Apparently, bacteriochlorophyll has a higher affinity for light than carotenoids. The maximum specific growth rate of *Rhodopseudomonas* culture was, however, independent of the wavelength used. In addition, the light-harvesting pigment content of the photosynthetic culture did not affect the affinity (K_I) or the uptake rate of light energy. The alteration in pigment content observed at different specific growth rates was interpreted as a physiological adaptation of the culture aimed at increasing photon absorption (Gobel, 1978).

Among the microalgae, the green algae have lower affinity for white light when compared to other groups of microalgae, the K_I values decrease in the order of chlorophytes > diatoms > dinoflagellates (Richardson *et al.*, 1983). This may explain the observation that diatoms and dinoflagellates are the predominant species in temperate and tropical seawater, respectively.

3.5.2 Continuous cultures

In continuous flow cultures, fresh culture medium is supplied to the homogeneously mixed culture and culture is removed continuously or intermittently.

The approach is based on the observations that substrates are depleted and products accumulate during growth. Eventually, culture growth ceases due to depletion of the growth limiting substrate or accumulation of a growth-inhibiting product. To sustain cell growth, the growth-limiting substrate needs to be replenished and the growth inhibitory product needs to be removed or diluted by adding fresh culture medium.

3.5.2.1 Principles of continuous flow culture

For simplicity, let us assume that the medium feed rate and the rate of removal of culture (F) is the same, and the culture volume is a constant, V (Fig. 3.1). A peristaltic pump is most suitable for delivery of medium into the culture, for the mechanical parts are not in direct contact with the medium.

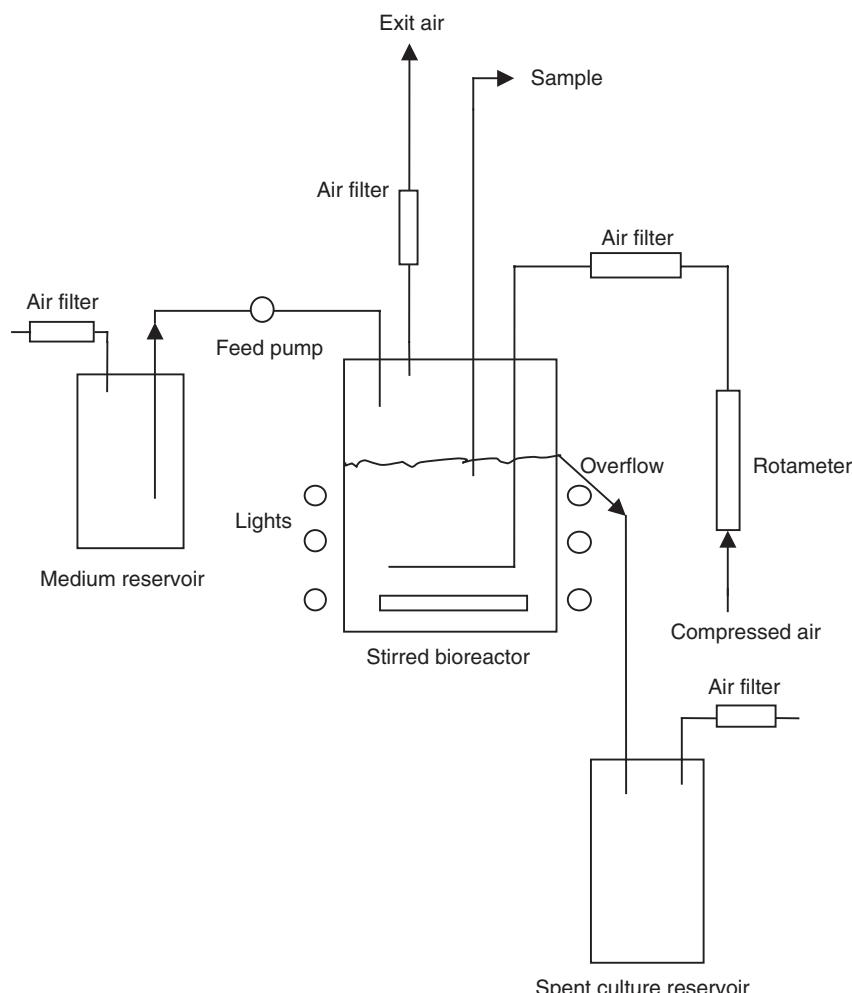


Fig. 3.1. Schematic diagram of a chemostat setup. Reprinted with permission from Kluwer Academic Publishers (*J. Appl. Phycol.*).

The culture could be removed by another peristaltic pump, or through an overflow located at the side of the culture vessel.

The increase in biomass in the culture can be expressed as follows:

$$\text{Net increase in biomass} = \text{Growth} - \text{Biomass removal}$$

For an infinitely small time interval dt , this balance for the culture could be written as,

$$Vdx = V\mu Xdt - FXdt$$

where,

V = Culture volume (l),

dx = Increase in biomass concentration (g l^{-1}),

μ = Specific Growth rate (1 h^{-1}),

X = Biomass concentration (g l^{-1}),

dt = Infinitely small time interval (h),

F = Culture flow rate (litre h^{-1}).

Thus, $dx/dt = (\mu - F/V)X$.

The term F/V represents the rate of dilution of the culture. For example, medium is added into and culture removed from a 5 l algal culture, at a flow rate of 10 l h^{-1} . The rate of dilution of the culture is $10/5 = 2 \text{ h}^{-1}$. That is, the culture is diluted two times every hour. The F/V is termed dilution rate (D) with the unit of 1 h^{-1} . Thus, the above equation could be written as,

$$dx/dt = (\mu - D)X$$

This equation suggests that at *steady state*, the specific growth rate equals the dilution rate ($\mu - D = 0$), $dx/dt = 0$. That is, no net increase in the biomass concentration takes place. This steady state condition is readily demonstrated experimentally.

The steady state is self-regulatory and history independent. That is, irrespective of the initial cell concentration and physiological conditions, the steady state is identical for a given set of conditions. In general, steady state of a chemostat could be reached after four-volume changes of the culture, i.e. for a culture of 1 l volume, steady state could be reached after 4 l of fresh culture medium has been pumped through. Theory indicates that it is possible to fix the specific growth rate of an algal culture at any value from zero to the maximum, by adjusting the dilution rate of the culture.

In a light limited continuous flow culture, where all incident photosynthetically available radiance is absorbed, the energy balance in the culture could be expressed as follows,

$$\begin{aligned} \text{Net increase in energy content} &= \text{Energy absorbed by biomass} \\ &\quad - \text{Energy in outflow biomass} \end{aligned}$$

For an infinitely small time interval, dt ,

$$VdE = IAdt - FX \cdot dt/Y$$

where, dE = Increase in energy content of the culture ($J\text{ l}^{-1}$)

$$dE/dt = IA/V - FX/YV$$

At steady state, $dE/dt = 0$ and $D = F/V$ hence,

$$DX = IA \cdot Y/V$$

This relationship implies that for any incident irradiance, the output rate of a light limited algal culture in steady state ($\mu X = DX$) should be a constant, if growth yield is a constant value. Any deviation from the constant value would suggest a change in the conversion efficiency of light energy to biomass.

3.5.2.2 Chemostat

The special type of continuous culture where the rate of addition of medium and the rate of removal of culture is the same, and culture volume is thus maintained at a constant level, is called chemostat (*constant chemical environment*). Chemostat is widely used in research, for it allows full adjustment of the cells' physiology to the prevailing culture conditions and maintaining the specific growth rates at pre-determined values (Pirt *et al.*, 1980; Iehana, 1983; Lee & Soh, 1991; Molina Grima *et al.*, 1994). Culture parameters such as temperature, pH and substrate concentration could be readily adjusted and studied at fixed specific growth rates. In a simple batch culture, a change in a culture parameter leads inevitably to altered specific growth rate. Such a batch culture could not differentiate between the effects of culture parameters and the specific growth rate.

3.5.2.3 Cyclostat

It has been suggested that algae are exposed to an intermittent supply of light during any 24 h period. To simulate the daily pattern of illumination, a culture may be subjected to alternating light and dark periods, referred to as cyclostat (*constant cyclical illumination*) (Rhee *et al.*, 1981).

3.5.2.4 Turbidostat

A turbidostat is a constant volume continuous culture, where the concentration of the culture is maintained at a pre-set value by adjusting the rate of medium flow through a feedback control loop, hence the term *constant turbidity*. A turbidostat is an elaboration of the chemostat and it is particularly useful for operating under conditions which are unstable in the simple chemostat, such as high irradiance, near maximum growth rate and presence of inhibitory substrates. A turbidostat is also useful for slow growing algae

and those with a complex cell cycle. In the case of *Haematococcus lacustris*, as one example, the maximum specific growth rate is very different at various stages in the cell cycle, resulting in complete washout (total removal of cells from the culture system through excessive dilution) of the culture in a fixed dilution rate chemostat (Lee & Ding, 1994).

The original turbidostat is a chemostat provided with a photoelectric cell for sensing the turbidity of the culture, adding medium when biomass concentration rises above a chosen level. Technically, turbidity measurements are difficult except in short-term cultures, largely because of cell adhesion to the surface of the optical cell. Other growth-linked parameters, such as O₂ output, CO₂ consumption, pH change or substrate concentration (including dissolved O₂ concentration) may be used as alternatives to turbidity measurements.

3.5.2.5 Fed-batch culture

In a fed-batch culture, the medium is added continuously or intermittently whereas the culture is harvested periodically, thus the culture volume may not be constant and the rate of dilution varies with the culture volume. A *quasi steady state* is reached when the biomass concentration and other culture parameters vary in a repeating pattern within a fed-batch cycle.

Fed-batch culture is the most widely used industrial continuous flow culture process, where concentrated culture medium (such as acetate) is fed continuously or intermittently, and the culture is harvested at the end of the cultivation cycle (Lee, 1997).

3.5.2.6 Cell recycled culture

Devices may be incorporated in a culture system to retain or to return cells back into the culture, in order to increase biomass concentration and productivity. This approach is particularly useful for cultures which have a very slow specific growth rate, cells being retained in the culture by a cell filter system (Chen & Johns, 1996). Alternatively, by simple gravity settling of the removed culture volume (de la Noue & Ni Eidhin, 1988), the concentrated cell suspension is returned to the culture.

3.5.3 Immobilized cultures

3.5.3.1 Entrapment

Various species of microalgae have been successfully entrapped in natural polysaccharide gels (calcium or barium alginate, agar, carrageenan) or synthetic polymers (acrylamide, photo-crosslinkable resin, urethane), and showed improved stability and productivity (Robinson *et al.*, 1986). Calcium alginate is widely used for the entrapment of algal cells, for it does not require heat treatment in the entrapment process and it is not toxic. The alginate beads provide a protective and stable microenvironment which allows the cells to grow at a faster maximum specific growth rate, at higher culture

temperature and in high shear conditions (Bailliez *et al.*, 1985; Tamponnet *et al.*, 1985; Ding & Lee, 1994). Moreover, when an entrapped algal culture is contaminated by an undesirable algal species, the contaminant could be removed by washing the beads with sterilized CaCl_2 solution or medium. The method has been successfully used in the cultivation of *Haematococcus* sp. at 32°C in a high shear airlift bioreactor (Ding & Lee, 1994).

An example of a protocol for the entrapment of microalgal cells in Ca-alginate beads is as follows: Sodium alginate (70% guluronic acid) is prepared in 4% (w/v) solution in distilled water, autoclaved prior to use. About 1% (w/v) of algal cells is mixed with sodium alginate solution at room temperature to a final concentration of 2% (v/v) alginate cell mixture. The well-mixed cell suspension is pumped through a peristaltic pump at a rate of 10 ml min⁻¹. The cell suspension is forced through a 0.5 mm diameter orifice placed at about 20 cm above a 0.1 M CaCl_2 solution stirred at 100 rpm. Once droplets of Na-alginate-cells fall into the CaCl_2 solution, Ca-alginate spheres of uniform size (~3 mm diameter) are formed and are allowed to stay in the solution for 30 min. Cells in alginate beads could be recovered by stirring the beads in 0.1 M sodium citrate solution.

A technical difficulty in using the cell entrapment system for long-term mass cultivation of microalgae is the low stability of the alginate beads. The presence of EDTA, phosphate and other cations (e.g. Mg^{+2}) in the culture medium progressively weakens the structural integrity of the alginate gel by removing Ca^{+2} ions through chelation and precipitation, leading to leakage of algal cells. The mechanical strength of the alginate beads needs to be periodically strengthened by washing the beads in 0.1 M CaCl_2 solution.

3.5.3.2 Absorption

Algal cells could be chemically or physically absorbed on solid supports, such as polyurethane foam and coral stone. For immobilization by absorption on polyurethane, the forms are prepared by mixing 1 ml of prepolymer with 1 ml of distilled water. After polymerization, the form is cut into small cubes (5 mm³), boiled in water for 30 min, washed five times with distilled water to remove toxic products from polymerization, and then dried at 80°C for one day. The dry foam pieces (0.5 g) are distributed in 50 ml conical flasks, each containing 19 ml culture medium. After being autoclaved, the flasks are inoculated with 1 ml of alga (5–8 g l⁻¹) and incubated with shaking and illumination for 14 days. Following this period, approximately 70% of algal cells are absorbed on the porous surface of the forms. The cells are recovered by squeezing and washing the forms in water. This method has been used for the immobilization of *Botryococcus braunii* (Largeau *et al.*, 1980) and *Porphyridium cruentum* (Thepenier *et al.*, 1985) on polyurethane forms and packed in column bioreactors.

Finally, it should be stressed that a method involving cell immobilization is not suitable in principle, for production of strictly photoautotrophic species, which cannot be grown at a high density that would bar the needed irradiance into the entire depth profile. Low density cultures exposed to low light intensities, render the entire system cost-ineffective.

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4 Environmental Stress Physiology

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4.1 Introduction

Algal ecology, physiology and biochemistry have been reviewed extensively over the decades. Contributions by Lewin (1962), Carr & Whitton (1973) and Fogg (1975) are just a few examples of textbooks which cover a wide range of topics related to the subject of this work, which is mainly devoted to the physiological response of algae to environmental stress.

Response to stimuli or change in its environment is an inherent characteristic of any living organism. Changes in environmental conditions may thus be defined on the basis of the response that the cell undergoes as a result of the sensed change, either a limiting- or a stress factor. For the sake of simplicity we define a limiting factor as one that determines the rate of growth or biochemical reaction, and that a change in its level will result in a change in the rate without any requirement for an acclimation process. Stress will thus be defined as an environmental condition that results in a metabolic imbalance that requires biochemical and metabolic adjustments before a new steady state of growth can be established.

Schematically the sequence of events associated with the response of living cells to an environmental change may be described as follows:

Environmental change → Sensing mechanism →
Response mechanism adaptation → New steady state

Outdoor algal cultures are exposed to a variety of changes in environmental conditions. These changes take place in two different timescales. One is the circadian cycle, which includes variation in light and temperature in a 24 h cycle. The other is a seasonal cycle that varies according to the climatic and geographical location of the particular habitat in which the algae are growing. In dense algal cultures used in algal biotechnology, a third cycle is imposed by the intensive mixing system, which mainly results in a light–dark cycle which fluctuates in terms of fraction of seconds as compared to the hours or months in the other two cycles.

Microalgae have indeed developed diverse mechanisms for sensing and acclimating to changes in their environment (for reviews see refs). Acclimation responses observed include the alteration of light-harvesting complex

synthesis and degradation in response to changes in light quality and intensity. Such alterations are aimed to help balance efficiently the absorption of excitation energy and the production of reducing power (NADPH) and chemical energy (ATP) with their utilization for growth and cell maintenance. Inability to maintain this balance due to excess excitation of the photosynthetic reaction centers may result in the production of toxic oxygen species that may lead to photo-oxidative death. As implied, many of the stress responses and adaptive process are associated with the photosynthetic apparatus.

4.2 Light and photosynthesis rate

4.2.1 P versus I curve

The light response curve (P/I) of microalgae has been used as a tool in analyzing the response of photosynthetically grown cells to the light environment and at the same time to analyze the response of the photosynthetic apparatus to environmental stress. The P/I curve can be divided into three distinct regions, i.e. a light-limited region, in which photosynthesis increases with increasing irradiance, a light-saturated region in which photosynthesis is independent of irradiance and a photoinhibited region in which photosynthesis decreases with further increase in irradiance. In addition, an intermediate region where there is either a gradual or abrupt transition from light-limited to light-saturated photosynthesis has been identified (Prioul & Chartier, 1977; Leverenz, 1987). A typical response of photosynthesis (CO_2 assimilation or O_2 evolution) to increasing irradiance is shown in Fig. 4.1. At low

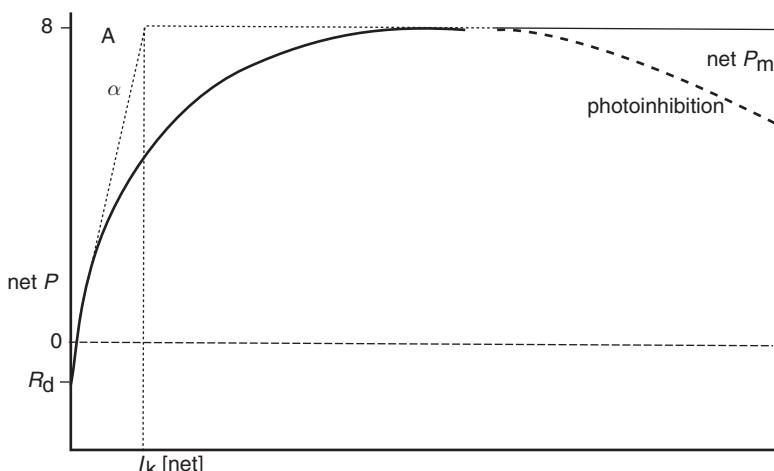


Fig. 4.1. A schematic diagram of photosynthesis (P) versus irradiance curve, showing the typical photosynthetic parameters. The light-saturated rate is denoted P_{\max} . At low irradiance, photosynthesis rate is approximately a linear function of irradiance, and the ratio between photosynthesis and irradiance is often denoted by the symbol α . The saturation irradiance I_k , is given as intercept between α and P_{\max} . At irradiance above the optimum, photosynthesis rates usually shows a decline from the light-saturate value. Dark respiration is denoted R_d .

irradiance, photosynthesis rates are linearly proportional to irradiance. In this region of the *P/I* curve, the rate of photon absorption determines the rate of steady-state electron transport from water to CO₂, and thus it is called the light-limited region. The initial slope of the *P/I* curve is usually denoted by the symbol α (Jassby & Platt, 1976). The slope can be normalized to chlorophyll biomass α^B and the units are (O₂ evolved or CO₂ fixed per unit chlorophyll)/(quanta per unit area). When the initial slope is measured on a leaf or in a dense microalgal culture in which all the photosynthetically active radiation is absorbed, the slope can be taken as a direct measure of the maximum quantum yield of photosynthesis. However, in natural phytoplankton communities or optically thin cultures of microalgae, light absorption is a small fraction of the incident light and the initial slope may not be used as a direct measure of the quantum yield. Yet, the initial slope is proportional to the quantum yield and can be used to compare cultures in which the light absorption has not changed. One means of deriving the maximum quantum yield from α^B is to measure the spectral irradiance and the spectral averaged optical absorption cross-section normalized to chlorophyll *a*, α^* (with units m² mg⁻¹ Chl *a*). This normalization is convenient as it allows the calculation of the absorbed light from measurements of chlorophyll *a* and incident spectral irradiance. From knowledge of α^* and α^B , the maximum quantum yield can thus be calculated:

$$\Phi_m = \alpha^B / \alpha^* \quad (4.1)$$

For exhaustive information on the definition and calculation of the optical cross-section in microalgae see Dubinsky (1992), Kromkamp & Limbeek (1993), Falkowski & Raven (1997).

At higher irradiances the relationship between absorbed light and rate of photosynthesis is not linear any more, consequently the quantum efficiency decreases. Eventually, photosynthesis becomes light saturated and the photosynthesis rate reaches the maximum (P_{max}) and remains constant with increasing irradiance. By definition, the rate of photon absorption at light saturation exceeds the rate of steady-state electron transport from water to CO₂. In addition to these fundamental parameters, some other derived parameters are widely used. The compensation irradiance for photosynthesis, $I_c = R_d/\alpha$, i.e. the light irradiance where no net oxygen evolution is observed. The parameter I_k represents the point on the *P/I* curve indicating the irradiance at which control of photosynthesis passes from light absorption and photochemical energy conversion to reductant utilization; it is a convenient indicator of photoacclimational status. I_k can be defined as $I_k = P_{max}/\alpha$. At irradiance less than I_k , electron transport capacity exceeds the rate at which photons are absorbed and delivered to PS II, and at irradiance values greater than I_k , the converse is true. Also, the predominant fluorescence quenching mechanism (see Chapter 3) at PFDs $< I_k$ is photochemical, i.e. photosynthetic, whereas above I_k it is non-photochemical, involving thermal dissipation. I_k has the advantage of being independent of the units used for expressing photosynthesis (O₂ or CO₂, weight, area, cell, carbon or chl *a* basis). As a result, it is tempting to use I_k as a parameter

for comparison of photoacclimation status both within and between species and to assume that its inverse relation to α implies a similar relation to maximum photosynthesis efficiency, that is ignoring the dependence of I_k on P_{\max} . As pointed out by Henley (1993) I_k is often a better indicator of P_{\max} than of α . In other words, a lower value of I_k does not imply necessarily a higher value of α (or quantum yield) but it could be simply due to a lower value of P_{\max} . For example, low temperature typically affects P_{\max} more than α , consequently, a low value of I_k , indicates an inefficient use of high light intensities rather than an efficient use of low ones (Fig. 4.2).

The quantitative description of the light-dependence of photosynthesis dates from Blackman's studies (1905) of limiting factors in plant productivity. Blackman considered the rate of plant production to be linearly dependent on the availability of a single limiting factor at low resource supply and independent of the availability of this factor above some threshold value. It was soon recognized that the transition between limiting and saturating resource availability may not be as abrupt as postulated by Blackman kinetics and a number of formulations of the P/I curve were proposed by plant physiologists, oceanographers and limnologists (Jassby & Platt, 1976). It has been frequently observed that some P/I data exhibit variability in the bending part of the curve (convexity), i.e. the sharpness of the transition from PFD limitation to saturation. Leverenz *et al.* (1990) demonstrated in *Clamydomonas reinhardtii* that the shape of the P/I curve depended on the degree of photoinhibition; the transition from light-limitation to light-saturation became less abrupt with increased photoinhibition. The convexity index θ (Leverenz *et al.*, 1990), originally termed M by Prioul & Chartier (1977) ranges from 1.0, when the curve is of the Blackman type, to 0.0 when the response is a rectangular hyperbola (Fig. 4.3). The latter situation may occur with stressed microalgae (Leverenz *et al.*, 1990).

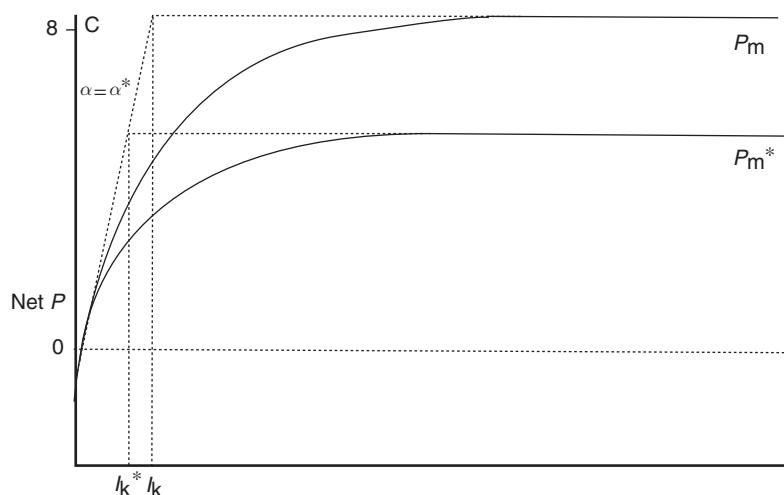


Fig. 4.2. Light response curves having same α but different P_{\max} and the resultant differences in I_k . This pattern may result from temperature changes.

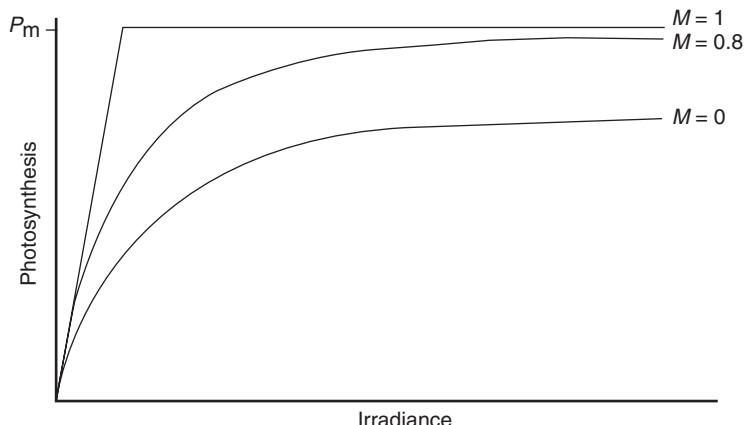


Fig. 4.3. Graphical display of P/I curves with different rates of convexity (M). Blackman curve ($M = 1$); non-rectangular hyperbola ($M = 0.8$), rectangular hyperbola ($M = 0.0$). P denotes the photosynthesis rate, I incident irradiance.

4.2.2 Photoacclimation

As mentioned previously, the rapid changes in ambient light quantity and quality to which microalgae are exposed resulted in the development of a remarkable capacity for photoacclimation. The timescale according to which the cells have to respond to those changes varies from seconds to days. As a result, algal cells have developed a few response mechanisms enabling them to deal with excess of energy. The state transition, and non-photochemical mechanisms (Chapter 3) operate to adjust the amount of light energy delivered to PS II on a timescale of seconds to minutes. Other processes reflecting changes in light quality or intensity may take longer and require structural and biochemical modifications. The long-term acclimation to irradiance is referred to as *photoacclimation*. This process involves changes at optical, biophysical, biochemical, ultrastructural, physiological and molecular levels (Falkowski, 1980; Richardson *et al.*, 1983; Escoubas *et al.*, 1995; Fisher *et al.*, 1996, 1998). A common trend characterizes the mechanism of photoacclimation, that is, an increase in chlorophyll *a*, and in other light-harvesting pigments, as growth irradiance decreases. The increase in pigment content during acclimation to low light, results in a decrease in the optical cross-section a^* ($\text{m}^2 \text{ mg}^{-1} \text{ Chl } a$), thus reducing the gain in light harvesting. Indeed, in a microalgal culture, a doubling of cellular chlorophyll does not bring about a doubling in the rate of light absorption (Dubinsky *et al.*, 1995). Conversely, cells acclimated to high irradiance generally show relatively high carotenoid content relative to chlorophyll *a*. Under stress conditions, some carotenoids such as β -carotene in *Dunaliella salina* and astaxanthin in *Hematococcus pluvialis* accumulate in globules outside of chloroplast, do not transfer excitation energy to reaction center and act as a screen to protect reaction center from excessive excitation, while xanthophyll cycle pigments in particular zeaxanthin, under high light can reduce excitation of PS II reaction center by dissipating excess of excitation energy via

non-photochemical quenching (Chapter 3). Because these carotenoids absorb light without a concomitant increase in the energy transfer to reaction center, organisms acclimated to high light often show lower maximum quantum yields.

The length of time required to accomplish the changes in pigmentation varies from species to species, from hours to several days. The rapid decrease in cellular chlorophyll after transition from low light (LL) to high light (HL) is assisted by the dilution of the pigment in the course of cell division, and does not result from active pigment destruction (Berner *et al.*, 1989). In contrast, the slower photoacclimation process during the transition from HL to LL is due to the fact that dilution of pigments as a result of cell division acts in the opposite sense to the direction of photoacclimation, which is directed to increase cellular pigmentation (Fisher *et al.*, 1996). Therefore, cells which are transferred from HL to LL reach the steady-state in cellular chlorophyll considerably later than those photoacclimating in the opposite sense.

Prezelin & Sweeney (1979); Falkowski & Owens (1980); Perry *et al.* (1981) and Ley & Mauzerall (1982) have suggested that phytoplankton responds to decreased light intensities by increasing either the size or the number of photosynthetic units (PSU)¹ within a cell. Prezelin & Sweeney (1979) have suggested that the two strategies of light-shade adaptation could be distinguished on the basis of the characteristics of photosynthesis-irradiance curves. A similar proposal has been also made for macroalgae (Ramus, 1981). The rationale for this proposal is that an increase in the size of photosynthetic units should result in compensation for low light by providing a constant amount of photons to reaction centers. In such a case, the maximum rate of cellular photosynthesis would remain constant under decreased light (Fig. 4.4A), less light will be required to saturate photosynthesis and PSUs will become more efficient. While an increase in the numbers of PSUs per cell, will result in an increased maximum photosynthesis rate, and more light will be required to saturate photosynthesis and hence a higher value of I_k (Fig. 4.4B). Both photoadaptation strategies have been observed in all algal classes studied so far. For example, it was found that *Dunaliella tertiolecta* (Chlorophyte) and *Nannochloropsis* (Eustigmatophyte) adapt to low light by increasing the number of photosynthetic units (Falkowski & Owens, 1980; Fisher *et al.*, 1996, 1998), while *Chlorella pyrenoidosa*, *Chlamydomonas reinhardtii* (Chlorophyte), and *Glenodinium* (Dinoflagellate) adapt to low light by increasing the PSU size (Meyers & Graham, 1971; Prezelin, 1976; Neale & Melis, 1986).

¹ The concept of photosynthetic units, originally proposed by Emerson and Arnold in the 1930s, was based on kinetic measurements of oxygen production. A photosynthetic unit has been operationally defined as the number of pigment molecules involved in the evolution of one molecule of O_2 , or the reduction of one molecule of CO_2 , when the chloroplast pigments are excited by one flash of light so short that the components involved in the process will not function twice during their lifetime and so strong that a further increase in flash intensity does not lead to an increase in the measured value (Falkowski, 1980). Schmid & Gaffron (1968) estimated from oxygen flash yield studies that the number of chlorophyll molecules per PSU ranges between 1800 and 2500.

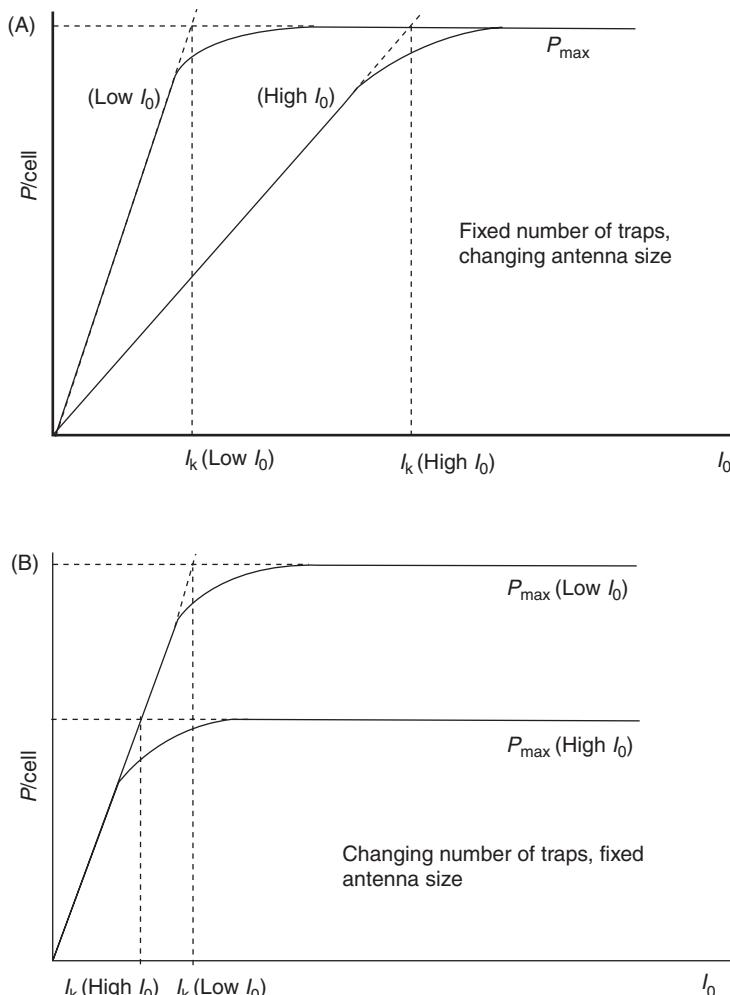


Fig. 4.4. Model of saturation curves. (A) Model of adjustment to low and high light irradiances by changing the size of a fixed number of PSUs. (B) Model of adjustment of the photosynthesis unit to low and high light by changing the number of PSUs and not their size. (From Ramus, 1981, with permission of Blackwell Scientific Publications.)

In cyanobacteria, the antenna system for PS I is totally different from that for PS II (Chapter 3). The light-harvesting antenna serving PS I is exclusively constituted of chlorophyll *a* while the one serving PS II consists mainly of phycobilisome. PS II, which is excited by phycobilisome has a relatively larger optical absorption cross-section compared to PS I which is excited by chlorophyll *a*. To balance the electron flow between PS II and PS I, cyanobacteria generally have more PS I reaction centers relative to PS II. This ratio can be altered both by light intensity and spectral distribution of irradiance. Cyanobacterial cells grown under high irradiance have a lower PS I/PS II ratio and lower phycobilisome content compared to cells grown under low light (Kawamura *et al.*, 1979; Murakami & Fujita, 1991; Hihara *et al.*, 1998;

Sonoike *et al.*, 2001). This change is considered as a compensation for PS II antenna size, since the size of the phycobilisome is preferentially reduced under highlight conditions. Campbell & Öquist (1996) using chlorophyll fluorescence, found that the light intensity to which the cells are acclimated could be predicted by the light response curve of the non-photochemical quenching parameter. In a wide range of cyanobacteria with different pigment contents, morphologies and light histories, non-photochemical quenching resulted minimum near the photon flux density in which the cells were grown. In cyanobacteria like in green plants, the intersystem redox state appears to direct the transcription of PS II and PS I reaction centers (Fujita *et al.*, 1988). The state transitions can thus be viewed as a short-term response to redox state of the plastoquinol pool, and photoacclimation can be viewed as a long-term response to the same control mechanism. The redox state of plastoquinone pool acts as a biological light meter that can signal the status of intersystem electron traffic and affect feedback responses in the light-harvesting systems (Escoubas *et al.*, 1995; Huner *et al.*, 1998).

4.2.3 Photoinhibition

4.2.3.1 Basic principles

Photoinhibition is defined as a light-induced depression of photosynthesis that is manifested as a decrease in the maximum quantum yield of photosynthesis, a decrease in the convexity of the photosynthetic light response curve and, in the case of prolonged exposure to excessive light, a decrease in the rate of light-saturated photosynthesis (Leverenz *et al.*, 1990; Long *et al.*, 1994). The term photoinhibition has also been used to mean damage to PS II and the term photoprotection to mean changes assumed to protect PS II against damage (Demmig-Adams & Adams, 1992). Photoprotection probably reflects a way of adjusting the rate of dissipation of absorbed radiation energy so that the excitation energy density in the PS II antenna is sufficient to drive photosynthesis at a rate that meets the demands of assimilatory reactions. This would reduce the possibility of overexcitation of the PS II reaction center. However, a clear distinction between damage and photoprotection is not always possible (Demmig-Adams & Adams, 1992; Ögren & Evans, 1992). The possibility of overexcitation of PS II increases when photosynthetic organisms are grown under suboptimal conditions; this would reduce the photosynthetic rate and lead to an increase in the dissipation of absorbed energy through nonradiative processes. Since the capacity for photoprotection is limited, certain conditions can lead to damage and loss of active PS II reaction centers. Environmental conditions that reduce carbon metabolism, such as chilling and freezing temperatures (Long *et al.*, 1983; Torzillo *et al.*, 1996; Vonshak *et al.*, 2001), high temperature (Bongi & Long, 1987), and nitrogen deficiency (Herzig & Falkowski, 1989), strongly increase the possibility of overexcitation of the PS II. Studies of photoinhibition on aquatic plants have been reviewed by Neale (1987) and by Long *et al.* (1994). In this section we will analyze how and to what extent the P/I curve can be modified by photoinhibition and the consequences of photoinhibition

to microalgal productivity outdoors. Initially, photosynthesis increases linearly with irradiance and the maximum quantum yield is determined from the initial slope of the curve. The shaded area in Fig. 4.5A above the light response curve indicates the amount of absorbed light that is not used for photosynthesis; while the dashed line in Fig. 4.5B represents a theoretical Blackman response curve (see previous section) where photosynthesis operates at the maximum quantum yield efficiency until light saturation occurs and photosynthesis is limited by the dark reactions. The shaded area enclosed by the Blackman curve and the effective light response curve of photosynthesis (solid line) indicates the absorbed light energy that could potentially be used for photosynthesis, but is lost due to intrinsic characteristics and regulatory processes of the photosynthetic apparatus (Baker, 1996). In this region of the P/I curve, changes occur within the thylakoids that result in a smaller proportion of absorbed light being used for photochemistry due to an increase in the rate at which energy absorbed by the antennae of PS II is dissipated as heat. This light-induced quenching of excitation energy is thought to occur in the light-harvesting antennae of PS II and is associated with the decrease in the pH of thylakoids lumen during the formation of the thylakoid ΔpH and the consequent conversion of the carotenoid violaxanthin to zeaxanthin via the xanthophyll cycle (Demmig-Adams & Adams, 1992). In Fig. 4.5C the effect of severe photoinhibition on the light response curve (lower solid line) is shown. The shaded area between the normal and photoinhibited curves represents the absorbed light energy lost to photosynthesis due to photoinhibition of the culture. In this case, decrease in the maximum quantum yield, in the convexity, and in the maximum photosynthesis rate (P_{\max}) are accentuated. This situation can occur when microalgal cultures are exposed to strong light (some ten times more than the growth irradiance) or to a combination of high light and other stress (e.g. low temperature). This behavior has been found, for example, in *Spirulina platensis* cultures following exposure to high irradiance (Torzillo &

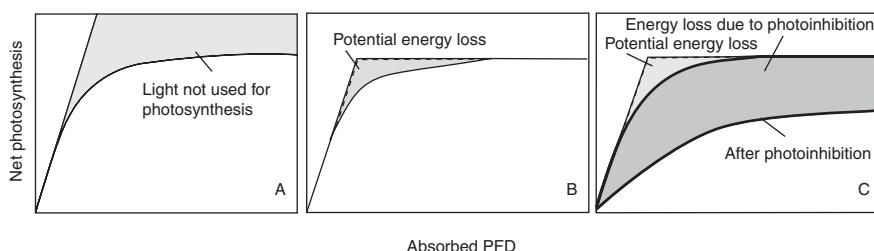


Fig. 4.5. (A) Typical photosynthesis response curve to increasing irradiance. The shaded area above the curve and the extrapolated initial slope, shows the amount of absorbed light that is not used for photosynthesis; (B) The shaded area enclosed between the Blackman curve and the effective light response curve, indicates the absorbed light energy that could potentially be used for photosynthesis, but is dissipated through non-photochemical quenching (heat) as a result of regulatory processes which are intrinsic of the photosynthetic apparatus; (C) The effect on P/I curve of severe photoinhibition which results in a decrease in both the quantum yield and P_{\max} . The shaded area under the normal P/I and the photoinhibited curves indicates the absorbed light energy lost to photosynthesis due to photoinhibition (modified from Baker, 1996). Reprinted with permission from Blackwell Science Publications.

Vonshak, 1994). However, in some cases the situation is not so clear and the term photoinhibition has been frequently interpreted in different ways. Confusion has been generated by the means used to estimate photoinhibition. Decrease in the maximum quantum yield and in the convexity of the P/I curve has been observed to precede decrease in P_{\max} , and a significant decrease in quantum yield may occur without any decrease in P_{\max} . Probably the original definition of photoinhibition given by Kok in 1956, that is, a light dependent reduction in photosynthetic efficiency, may still provide the most useful working definition of photoinhibition of photosynthesis for leaves and whole organisms.

For both microalgae and terrestrial plants, photodamage to PS II reaction centers can be detected with high sensitivity from changes in variable chlorophyll fluorescence (Baker & Horton, 1987; Björkman, 1987; Neale, 1987; Long *et al.*, 1994; Vonshak *et al.*, 1994; Torzillo *et al.*, 1996). The F_v/F_m ratio (variable to maximum fluorescence) is a convenient measure of the potential maximal quantum yield of PS II, and it has been assumed as an index of photoinhibition (Björkman & Demmig, 1987). Decrease in the F_v/F_m ratio has been found highly correlated to reduction in the quantum yield of oxygen evolution or CO_2 uptake (Genty *et al.*, 1989). In the field, F_v/F_m frequently exhibits diurnal depression that are roughly symmetric to light intensity and are mirrored by corresponding changes in the quantum yield of photosynthesis (Neale, 1987; Long *et al.*, 1994; Torzillo *et al.*, 1996). However, in aquatic systems as well as in some microalgal cultures, short-term photosynthesis measurements may often indicate maximum light-saturated photosynthesis rates (P_{\max}) at noon time, that is, in correspondence to the lowest value of F_v/F_m and quantum yield. This paradoxical co-occurrence of midday maxima in both photoinhibition and photosynthesis appears inconsistent with the debilitating effect of PS II damage as evidenced by the decrease in F_v/F_m and quantum yield of photosynthesis. An explanation to this paradoxical situation has been furnished in an elegant recent paper (Behrenfeld *et al.*, 1998). The effect of photoinhibition depends upon which step in the photosynthetic electron transport chain is rate-limiting at a given incident irradiance. Photosynthetic activity at sub-saturating light irradiance is rate-limited by light absorption and excitation energy transfer to PS II reaction centers is a near-linear function of irradiance. Conversely, at saturating light intensities, photosynthesis is limited on the acceptor side of PS II, generally by the capacity of enzymatic processes in the Calvin cycle (Sukenik *et al.*, 1987) which in effect restricts electron turnover through PS II. Behrenfeld *et al.* (1998) have demonstrated in *Thalassiosira weissflogii*, adapted to low light intensity, that changes in carbon fixation are not observed until rate limitation is shifted from the Calvin cycle reactions to electron transport through PS II. In *T. weissflogii* changes in P_{\max} were not observed until the reduction of active reaction centers had reached 50% of initial. These findings demonstrate that photoinhibition leads indeed, first of all, to a reduction in the quantum yield of photosynthesis which is mirrored in a decline of the F_v/F_m ratio, nevertheless, the overall electron transport (P_{\max}) can remain virtually unaltered despite substantial PS II photodamage (Kok, 1956;

Leverenz *et al.*, 1990; Behrenfeld *et al.*, 1998; Vonshak *et al.*, 2001). What then could be the expected consequences of these findings to microalgal biomass yield?

It must be pointed out that algal productivity depends primarily on light energy conversion efficiency, i.e. the absorption and utilization of light by the photosynthetic apparatus to assimilate CO₂ into dry matter. Dense algal cultures are therefore predominantly grown at light limitation and, consequently, their photosynthetic performance would be more dependent on the initial slope rather than on the light-saturated portion of the P/I curve. Hence, a reduction in the F_v/F_m ratio due to excessive light absorption in the top layers of an outdoor algal culture will result in a decrease in the biomass yield.

4.2.4 Photoinhibition in the field

Traditionally light has been referred to as the main limiting factor in mass culturing of microalgae (Burlew, 1953; Richmond & Vonshak, 1978). This assumption was based on the observation that outdoor algal cultures are kept in a dense suspension where light penetrates only through a small fraction of the culture. Also, for practical reasons, it was obvious that none of the mixing devices used in such systems could be able to induce a light-dark cycle to overlap with the timescale of the flashing light effect demonstrated in the lab. The first to demonstrate that dense *Spirulina platensis* cultures grown outdoors are undergoing a photoinhibitory process were Vonshak & Guy (1992). They clearly demonstrated that by shading the cultures and preventing them from full exposure to solar radiation a higher productivity could be maintained.

Later on by the use of in situ chlorophyll fluorescence technique it was demonstrated that in dense *Spirulina* cultures grown under optimal conditions, exposure to full sunlight results in a midday decrease in F_v/F_m (i.e. the maximum quantum yield achievable) of approximately 10% of the early morning value; the depressing effect of high light increase to 20% when the ΔF/F' _m (i.e. the effective quantum yield of PS II) is measured (Torzillo *et al.*, 1996). These and other results indicate that a reduction in the efficiency of photosynthesis in the middle of the day cannot be avoided even with ultra dense cultures grown at the optimum biomass concentration (Hu *et al.*, 1996). This kind of photoinhibition has been called *diurnal photoinhibition* (Ögren & Evans, 1992) in which recovery of F_v/F_m is usually completed by dusk. In a later study, Lu & Vonshak (1999) studied the response of outdoor cultures of *S. platensis* using the polyphasic rise of chlorophyll fluorescence transients, which provide information on the primary photochemistry of PS II. The maximum efficiency of PS II photochemistry (F_v/F_m) declined in response to daily increasing irradiance and recovered as daily irradiance decreased. The greatest inhibition (15%) in F_v/F_m was observed at 12:00 which responded to the highest irradiance. The daily change in the concentration of PS II reaction centers followed the same pattern as F_v/F_m. However, no significant changes in the probability of electron transport beyond Q_A (Ψ_o) were observed during the day. The results suggest that the decrease in F_v/F_m

induced by photoinhibition in outdoor *Spirulina* cultures was a result of an inactivation of PS II reaction centers.

In higher plants and green algae diurnal photoinhibition has been frequently correlated with increased zeaxanthin formation and increased thermal energy dissipation from PS II, as the assumed cause of depression of F_v/F_m and quantum yield of photosynthesis (Demmig-Adams & Adams, 1992). However, the correlation is not perfect and in some species recovery of F_v/F_m has required more time than that required to convert zeaxanthin back to violaxanthin indicating that the xanthophyll cycle is only partly responsible of the F_v/F_m decline (Masojidek *et al.*, 1999). In both laboratory and outdoor cultures of microalgae, it was found that the activation of the maximum non-photochemical quenching takes about 30–60 min of high irradiance. This may represent a problem for microalgae, even in response to diurnal changes of irradiance (in the morning), not to mention the fluctuating irradiance on cloudy days. Thus responses to such conditions can result in a significant loss of photosynthetic performance as a consequence of slow regulation. The opposite effect may occur in the afternoon, i.e. the rate of irradiance decrease is faster than that of the non-photochemical quenching relaxation.

4.2.4.1 Effect of sub-optimal temperature on photoinhibition and culture productivity

Even photon fluxes equivalent to about half of full sunlight frequently produce a persistent photoinhibition when applied in controlled environments in combination with other environmental stress limiting the C-metabolism, i.e. low temperature and/or high oxygen concentration in the cultures.

Algal cultures, grown outdoors, are usually exposed to a combination of environmental stress. The most common combination is high light and low temperature. It happens that while fluctuations in light intensity occur in a range of 1–2 h, the increase of temperature is a much slower process and takes about 4–5 h. This kind of de-synchronization between the two most important environmental factors, which affect photosynthesis and growth of outdoor algal cultures, results in a unique stress condition under which photoinhibition may indeed be induced at relatively low light intensity due to the sub-optimal temperature conditions (Vonshak *et al.*, 2001).

The effect of low temperature on photosynthesis and growth of outdoor cultures of *Spirulina* in tubular reactors has been investigated using saturating pulse fluorescence (Torzillo *et al.*, 1996). Diurnal changes in maximum photochemical quantum yield of PS II of dark adapted cultures, F_v/F_m ratio, or in the effective photochemical quantum yield of PS II, $\Delta F/F'_m$ were measured under steady-state photosynthesis in cultures grown at 25°C (that is 10°C below the optimum). A reduction of 30% in the F_v/F_m ratio was found in the middle of the day in the culture grown at 25°C (Fig. 4.6); at the same time of the day $\Delta F/F'_m$ decreased up to 52% in the culture grown at 25°C. In the evening, recovery of the morning values of F_v/F_m and $\Delta F/F'_m$ ratios was incomplete. Photoinhibition reduced the daily productivity of the

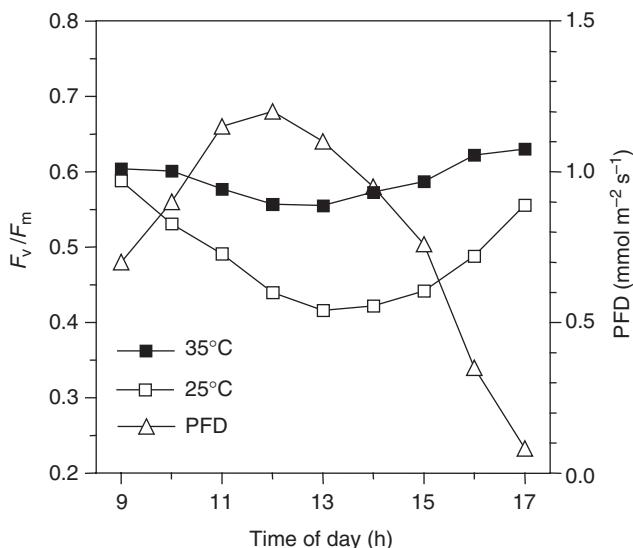


Fig. 4.6. Diurnal changes in the F_v/F_m ratio of *Spirulina platensis* cultures grown outdoors in tubular photobioreactors at two temperatures.

culture by 33% with respect to another culture grown at 35°C . These results strongly support the hypothesis that photoinhibition can take place even in relatively dense outdoor cultures of *Spirulina* when they are exposed to the combination of high light and sub-optimal temperature (Torzillo *et al.*, 1996).

The effect of low temperature on photoinhibition was also investigated in outdoor cultures of *Monodus subterraneus* (Eustigmatophyta) by Vonshak *et al.* (2001) by measuring the diel changes in photosynthetic oxygen evolution and several photochemical parameters. Cultures were maintained at two temperature regimes. In one, the rise in temperature was initiated in the morning as a result of the increase in solar radiation up to the optimal temperature of 28°C , while in the other culture a heating device was used to increase the rate of warming up in the early morning. It was found that, although the two cultures were maintained most of the day at the same temperature and light intensity, cultures exposed to sub-optimal morning temperature, for only a short time, showed a larger decrease in almost all the photosynthetic parameters. By comparing the diel changes in maximal photochemistry efficiency of PS II, the relative electron transport rate, photochemical and non-photochemical chlorophyll fluorescence quenching of the cultures, they concluded that even a relatively short exposure to sub-optimal morning temperatures induced a photoinhibitory damage. The higher photochemical activity of the heated culture was also reflected in a 60% increase in productivity compared to the nonheated one.

Increased susceptibility to photoinhibition can be caused by several mechanisms: (i) low temperature slows the rate of CO_2 fixation thus causing the overreduction of electron transport compounds at a given photon fluence rate; (ii) low temperature inhibits the scavenging of active oxygen species, known to protect PS II against photoinhibition; (iii) low temperature inhibits

the PS II repair cycle, i.e. the interplay between degradation and *de novo* synthesis of the D1 protein degraded during photoinhibition.

4.2.4.2 Effect of high oxygen concentration and low temperature on photoinhibition and culture productivity

A number of studies have suggested that reaction of dioxygen (O_2) resulting in active oxygen species initiate early destructive processes of photoinhibition (see Krause, 1994 for review). The photosynthetic electron transport system represents the major source of active oxygen species having the potential to generate singlet oxygen, hydrogen peroxide and the superoxide radical (Asada, 1994). However, the role of O_2 during high light stress is dualistic. Oxygen can protect the photosynthetic apparatus from photoinhibition by dissipation of excessive energy through photorespiration or via the Mehler reaction (Krause & Cornic, 1987; Wu *et al.*, 1991), but harmful oxygen radicals generated in the latter reaction can have a potentially deleterious effect on photosynthetic structures (Asada & Takahashi, 1987). When scavenging of potentially damaging oxygen species is insufficient, photoinhibition can occur.

Conditions of high O_2 and low CO_2 concentrations are known to stimulate photorespiration in terrestrial plants, but there is no evidence that this process takes place in cyanobacteria (Colman, 1989). The active intracellular accumulation of inorganic carbon developed by cyanobacteria may be sufficient to inhibit ribulose-1,5-bisphosphate oxygenase activity and therefore to suppress photorespiration.

The first evidence of oxygen inhibition on *Spirulina maxima* growth was reported by Torzillo *et al.* (1984). They showed that laboratory cultures grown under an oxygen partial pressure of 0.7 atm caused a growth decrease of about 40% compared to a culture grown at 0.2 atm of O_2 . Similar results have been also reported by Marquez *et al.* (1995). They clearly showed that the inhibition caused by O_2 on the growth was dependent on the oxygen concentration tested. Since both photosynthesis and growth of *Spirulina* cultures were inhibited to a comparable extent the authors suggested that inhibition of oxygen may cause some damage to the photosynthetic apparatus. The effect of high oxygen concentration on the photosynthesis and growth of *Spirulina* was further studied using chlorophyll fluorescence on laboratory cultures bubbled with pure oxygen so that the O_2 concentration reached 36 mg l^{-1} (Vonshak *et al.*, 1996). It was found that the effective photochemical efficiency of PS II ($\Delta F/F'_m$) was much more depressed than maximum photochemical quantum yield of PS II, F_v/F_m , and that the decline of these two parameters occurred according to two very different kinetics. These facts might indicate that photoinhibition and photooxidation in *Spirulina* could occur as parallel processes and not be necessarily sequential.

Attempts to investigate the mode and type of oxygen species involved in the oxidative damage of *Spirulina* cells have also been performed (Singh *et al.*, 1995). The results showed that the singlet oxygen was the most predominant

oxygen species generated during high light stress, while superoxide and hydroxyl radicals played a minor role in the photodynamic damage of *Spirulina* cells.

The combination of high oxygen concentration and high light intensity is very frequent in outdoor cultures of *Spirulina*, particularly when grown in closed systems. For example, in photobioreactors made with tubes of about 5 cm internal diameter, in well-growing cultures, the oxygen concentration can increase at a rate of $2\text{--}3 \text{ mg l}^{-1} \text{ min}^{-1}$. This results in an oxygen concentration of up to $70\text{--}80 \text{ mg l}^{-1}$ even with a gas exchange with air every 50 seconds and with a relatively high turbulence rate (Vonshak *et al.*, 1996; Torzillo *et al.*, 1998).

The combination of high oxygen concentration and low temperature in outdoor cultures can occur at the beginning of the cooler season, when the culture temperature drops much below the optimum, but irradiance is still enough to drive photosynthesis at an appreciable rate. Such conditions can be very common in desert areas where the morning temperature of the culture is far below the optimum while light intensity is high enough to induce photo-inhibition. The synergistic effect of high oxygen concentration and low temperature was studied in outdoor cultures of *Spirulina* grown in tubular photobioreactors by using an online chlorophyll fluorescence technique (Torzillo *et al.*, 1998). The results have shown that the combination of low temperature and high oxygen concentration had a considerable impact on PS II photoinhibition measured as changes in the F_v/F_m ratio, resulting in a strong reduction of the growth of the culture where a mere reduction of 10°C below the optimum was imposed and the oxygen concentration was allowed to rise to $70\text{--}80 \text{ mg l}^{-1}$ (Fig. 4.7). A great part of the radiation absorbed by the photosynthetic apparatus was dissipated since a significant reduction in the effective photochemical quantum yield of PS II ($\Delta F/F'_m$) was observed during the day. However, while in the low oxygen culture, recovery

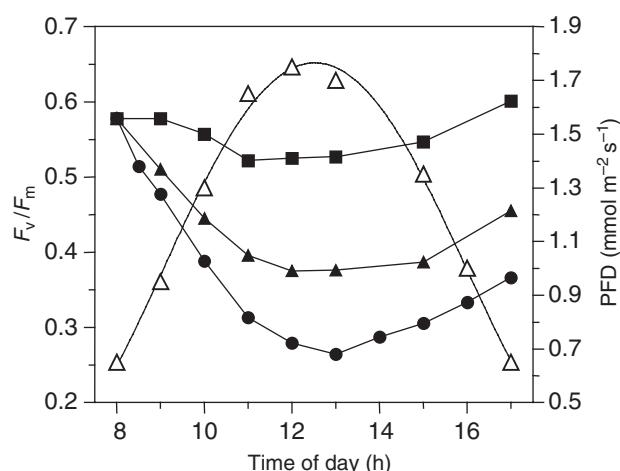


Fig. 4.7. Effect of oxygen concentration and temperature on the F_v/F_m ratio of *Spirulina platensis* cultures grown outdoors in photobioreactors. (■) Low oxygen-Optimal temperature; (▲) High oxygen-Optimal temperature; (●) High oxygen-Low temperature; (Δ) PFD.

Table 4.1. Effect of oxygen concentration and temperature on biomass productivity and chlorophyll synthesis in *Spirulina platensis* cultures grown outdoors in photobioreactors.

Culture conditions	Oxygen concentration (mg l^{-1})	Temperature ($^{\circ}\text{C}$)	Chlorophyll synthesis ($\text{mg l}^{-1} \text{d}^{-1}$)	Biomass synthesis ($\text{mg l}^{-1} \text{d}^{-1}$)
LO-OT	22 ± 2	35	6.02 ± 0.18	570 ± 28
HO-OT	60 ± 19	35	2.57 ± 0.05	380 ± 18
HO-LT	58 ± 16	25	0.22 ± 0.01	230 ± 10

SD, standard deviation of triplicate experiments; LO-OT, low oxygen-optimal temperature; HO-OT, high oxygen-optimal temperature; HO-LT, high oxygen-low temperature.

was complete by dusk, in the high oxygen cultures persistent photoinhibition took place since recovery of both F_v/F_m and $\Delta F/F'_m$ was incomplete at the end of the day. Photoinhibition reduced the daily productivity of the culture grown under high oxygen stress by about 33%, and that of the culture grown under high oxygen-low temperature stress by 60% (Table 4.1).

There is little doubt that oxygen accumulation in the culture represents the main obstacle to the development of closed photobioreactors in industrial scale unit. Indeed, in this system the advantage to grow algal cultures at a very high biomass concentration is greatly reduced by oxygen accumulation. On the other hand, the fragility of the cells usually dictates strict limitation of the culture circulation speed, which aims at increasing gas exchange with air and increasing the turbulence of the culture. Therefore, the application of an efficient degassing system to prevent high oxygen accumulation is a prerequisite for successful design of industrial scale photobioreactors.

4.2.5 Some practical considerations

Maximum quantum yield of PS II (F_v/F_m) in *healthy* microalgal cultures ranges from 0.7 to 0.8. Once exposed to full solar radiation at midday a decline of up to 90% of the initial value may be observed. This means that photon use efficiency drops to about 10% of that observed in the early morning. The reason for this decline in efficiency is explained in that even under intensive mixing the first layers of cells absorb light in excess to what they can use in the photosynthetic process, resulting in a need for dissipation of energy through non-photochemical quenching. Moreover, if the energy dissipation mechanism through non-photochemical quenching gets saturated, photoinhibition will take place. The problem has been figured out already, 50 years ago, and basically three types of approaches have been proposed:

1. Increase of cell density and the mixing rate of the cultures in order to prevent the saturation effect;
2. Use of special designs of photobioreactors in which it is possible to improve light distribution in the culture;
3. Search for strains having small antenna size and thus higher photosynthesis saturation levels.

The first strategy has been pursued by Richmond and co-workers since the beginning of algal biotechnology outdoors, and particularly in the recent years with the use of ultra high cell densities of *Spirulina* and other microalgae in flat photobioreactors. However, even when *Spirulina* cultures were grown at the optimal cell density, and with very high mixing rates, it was not possible to prevent a reduction in the F_v/F_m ratio in midday (Hu *et al.*, 1996).

Special designs of photobioreactors have been studied quite intensively in Florence (Italy). Carlozzi & Torzillo (1996) attacked the problem by devising and constructing a strongly curved tubular photobioreactor for mass culture of *Spirulina*. This tubular photobioreactor was studied to create convective mixing in the tube lumen so as the cells of the core region are carried towards the tube wall to receive illumination. Considering the higher power required to support such a convective mixing, however (about 40% higher than conventional reactors), the small increase in biomass yield obtained (17%) did not justify this device. Other special designs have been tested by Laws *et al.* (1987). Finally photobioreactor designs devised to promote *light dilution* have been proposed by Torzillo *et al.* (1993) and more recently by Tredici & Chini Zittelli (1997). Most of these solutions are cumbersome and difficult to scale-up at industrial level in which a low cost production system is desired.

The third approach in search for algal strains with small antenna is based on the following rationale. Strains having a small antenna size will minimize absorbance of light by the outer layers of cells, thereby reducing the dissipation of light through non-photochemical quenching and the risk of photo-inhibition. This should result in a higher overall photosynthetic productivity in outdoor cultures. Indeed, small antenna-size cells are characterized by higher photosynthesis rates (Nakajima & Ueda, 1997, 2000; Neidhardt *et al.*, 1998; Melis *et al.*, 1999). In this fashion, small antenna size will permit the increase of culture density and/or of the culture depth, and consequently an increase in biomass output rate.

Small antenna size may be obtained through acclimation of cells to strong light in the laboratory, however, once cells are transferred outdoors the antenna size would readily revert to that of normally pigmented cells upon lowering of light intensity during the day and as a result of the increased cell density due to daily growth. Since no small antenna-size species have been found in nature, it seems necessary to develop mutants with stable characteristics, i.e. cell-type with an antenna size that is similar to that observed in high light acclimated cells. These findings may have profound and immediate consequences on the industrial scale biomass production of microalgae where an increase in population density and productivity would allow a reduction of costs.

4.3 Salinity stress

The response of algae and cyanobacteria to changes in the osmotic environment have attracted considerable attention since they are inhabitants of many of the biotopes characterized by big variations in salinities and may thus serve as model organisms for the study of the response of photosynthetic

organisms to osmotic stress. The fundamental aspects of salt adaptation were intensively reviewed: Kirst (1990) dealing with tolerance of marine macroalgae and phytoplankton species to salinity; Oren (1999) reviewed the energetic costs of salt adaptation. Salt acclimation of cyanobacteria was reviewed by Reed & Stewart (1988), and more recently by Erdmann & Hagemann (2001). This section of the chapter will mainly deal with the interaction of the photosynthetic apparatus of microalgae and salinity stress.

Photosynthesis of algae is inhibited by osmotic stress (Vonshak & Richmond, 1981; Gilmour *et al.*, 1984; Kirst, 1989; Endo *et al.*, 1995). Such a decrease in photosynthesis may be associated with the inhibition of PS II activity (Gilmour *et al.*, 1984, 1985; Endo *et al.*, 1995). In *Dunaliella tertiolecta*, the osmotic stress inhibits the non-cyclic electron transport and stimulates the cyclic electron transport and the fluorescence emission arising from PS I at 77K, suggesting that the inhibition of PS II activity results from the ΔpH -dependent down-regulation and the state II transition (Gilmour *et al.*, 1984, 1985). Endo *et al.* (1995) have recently shown that in *Chlamydomonas reichardtii* the inhibition of quantum yield of PS II photochemistry by osmotic stress is due to an increase in non-photochemical quenching which is attributable to a state II transition. In the red alga *Porphyra perforata*, Satoh *et al.* (1983) demonstrated that the decrease in excitation energy reaching PS II reaction centers and the inhibition of the oxidizing side of PS II by salt stress resulted in a decrease in PS II activity. It seems that many cyanobacteria are capable of compensating the reduction of energy supply from the photosynthetic pathway by significantly increasing their respiratory activity (Vonshak *et al.*, 1988; Gabbay-Azaria *et al.*, 1992; Peschek *et al.*, 1994; Zeng & Vonshak, 1998). Studies on the kinetics of the response of *Spirulina platensis* to salinity stress (Lu *et al.*, 1999) revealed that the responses of the maximal photochemical efficiency of PS II to high salinity were composed of two phases. The first phase took place in the first 4 h characterized by an immediate decrease in F_v/F_m , in the first 15 min after exposure followed by a recovery to around 90% of initial value in about 2–4 h. This phase was independent of light. The second phase proceeded after 4 h, in which F_v/F_m declined to 70% of initial value at 12 h in the light, but no further decrease in F_v/F_m was observed in the dark, indicating that photoinhibition was induced by salinity stress. While the ability to respond immediately to a change in the osmotic environment is a prerequisite to enable the algal cells to survive the change, the next step is the ability of the cells to adapt and establish a new steady state of growth. In many of the algal systems studied, a decline in productivity is observed once adapted to excessive salinity and clearly associated with a decrease in their photosynthetic capacity. It was reported that one of the primary sites of damage to the photosynthetic apparatus by environmental stress is located in PS II (Baker, 1991). In cyanobacteria, the effect of salt stress on PS II has not been studied as intensively as in higher plants. Jeanjean *et al.* (1993) reported that no significant changes were noticed in the activity of PS II electron transport in *Synechocystis* sp. PCC 6803 adapted to 0.55 M NaCl. However, in cells adapted to a higher level of salinity (0.684 M), PS II activity decreased

(Schubert & Hagemann, 1990). Using transients in chlorophyll (Chl) *a* fluorescence analysis, Lu & Vonshak (2002) and Lu *et al.* (1998) analyzed the modifications that took place in *S. platensis* cells adapted to elevated salinity.

The findings pointed out that after the initial decline in all the photosynthetic activities some of them were restored. No modification was induced by salt stress at the donor side of PS II. This finding also suggests that the decrease in PS II activity, seen during adaptation to salt stress, may be due to damage at the acceptor side of PS II and/or in the PS II reaction centers. Since no significant decrease in the probability of electron transport beyond Q_A (Ψ_o) was observed, it may as well indicate that the acceptor side also was not the main site of damage induced by salt stress. It therefore appears most likely that salt stress causes damage to the PS II reaction center itself. The decrease in the rate constant of energy trapping by PS II reaction centers in salt-adapted cells, derived from the fluorescence parameter $(1/F_o) - (1/F_m)$, also suggests that the main effect induced by salt stress lies in the PS II reaction centers. An increase in the complementary area above the fluorescence induction curve in salt-adapted cells exposed to 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) clearly demonstrated that salt stress resulted in an increase in the proportion of closed PS II reaction centers.

Another modification observed in the salt-adapted cells was an increase in the respiratory rate thereby affecting PS II, since the respiratory electron transport chain is often coupled with the photosynthetic electron transport chain in cyanobacteria. However, the higher excitation pressure that this would place on PS II could be overcome by a decrease in the absorption cross-section of PS II (as reflected by a decrease in the ratio of phycocyanin/Chl), a decrease in the rate constant of excitation energy trapping by PS II reaction centers and by increased PS I activity. This would result in a decrease in energy transfer between phycobilisomes and PS II, and shift the distribution of excitation energy more in favor of PS I. Enhancement in PS I activity should increase cyclic electron transport. Several reports have shown that cyclic electron flow increases under salinity stress (Jeanjean *et al.*, 1993; Hibino *et al.*, 1996). Thus, it seems that an increase in PS I activity in salt-adapted cells may protect PS II from excessive excitation energy under salt stress. On the other hand, the increases in PS I activity and in the respiratory rate of salt-adapted cells may provide more energy for the synthesis of organic osmolytes and for the extrusion of Na⁺ in cells to maintain osmotic balance.

It was thus suggested that adaptation of the PS II apparatus to salt stress in *Spirulina* cells appears to involve a decrease in the absorption cross-section (decreased ratio of phycocyanin/Chl), and in modifications to PS II photochemistry. An increase in PS I activity parallels the decrease in the maximum quantum efficiency of PS II photochemistry and may regulate excitation energy equilibration to maintain balanced electron transport in salt-adapted *Spirulina* cells. Through an increase in the proportion of closed PS II reaction centers, the PS II apparatus was thus protected from further excess excitation energy.

4.4 Concluding remarks

A better understanding of the mechanism of how excess solar energy is dissipated by the photosynthetic apparatus under high-irradiance stress is a major problem, not only for researchers studying fundamental aspects of photosynthesis, but also for applied photosynthesis research. Indeed, the optimization of growth conditions in outdoor cultures for mass production necessarily requires a knowledge of the fate of light energy within the photosynthetic apparatus. On the other hand, in algal biotechnology the warning signals must be recognized as soon as possible in order to prevent a significant reduction in daily productivity or situations which, in few days, may culminate in the loss of the culture. Since environmental stress affects the function of PS II, directly or indirectly, chlorophyll fluorescence technique represents a useful tool to get rapid evidence of stress conditions affecting the photosynthetic activity of the culture and to get a quantification of the effect of stress on biomass yield.

Outdoor dense microalgae cultures may experience large variations in light intensity due to the changes in daily irradiance and mixing. Although turbulent mixing is aimed to expose the cells to average, uniform irradiance, relatively long exposure of cells to excess light cannot be avoided. On the other hand, a strategy striving to counteract photoinhibition at midday, through an increase in cell concentration, would lead to acclimation of the cells to low irradiance, which may result in an increase in the PS II antenna size and thus to an increased risk of over-excitation (Falkowski & Raven, 1997; Neidhardt *et al.*, 1998). Moreover, the increased fraction of cells deprived of light in the deeper layers may bring about an increase in the energy dissipated through respiration.

The effect of photoinhibition on the productivity of microalgal cultures substantially increases if additional stress, e.g. sub-optimal temperatures or high oxygen concentration are superimposed. Thus, a larger proportion of the radiation absorbed by the photosynthetic apparatus is dissipated through non-photochemical pathways resulting in a reduced biomass yield. Production of genetically modified strains with small antennae size seems to be the most promising way to circumvent the problem of light penetration into the culture and at the same time to reduce the impact of photoinhibition on biomass productivity of outdoor microalgal cultures.

4.5 Summary

Microalgae represent a unique experimental system to study stress responses of photosynthetic organisms. In higher plants, response and adaptation to stress takes place in two levels: The metabolic level and the morphological/structural level. In many cases it is difficult, if not impossible, to determine which is the initial response and which one is just a result of the initial modification. Since microalgae lack the morphological structure that characterizes higher plants they may be used as a unique experimental system to study metabolic and molecular processes associated with the response and adaptation of photosynthetic organisms to stress. The study of stress

physiology and adaptation of microalgae also has an important application in further development of the biotechnology for mass culturing of microalgae.

- When culturing algal cells under outdoor conditions cells are exposed to severe changes in light and temperature much faster than the timescale required for the cells to be able and adapt. A better understanding of those parameters and the ability to monitor those conditions will provide the growers with a better knowledge on how to optimize growth and productivity.
- Induction of accumulation of high value products is associated with stress conditions. A better understanding of the physiological response may help in providing a better production system for the desired product and at a later stage give an insight of the potential for genetic modification of desired strains.
- The potential use of microalgae as part of a biological system for bioremediation/detoxification and waste treatment is also associated with growing the cells under stress conditions.

Understanding the process associated with these unique environmental conditions may help in choosing the right culture conditions as well as selecting strains in order to improve the efficiency of the biological process.

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5 Environmental Effects on Cell Composition

Qiang Hu

5.1 Introduction

Over 40 years ago Parsons *et al.* (1961) analyzed 11 different species of marine plankton that represented six taxonomic classes, all grown under similar physical and chemical conditions and harvested in the exponential phase. Their study revealed that the composition of the cells was generally similar when comparison was made by expressing the amounts of major fractions in terms of total organic carbon in the cells. Indeed, microalgae of different origins have a tendency, albeit with certain exceptions, to resemble each other in terms of cell composition, particularly in the relative amounts of crude protein, lipids, and carbohydrate that they contain when grown under more or less optimal growth conditions. For a single species, on the other hand, the variation in cell composition may differ many fold, according to the culture conditions under which it is grown. For example, *Chlorella* sp., *Botryococcus braunii*, and *Dunaliella salina*, which are all classified under Chlorophyceae, Volvocales, show typical biochemical composition: 30–50% proteins, 20–40% carbohydrate and 8–15% of lipids under favorable environmental conditions. These species, however, can accumulate under unfavorable environmental conditions up to 80% of fatty acids, 80% of hydrocarbons, and 40% of glycerol, respectively, on the basis of the dry weight. Clearly, environmental factors, particularly light, temperature, nutrient status, and salinity, not only affect photosynthesis and productivity of cell biomass, but also influence the pattern, pathway and activity of cellular metabolism and thus dynamic cell composition. The effects on the latter have far-reaching biotechnological implications and consequences. This chapter will describe some general trends of the cellular responses of microalgae, in terms of cell composition, to the major environmental factors, and then address how manipulation of algal cultures with various environmental factors could achieve specific biotechnological goals.

5.2 Environmental factors

5.2.1 Light

Effects of light on biochemical composition of photosynthetic algae are largely controlled by the process called photoacclimation or photoadaptation. In this process, algal cells undergo dynamic changes in cell composition,

along with alterations in ultrastructural, biophysical and physiological properties to augment photosynthesis and algal growth (Dubinsky *et al.*, 1995). A common trend of cellular response to decreasing light intensity is to increase chlorophyll *a* and other light-harvesting pigments (such as chlorophyll *b*, chlorophyll *c*, phycobiliproteins and primary carotenoids). On the other-hand, in response to high light intensity chlorophyll *a* and other pigments directly involved in photosynthesis decrease, while the secondary carotenoids (e.g. zeaxanthin, β -carotene, astaxanthin), which serve as photoprotective agents, increase. These carotenoids often accumulate in special structures, such as plastoglobuli of plastids (Ben-Amotz *et al.*, 1982) or cytoplasmic lipid bodies (Vechtel *et al.*, 1992), thereby playing their roles in preventing excess light energy from reaching the photosynthetic machinery. Carotenoid accumulation may, in general, result from the alternation of carbon and nitrogen flows within the cells under stressful conditions.

High light intensities tend to enhance polysaccharide production in algal cells. Friedman *et al.* (1991) reported that 0.6- and 3-fold increases in polysaccharide were obtained in cultures of *Porphyridium* sp. and *Porphyridium aerugineum*, respectively, when growth light intensity increased from 75 to $300 \mu\text{mol m}^{-2} \text{s}^{-1}$. Tredici *et al.* (1991) demonstrated that carbohydrate synthesis in *Spirulina platensis* grown outdoors was significantly higher on sunny days than on cloudy days.

Numerous studies with microalgae of various groups suggest that the cellular content of lipids and total polyunsaturated fatty acids (PUFA), including eicosapentaenoic acid (EPA, 20 : 5 ω 3), is inversely related to growth light intensity (Cohen, 1999). Sukenik *et al.* (1989) showed that *Nannochloropsis* cells were characterized by a high lipid content and high proportions of EPA under light-limiting conditions, whereas 16:0 and 16:1 species predominate as light intensity increased to a saturated level. Since PUFA are the major constituents of the thylakoid membranes, low light-enhanced production of PUFA is usually coupled with a concomitant increase in total thylakoid membranes in the cells (Burner *et al.*, 1989). There are, however, some exceptions. Strong light intensity was observed to increase the PUFA levels in certain species, possibly resulting from an increase in oxygen-mediated lipid desaturation under high light conditions (Molina Grima *et al.*, 1999).

5.2.2 Temperature

The effect of temperature on biochemical reactions makes it one of the most important environmental factors influencing the biochemical composition of algae. A well-studied subject is the effect of temperature on membrane lipid composition and content. A decrease in growth temperature below an optimal level generally increases the degree of unsaturation of lipids in membrane systems. Enhanced stability and fluidity of cellular membranes, particularly thylakoid membranes (through increased levels of unsaturated fatty acids in membrane lipids) protect the photosynthetic machinery from photoinhibition at low temperatures (Nishida & Murata, 1996). It was found that upregulation of the expression of the genes encoding acyl-lipid desaturases is responsible for low-temperature-induced desaturation of fatty

acids in *Synechocystis* sp. PCC6803 (Murata, 1989). It seems that temperature, in a physiologically tolerant temperature range, may exert more significant effect on the relative cellular content of lipid classes and/or the relative composition of molecular species within a lipid class rather than on total lipid content in the cells. For instance, no consistent trends of lipid content per cell with temperature were found over eight species of marine phytoplankton (Thompson *et al.*, 1992). It was also found that decreases in growth temperature below the optimal range might result in increasing enzyme production as an adaptive mechanism for maintaining rates of photosynthesis and respiration. In addition, low temperatures induce cellular accumulation of polyols and amino acids or amino acid derivatives as compatible solutes, which might contribute to the sensitivity or tolerance of microalgae to chilling.

Tjahjono *et al.* (1994) reported that astaxanthin formation in *Haematococcus* cells increased threefold when growth temperature increased from 20°C to 30°C. Liu & Lee (2000) confirmed the same phenomenon with another green alga *Chlorococcum* sp. The total carotenoid content almost doubled (from 4.4 mg g⁻¹ to 8.4 mg g⁻¹ dry weight) when growth temperature increased from 20°C to 35°C. Temperature also exerts effect on carotenoid composition. Some threefold increase in astaxanthin content at the expense of mainly β-carotene and cantaxanthin was obtained at 35°C relative to 25°C of cultures of *Chlorococcum* sp. (Liu & Lee, 2000). It was suggested that higher temperatures may induce the formation of active oxygen radicals in algal cells, resulting in oxidative stress-induced carotenogenesis (Tjahjono *et al.*, 1994), and/or enhance temperature-dependent enzymatic reaction involved in carotenogenesis (Liu & Lee, 2000).

Growth temperature has been assumed to influence the pattern of cellular carbon and nitrogen quotas as well as cell volume, which can be best described as a U-shaped response. An optimal temperature for growth may result in algal cells having minimal cell size, cellular carbon and nitrogen contents, whereas a temperature below or above the optimal level may lead to increases in cell volume and biochemical content (Goldman & Mann, 1980; Rhee, 1982; Harris, 1988). In other words, it requires more carbon and nutrients to produce a cell at the same growth rate at a non-optimal temperature (Darley, 1982).

5.3 Nutritional factors

5.3.1 Nitrogen

Nitrogen, which generally accounts for about 7–10% of cell dry weight, is an essential constituent of all structural and functional proteins in algal cells. In general, microalgae have a limited ability to produce nitrogen storage materials when growing under nitrogen-sufficient conditions, exceptions are cyanophycin (Simon, 1971) and phycocyanin (Boussiba & Richmond, 1980), found to be nitrogen storage compounds in many cyanobacteria. When microalgae are grown under nitrogen-limited conditions, the most striking effect is the active and specific degradation of phycobilisomes (Collier &

Grossman, 1992). Until cell nitrogen falls below a threshold value, photosynthesis still continues, albeit at a reduced rate. The flow of carbon, fixed in photosynthesis, under these circumstances is diverted from the path of protein synthesis to that leading to either lipid-or carbohydrate synthesis.

Numerous studies show that the biosynthesis and accumulation of lipids is enhanced in nitrogen-limited or deprived cultures of microalgae of various taxonomic groups. In contrast to the polar lipids of nitrogen-sufficient cells, neutral lipids in the form of triacylglycerols become the predominant components of lipids from nitrogen-depleted cells (Thompson, 1996).

Yet, some algal species increase their carbohydrate rather than their lipid content under nitrogen depletion conditions, e.g. many *Dunaliella* strains, in which large quantities of glycerol can be accumulated along with increased mono-, di- and polysaccharides under nitrogen deprived growth conditions (Borowitzka & Borowitzka, 1988). Whether synthesis of neutral lipids or carbohydrates under nitrogen-limited conditions is species-specific, and has physiological significance, is not clear. Within a single genus of *Chlorella*, for instance, some strains were found to accumulate large amounts of starch, under nitrogen starvation, whereas others accumulated neutral lipids instead (Richmond, 1986).

Accumulation of secondary carotenoids is another main characteristic of many algae when growing under nitrogen-limited conditions, which is often accompanied by a decrease in the chlorophyll content of the cells. Ben-Amotz *et al.* (1982) showed that enhanced production of β-carotene occurred in *Dunaliella* cells starved of nitrogen. Borowitzka *et al.* (1991) showed that low nitrogen concentration is a major factor in stimulating the synthesis and accumulation of astaxanthin and its acylesters in *Haematococcus pluvialis*. More recently, Zhekisheva *et al.* (2002) reported that under nitrogen depletion conditions, *Haematococcus pluvialis* produced, for each picogram of astaxanthin, five picograms of fatty acids, particularly oleic acid-rich triacylglycerols, suggesting that these two processes are interrelated and that they enable the oil globules to maintain the high content of astaxanthin esters.

5.3.2 Phosphorus

Phosphorous is another major macronutrient that plays an important role in cellular metabolic processes by forming many structural and functional components required for normal growth and development of microalgae. When nutrients are provided in excess and light is the growth-limiting factor, most algal species display a remarkable consistency in their cellular phosphorous content, ca. 1% of dry weight (Goldman, 1980). Orthophosphate is incorporated into organic components through various types of phosphorylation. In addition, inorganic phosphate may also occur in the cells in the form of polyphosphates, accumulating in distinct polyphosphate granules. These polyphosphate granules often appear in normal cells under phosphate-sufficient growth, but disappear once phosphate becomes deficient (Healey, 1982).

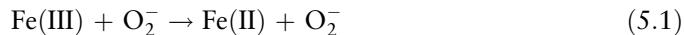
Some of the symptoms of phosphorous depletion are similar to those observed in nitrogen-deficient cultures. The contents of chlorophyll *a* tend

to decrease while carbohydrate content increases in eukaryotic and prokaryotic cells (Healey, 1982). In contrast to nitrogen depletion, little phycobilisome degradation occurs during phosphorous depletion. The decrease in cellular phycobilisome level is due to cell division while new phycobilisome synthesis ceases (Collier & Grossman, 1992). Phosphorous depletion was reported to result in the accumulation of β -carotene in *Dunaliella* cells (Ben-Amotz *et al.*, 1982), and astaxanthin accumulation in *Haematococcus* cells (Boussiba *et al.*, 1992), but not markedly so when compared to nitrogen deficiency.

5.3.3 Iron

Among essential trace mineral elements, iron plays an important role in cellular biochemical composition because of its redox properties and implication in fundamental processes such as photosynthesis, respiration, nitrogen fixation and DNA synthesis. Iron deficiency was found to induce various biochemical changes. On the one hand, c-phycocyanin and chlorophyll *a* may be degraded when Fe becomes limiting (Hardie *et al.*, 1983). On the other hand, accumulation of *iron-stress-induced* protein (*isiA*), which associates with PS I to form a complex that consists of a ring of 18 *isiA* molecules around a PS I trimer, was found in some cyanobacteria exposed to iron deficiency (Bibby *et al.*, 2001). This significantly increased size of the light-harvesting system of PS I was thought to increase the flexibility of cyanobacterial light-harvesting systems to compensate for the lowering of phycobilisome and PS I levels in response to iron deficiency. In many but not all microalgae and cyanobacteria it was noted that Fe-containing ferredoxin, a component of the photosynthetic electron transport chain, dropped markedly whereas the content of flavodoxin, an electron carrier that does not contain Fe, increased in cells during periods of low Fe availability (Bottin & Lagoutte, 1992; McKay *et al.*, 1999).

Excess iron in algal cultures, on the other hand, may elicit an oxidative stress leading to a variety of physiological changes. At the cellular level, Fe^{2+} can react with H_2O_2 , which is produced by microalgal cells, to generate hydroxyl radicals (OH^\cdot) by the Fenton reaction:



Kobayashi *et al.* (1993) reported that acetate-induced astaxanthin formation in *Haematococcus pluvialis* was markedly enhanced by the addition of iron (Fe^{2+}). They suggested that Fe^{2+} might possibly function as an OH^- generator via the iron-catalyzed Fenton reaction, and that OH^- or other active oxygen species (${}^1\text{O}_2$, O_2^- , H_2O_2 , or AO_2^-) may play an essential role(s) in the enhanced carotenogenesis in the alga. Recently, an antioxidant role of astaxanthin in *Haematococcus* cells under oxidative stress was confirmed by measuring the capacity for *in vivo* conversion of 2',7'-dichlorofluorescein diacetate to the fluorescent dye dichlorofluorescein in vegetative cells, as well as immature and mature cysts cells (Kobayashi, 2000).

5.4 Salinity

Many microalgae are capable of accumulating small molecules as osmoregulatory substances or *osmoticants* in response to an increase in salinity or osmotic pressure of the environment (*salinities* in this chapter are given as % NaCl [w/v]). Among the osmoticants found in microalgae, the polyols are most important. The common polyols of algae include: glycerol, mannitol, galactitol, sorbitol, glycerol galactoside, sucrose and trehalose. Glycerol content of up to 50% of the dry weight was detected in *Dunaliella* grown in elevated salinity conditions. It was believed that starch breakdown might somehow account for the glycerol biosynthesis, although it is not known how the molecular mechanism of carbon distribution between starch and glycerol is regulated in this organism under salinity conditions (Brown & Borowitzka, 1979).

Increase in salinity may result in slightly increased total lipid content of algae, as observed in cultures of *Monodus subterraneus* (Iwamoto & Sato, 1986), and *Dunaliella* spp. (Borowitzka & Borowitzka, 1988). The proportion of unsaturated fatty acid EPA in total fatty acids of *M. subterraneus* and *N. aculada* decreased under these conditions. Variations in salinity may exert a very limited influence on the carotenogenesis of microalgae (Cifuentes *et al.*, 2001), although significant increases in carotenoid concentrations were observed with increasing salinity in certain *Dunaliella* strains (Borowitzka & Borowitzka, 1988).

5.5 Synergistic effects of combinations of chemical and physical factors on cell composition

Although numerous reports have shown that cell composition of microalgae can be affected by a single chemical or physical factor, the effectiveness of such treatment is usually poor, and the change is slow. Cifuentes *et al.* (2001) investigated growth responses and pigment content of nine strains of *Dunaliella* in a wide range of salinity [1–30% (w/v) NaCl] and found none turning orange or red in color under the wide range of salt concentrations. The total carotenoid to chlorophyll ratios were always lower or equal to 1.0. However, the application of high salinity, in conjunction with high light intensity and high temperature, is often observed to yield high cellular contents of β-carotene in this organism (Borowitzka & Borowitzka, 1988).

A good example of synergistic effects of a combination of multiple chemical and physical factors on algal pigmentation was given by Steinbrenner & Linden (2001) who studied stress-induced astaxanthin formation in the green alga *Haematococcus pluvialis*. They observed that the application of a single stress factor (either high light, salt, or iron) to *Haematococcus* cultures only resulted in a moderate induction of astaxanthin synthesis and accumulation. A sustained increase in pigment synthesis occurred when a combination of two stress factors (high light plus salt, high light plus iron or salt plus iron) was applied to the culture. The highest production of astaxanthin was obtained in cultures in which all three factors were introduced simultaneously.

5.6 Biotechnological approaches to control cell composition

Cell composition of microalgae can be optimized readily by photobioreactors that control environmental conditions and through the use of multistage (or multiphase) cultivation strategy.

Of the numerous design parameters, the length of light-path (culture depth) of a photobioreactor is the most critical factor affecting the light regime to which the average single cell in the culture is exposed. Photoacclimation of algae to specific light regime usually results in changes in biochemical composition. As long as operational parameters are concerned, cell concentration (or population density) of algal culture is the single most effective biological factor affecting the biochemical composition of algal biomass (Dubinsky *et al.*, 1995; Hu *et al.*, 1996). Hu *et al.* (1997) determined that the highest cellular content of EPA coincided with maximal productivity of cell mass in *Monodus subterraneus*. Similar findings were reported by Chini Zittelli *et al.* (1999) and by Zou *et al.* (2000) with cultures of *Nannochloropsis* sp. These observations reveal one important advantage of manipulating cell concentration over some chemical and/or physical parameters, to affect cell composition. Control over cell concentration of the culture would permit essentially all other factors to function at their optimum. In contrast, introduction of salinity, nutrient depletion, and/or unfavorable temperature to algal cultures aiming to induce the highest content of specific product may not only reduce the overall biomass productivity but also introduce potential instability of the culture which is often responsible for contamination and culture collapse (Hu *et al.*, 1997).

The concept of the multistage cultivation strategy is to ensure maximum production of biomass in one stage and maximum induction and accumulation of desired products in the other. This concept has been successfully applied to outdoor mass culture of *Dunaliella* in open raceways for enhanced growth and carotenogenesis (Ben-Amotz, 1995, 1999). In the conventional one-phase type of cultivation, the productivity of β -carotene was below $200 \text{ mg m}^{-2} \text{ d}^{-1}$. In the two-phase culture mode, the alga was maintained in stage one for optimizing biomass production of cells containing a low β -carotene to chlorophyll ratio. The culture was then transferred to stage two, diluted to about one-third for inducing carotenogenesis. As a result, the β -carotene productivity increased to $450 \text{ mg m}^{-2} \text{ d}^{-1}$ in stage one and to $300 \text{ mg m}^{-2} \text{ d}^{-1}$ in stage two. A similar approach has also been applied to *Haematococcus* cultures grown in tubular photobioreactors to maximize astaxanthin production (Boussiba, 1996; Harker *et al.*, 1996).

Yet, another type of multiphase cultivation strategy is the application of integrated photobioreactor systems of various designs. It should include, but not be limited to, the combination of (1) open pond and closed reactor; (2) flat panel- and tubular reactors; (3) flat panels of various optical paths, or tubular reactors of various diameters; and (4) photobioreactor and heterotrophic fermenter. Richmond (1987) proposed the concept of a combined cultivation system made of a tubular reactor connected to an open raceway. As culture temperatures increase rapidly in the tubular reactor in the morning, overheating would be prevented at noon and afternoon by circulating the culture in the open raceways (exposed to evaporative cooling) prior to

returning to the tubular reactor. Boussiba (1996) described an integrated system consisting of a flat panel reactor and a tubular reactor for outdoor mass cultures of *Haematococcus pluvialis*. During the first stage, the *Haematococcus* cells were maintained in a flat panel reactor for achieving maximum rates of algal growth and a high cell concentration. For the second stage, the culture suspension is transferred into a tubular reactor for enhanced carotenogenesis in the cells.

A modular reactor system with identical configuration but varying in thickness or diameter of individual modules could be an alternative approach for manipulation of cell composition. For production of low-light enhanced products, such as phycobiliproteins and chloroplast membrane-bound PUFAs, a thinner/smaller diameter, yet optimized reactor unit should be used to increase the growth rate. When the algae reach the stationary phase of growth, the culture can be transferred into another reactor unit with a longer light path to affect the accumulation of desired product. In contrast, a reactor with a further reduced light path may be used at the second stage to stimulate high light-induced product formation, such as secondary carotenoids.

High cell density and biomass yield may be readily obtained through heterotrophic cultivation of selected algal species in conventional fermentors (Chen, 1996). This cultivation mode may be less effective, however, for producing light-induced metabolites. One way to overcome the problem is to transfer the culture at the end of the heterotrophic culture phase, in which cell density has reached its maximum, to a photobioreactor (open raceway or enclosed system) to induce product formation (Ogbonna & Tanaka, 2000).

Finally, it should be kept in mind that the potential yield of specific products from microalgae is a function of cell composition, cell density and specific growth rate. As described earlier in this chapter, culture conditions favoring desired product formation are not usually compatible with those required for maximal growth and biomass production. It is critical, therefore, to quantitatively determine the yield potential of desired products from microalgae when grown under conditions favoring modifications in cellular chemical composition, at the expense of attaining maximum biomass yield (Goldman, 1980).

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Part II

Mass Cultivation of Microalgae

6 Algal Nutrition

Mineral Nutrition

Johan U. Grobbelaar

6.1 Nutritional modes

It is mainly to the credit of Justus von Liebig (1803–1873) that the importance of mineral nutrients for plant growth, and nutrition in general was recognised. The need to supply mineral nutrients and other growth requirements to algae in culture has thus been known for a long time. Algae are capable of many kinds of trophic (nourishment) centred on both major forms of nutrition, namely autotrophy (phototrophy) and heterotrophy (phagotrophy), of which autotrophy is by far the most important.

The possible nutritional routes for algae are shown in Fig. 6.1. Autotrophic organisms obtain their energy through the absorption of light energy for the reduction of CO_2 by the oxidation of substrates, mainly water, with the release of O_2 . Photoautotrophic organisms only require inorganic mineral ions and obligate photoautotrophs are those that cannot grow in the dark. By far, most algae belong to this category, although many require minimal quantities of organic compounds for growth, such as vitamins.

Heterotrophic organisms obtain their material and energy needs from organic compounds produced by other organisms. Several algal species can be grown exclusively on organic substrates and this has become a viable option in conventional closed bioreactor production systems for biomass and biocompounds, produced by certain species under specific growth conditions (see Chapter 7).

Photoheterotrophic organisms require light as energy source to use organic compounds as nutrients. The organic compounds may also satisfy the energy requirements of the algae. Auxotrophy is where the algae require only small quantities of essential organic compounds such as vitamins and amino acids. Mixotrophic or amphitrophic growth is equivalent to autotrophy and heterotrophy, where both organic compounds and CO_2 are necessary for growth. A definite switch between autotrophy and heterotrophy is not manifested and both processes are present, except in total darkness. Lee *et al.* (1996) reported the heterotrophic growth of *Chlorella sorokiniana* at night on glucose, while during the day they were mixotrophic utilising both glucose and CO_2 (see Section 6.2).

No clear distinction is possible, except for the obligate trophic types, and some interchange between the various trophic possibilities is likely under most growth conditions. Unclear is the role of excreted organics in the trophic

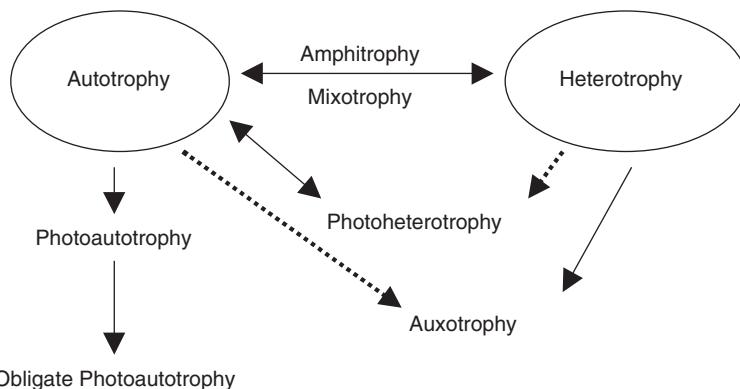


Fig. 6.1. Various trophic possibilities for algae, where autotrophic and heterotrophic growth are the most important one's.

of algae. The release of glycolic acid by algae has been known for a long time, but its function and role is uncertain (Fogg, 1966). Well known is the excretion of alkaline phosphatases by algae especially when phosphorus is growth limiting (Grobbelaar, 1983). This mobilises adsorbed organic-P rendering it available for the algae. Exudates are known to inhibit algal growth especially as a means of limiting competition amongst species and as defence against predation. Richmond (2000) has suggested that the production of autoinhibitors, taking place in ultrahigh-density mass algal cultures may be one of the most important factors which prevent attaining the maximal productivity potential, resulting in reduced yields. Grobbelaar (1985) speculated that the excreted organics serve as an energy source for the algae, especially at night, following photosynthesis and growth, during daylight when the alga is capable of mixotrophic growth. The production of extracellular organics follows the diurnal growth curve but is out of phase with about 6 h of the growth increment of the particulate biomass (Fig. 6.2). The results clearly show a decrease during the dark period, indicating re-absorption. The fact that the algae act as *organic pumps* and that appreciable quantities are being excreted, suggests that a significant part of the overall productivity does not form part of the particulate fraction, which is normally measured when yields are determined. These excreted organics are often ignored when areal production rates or yields are assessed.

6.2 Nutrient requirements

Soil water extracts initially formed the basis of culture solutions (Pringsheim, 1950), but thereafter many studies were conducted to determine the optimal nutrient concentrations for various algal species. Vonshak (1986) summarised the requirements for developing nutrient recipes for algal cultivation as follows:

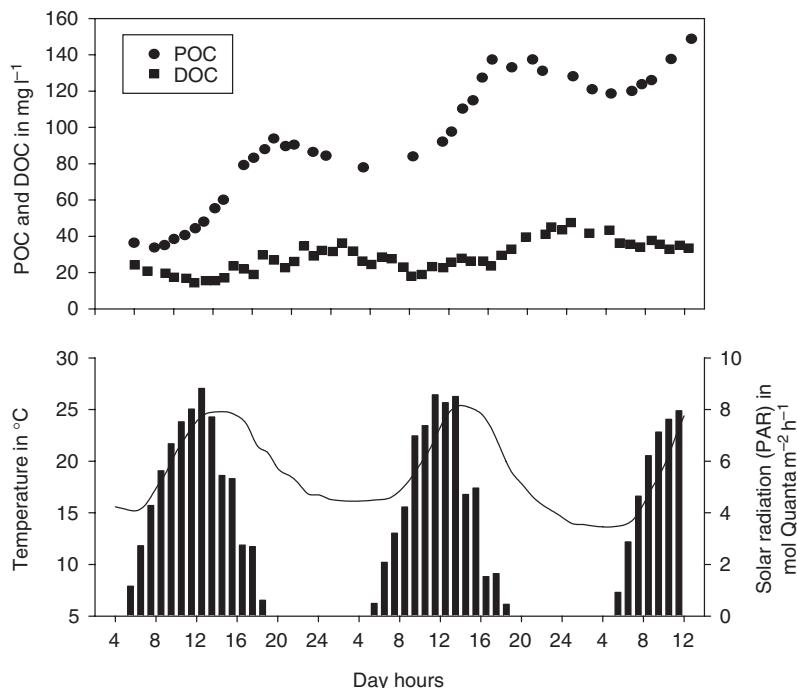


Fig. 6.2. Biomass (POC) and extracellular dissolved organic carbon (DOC) variations as measured in a batch culture of *Scenedesmus obliquus* (J.U. Grobbelaar unpublished data).

- (i) The total salt content, which is determined by the habitat from where the algae originates.
- (ii) Cell composition in terms of the major ionic components such as K^+ , Mg^{2+} , Na^+ , Ca^{2+} , SO_4^{2-} and Cl^- (Table 6.1).
- (iii) The nitrogen sources, especially nitrate, ammonia and urea.
- (iv) Carbon source either CO_2 or HCO_3^- .
- (v) pH.
- (vi) Trace elements and some chelating agent such as ethylenediamine-tetraacetic acid (EDTA).
- (vii) Vitamins.

Further to the above it is also important to consider:

- (i) The trophic route focussing on whether inorganic carbon or organic carbon should form the carbon skeletons for further synthesis. Organic sources often used are glucose, fructose, and acetic acid. The advantage of acetic acid as a source of CO_2 is that it could be supplied on pH demand.
- (ii) The purpose for which the algae will be cultured. Maintenance of cultures in a culture collection, versus growth for optimal biomass yields, versus stress for optimal biosynthesis of valued biocompounds, would require very different formulations of the nutrient recipes. Carotenogenesis is initiated in *Dunaliella salina* following amongst other mainly nitrogen and salt stress (Ben-Amotz & Avron, 1989). Normally stress would not be applied when maintaining algae in culture collections. The

Table 6.1. A list of nutrients required by algal cells and elementary composition of algae.

Element	Compounds	Conc range/liter media	Cell composition range µg/mg dry weight	Relative no. of atoms
C	CO_2 , HCO_3^{2-} , CO_3^{2-} , organic molecules	g	175–650	4460000
O	O_2 , H_2O , organic molecules	g	205–330	2120000
H	H_2O , organic molecules, H_2S	g	29–100	8140000
N	N_2 , NH_4^+ , NO_3^- , NO_2^- , amino acids, purines, pyrimidines, urea, etc.	g	10–140	487000
Na	Several inorganic salts, i.e. NaCl , Na_2SO_4 , Na_3PO_4	g	0.4–47	32500
K	Several inorganic salts, i.e. KCl , K_2SO_4 , K_3PO_4	g	1–75	55000
Ca	Several inorganic salts, i.e. CaCO_3 , Ca^{2-} (as chloride)	g	0.0–80	27500
P	Several inorganic salts, Na or K phosphates, $\text{Na}_2\text{glycerophosphate} \cdot 5\text{H}_2\text{O}$	g	0.5–33	43800
S	Several inorganic salts, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, amino acids	g	1.5–16	23800
Mg	Several inorganic salts, Co_2^{3+} , SO_4^{2-} , or Cl^- salts	g	0.5–75	28700
Cl	As Na^+ , K^+ , Ca^{2+} , or NH_4^+ salts	g	*	*
Fe	FeCl_3 , $\text{Fe}(\text{NH}_4)_2\text{SO}_4$, ferric citrate	mg	0.2–34	13800

Zn	SO_4^{2-} or Cl^- salts	mg	0.005–1.0	540
Mn	SO_4^{2-} or Cl^- salts	mg	0.02–0.24	138
Br	As Na^+ , K^+ , Ca^{2+} , or NH_4^+ salts	mg	*	*
Si	$\text{Na}_3\text{SiO}_3 \cdot 9\text{H}_2\text{O}$	mg	0–230	237 000
B	H_3BO_3	mg	0.001–0.25	350
Mo	Na^+ or NH_4^+ molybdate salts	μg	0.0002–0.001	1
V	$\text{Na}_3\text{VO}_4 \cdot 16\text{H}_2\text{O}$	μg	*	*
Sr	SO_4^{2-} or Cl^- salts	μg	*	*
Al	SO_4^{2-} or Cl^- salts	μg	*	*
Rb	SO_4^{2-} or Cl^- salts	μg	*	*
Li	SO_4^{2-} or Cl^- salts	μg	*	*
Cu	SO_4^{2-} or Cl^- salts	μg	0.006–0.3	200
Co	Vitamin B ₁₂ , SO_4^{2-} , or Cl^- salts	μg	0.0001–0.2	125
I	As Na^+ , K^+ , Ca^{2+} , or NH_4^+ salts	μg	*	*
Se	Na_2SeO_3	ng	*	*

* Insufficient information.

Adapted from: Healy, F.P., CRC Critical Reviews in Microalgae, CRC Press, Boca Raton, Florida, 1973 and from Aaronson, S., Experimental Microbiological Ecology, Academic Press, New York, 1970.

nutrient supply could determine the cultivation process. For example, when producing β -carotene from *Dunaliella* dual-phase culture techniques could be used, where the first photobioreactor is used for optimal biomass production and a second is used for optimal β -carotene production. The nutrient composition and supply would be very different (Ben-Amotz, 1995), the first reactor would have excess nitrogen and low salinity to allow high biomass yields, while the second would have little nitrogen and high salinity for maximal carotenogenesis.

For autotrophic growth the supply of about 30 elements (Table 6.1) is important and the review by Kaplan *et al.* (1986) gives a comprehensive overview of the subject. Macronutrients are supplied at concentrations of g/l and the micronutrients in mg l⁻¹ (see selected recipes, Table 6.2). The literature is extensive, but for the purposes of this chapter the focus will be on considerations that are important for determining the nutrient supply in applied phycology (algal biotechnology). Accordingly, the assumption is made that in most applied phycological processes, high biomass yields per reactor volume or illuminated surface area are of prime importance. The physiological and technological considerations necessary for achieving high yields have been elucidated (Grobbelaar, 2000). Richmond & Becker (1986) stated that, when the nutritional requirements of mass cultured algae are satisfied and the environmental conditions are not growth-limiting, mixing designed to create a turbulent flow constitutes the most important requisite for consistently obtaining high yields of algal mass. Such turbulence is important not only for enhancing exchange rates of nutrients and metabolites between the cultured cells and their growth medium, but the increased light/dark frequencies result in increased productivity and photosynthetic efficiencies (Grobbelaar, 1994). Also, increased turbulence affects the boundary layer and, therefore, the diffusion gradient for nutrients and metabolites.

The three most important nutrients for autotrophic growth are C, N and P and their supply is central to algal biotechnology.

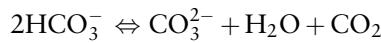
6.2.1 Carbon

For high rates of autotrophic production, supply of CO₂ and HCO₃⁻ is most important. Contrary to land plants, atmospheric CO₂ cannot satisfy the C-requirements of high yielding autotrophic algal production systems. Diffusion rates for CO₂ from the atmosphere into open ponds can at most sustain productivities around 10 g (dw) m⁻² d⁻¹. Rates as high as 70 g (dw) m⁻² d⁻¹ have been reported giving a carbon shortfall of about 30 g C m⁻² d⁻¹ (assuming a 50% C content of the biomass) (Lee *et al.*, 1995). The CO₂–H₂CO₃–HCO₃⁻–CO₃²⁻ system is the most important buffer generally present in freshwaters and it is the best means available to control and maintain specific pH levels that are optimal for mass-cultivated species.

The bicarbonate–carbonate buffer system can provide CO₂ for photosynthesis through the following reactions:

Table 6.2. Recipes of a few selected growth media used for growing different algae. All concentrations are in g L⁻¹, unless indicated otherwise and the quantities are for 1 litre of culture solution.

Substance	Nutrient solution					Ben-Amotz & Avron (1989)
	BG11	Modified Allen's	Bold's Basal	Sorokin/Krauss (1956)	Zarrouck (1966)	
NaNO ₃	1.5	1.5	0.25		2.5	
KNO ₃						0.505
K ₂ HPO ₄ • 3H ₂ O	0.04	0.039	0.075		0.5	0.014
KH ₂ PO ₄						
MgSO ₄ • 7H ₂ O	0.075	0.075	0.075	1.25	1.0	
MgCl ₂ • 4H ₂ O					0.2	1.2
CaCl ₂ • 2H ₂ O	0.036	0.025	0.084	0.04	0.08	0.1
Ca(NO ₃) ₂ • 4H ₂ O						0.033
Na ₂ SiO ₃ • 9H ₂ O						
Citric acid	0.006	0.006				
Fe-Ammonium citrate	0.006					
FeCl ₃	0.002					
FeSO ₄ • 7H ₂ O		0.001	0.001	0.00498	0.05	0.01
EDTA, 2Na-Mg salt			0.05	0.5	0.01	
NaHCO ₃					16.8	1.7
Na ₂ CO ₃	0.02	0.02	0.025			
NaCl					1.0	
K ₂ SO ₄			0.025		1.0	117.0
KOH			0.031			
Tris-HCl						6
H ₃ BO ₄ (µg l ⁻¹)	2.86	2.86	11.42	114	2.86	
MnCl ₂ • 4H ₂ O (µg l ⁻¹)	1.81	1.81	1.14	14	1.81	
ZnSO ₄ • 7H ₂ O (µg l ⁻¹)	0.2222	0.222	8.82	88	0.222	
ZnCl ₂						14
Na ₂ MoO ₄ • 2H ₂ O (µg l ⁻¹)	0.391	0.391	1.57	16	0.08	
CuSO ₄ • 5H ₂ O (µg l ⁻¹)	0.079	0.079				
CuCl ₂ • 2H ₂ O						
Co(NO ₃) ₂ • 6H ₂ O (µg l ⁻¹)	0.0494	0.0494	0.49	5		4.8
CoCl ₂ • 6H ₂ O (µg l ⁻¹)						
MoO ₃ (µg l ⁻¹)			0.71	7	0.01	
Adjust final pH	7.4	7.8		6.8		7.5



These reactions imply that during photosynthetic CO_2 fixation, OH^- accumulates in the growth solution leading to a gradual rise in pH. It is not uncommon to measure pH's as high as 11 in high algal density production systems where no additional CO_2 has been supplied (Richmond & Grobbelaar, 1986). pH-static control via direct CO_2 sparging into the culture media is the best and most convenient method of pH control and at the same time supplying CO_2 for high yield in mass algal cultures. This also applies to growth media rich in HCO_3^- such as for *Spirulina* growth. Since active photosynthesis results in an increase in pH, the opposite is true for CO_2 release during respiration, according to the above reactions. The overall influence is little since as a general rule dark respiration is less than 10% of total photosynthetic production (Grobbelaar & Soeder, 1985) (see Section 8.11.3). As already mentioned, in certain species carbon could also be supplied organically as sugars, acids and alcohols for mixotrophic growth. For example, the supply of acetic acid can be used for pH control in such cultures.

6.2.2 Nitrogen

After carbon, nitrogen is the most important nutrient contributing to the biomass produced. The nitrogen content of the biomass can range from 1% to more than 10% and it not only varies between different groups (e.g. low in diatoms) but within a particular species, depending on the supply and availability. Typical responses to nitrogen limitation is discolouration of the cells (decrease in chlorophylls and an increase in the carotenoids) and accumulation of organic carbon compounds such as polysaccharides and certain oils (PUFAs) (Becker, 1994).

Nitrogen is mostly supplied as nitrate (NO_3^-), but often ammonia (NH_4^+) and urea are also used, with similar growth rates recorded (Kaplan *et al.*, 1986). A variety of organic N compounds are utilised by algae, several of which can serve as the only source of N. Ammonia nitrogen is often the preferred N-source for microorganisms and the assimilation of either NO_3^- or NH_4^+ is related to the pH of the growth media. When ammonia is used as the sole source of N, the pH could drop significantly during active growth, due to the release of H^+ ions. This phenomenon has been used as a control measure to check herbivorous invertebrate growth following their invasion of mass algal cultures. An increase in pH occurs when nitrate is supplied as the only N-source. A factor that may be important when deciding whether to supply either nitrate or ammonia, is that the latter could be lost from the growth media due to volatilisation, particularly when the pH increases. Whether the supply of either NO_3^- or NH_4^+ is energetically beneficial for mass algal growth is not resolved and production rates are similar.

Some cyanobacteria are capable of utilising elemental nitrogen by the reduction of N₂ to NH₄⁺, a process catalysed by the enzyme nitrogenase. Although significant quantities could be fixed on an extensive basis, such as rice paddies or in natural ecosystems, the quantities are too low for high rate algal production systems. The possibilities that nitrogen-fixing species will become dominant in high rate algal production systems where N is limiting is highly unlikely and N-fixation would only apply to extensive production systems.

The important consideration in algal biotechnology aimed at achieving high yields is to ensure the adequate supply of this important nutrient. In fact culture media are formulated such that nutrients are supplied in excess to ensure that they never become the rate-limiting factor. For specific applications, however, nutrients are purposefully supplied in limiting concentrations, as an example for maximum carotenogenesis in *Dunaliella* for β-carotene production (Ben-Amotz & Avron, 1989), or carbohydrates, oils and fats in a variety of algae (Borowitzka, 1988), or polyunsaturated fatty acids (Garcia *et al.*, 2000) in algae grown mixotrophically.

6.2.3 Phosphorus

Phosphorus is essential for growth and many cellular processes such as energy transfer, biosynthesis of nucleic acids, DNA, etc. The preferred form in which it is supplied to algae is as orthophosphate (PO₄²⁻) and its uptake is energy dependant. Although algal biomass contains less than 1% P, it is often one of the most important growth limiting factors in algal biotechnology. This is because it is easily bound to other ions (e.g. CO₃²⁻ and iron) resulting in its precipitation and consequently rendering this essential nutrient unavailable for algal uptake. Algae are also able to store excess P in polyphosphate bodies during the so-called *luxury uptake*. This may be used when the external supply becomes limiting and is crucial in understanding the supply and uptake of nutrients, especially when determining the cell quota (see Section 6.4).

The supply of P also influences the composition of the produced biomass. It is especially the lipid content and carbohydrates that are affected by external and internal supplies of P (Borowitzka, 1988). Also important is the ratio of N:P in the growth media as this not only determines the potential productivity but it is important in maintaining the dominance of the candidate species in culture.

6.2.4 Other macro- and micronutrients, chelates and water

As stated above about 30 inorganic elements and at least as many organic compounds can be utilised by algae for nutrition (Kaplan *et al.*, 1986; Becker, 1994) and other than C, N and P, of importance are also S, K, Na, Fe, Mg, Ca and trace elements such as B, Cu, Mn, Zn, Mo, Co, V and Se, as reflected in the elementary composition of algae (Table 6.1). Detailed descriptions are readily available regarding mineral nutrition, their role and importance, and will not be presented here (Salisbury & Ross, 1992; Marschner, 1995). Many of the trace elements are important in enzyme reactions and for the biosynthesis

of many compounds, e.g. Co is essential for vitamin B₁₂ production. Their supply and availability should be monitored since as with P they are prone to binding with other growth media constituents resulting in precipitation and consequently rendering them unavailable. The addition of metal chelators such as EDTA and especially the disodium salt that readily dissolves in water has alleviated this problem. Iron chelated with trisodium hydroxyethylene-diaminetriacetate is more stable under alkaline conditions than Fe-EDTA. Nitrilotriacetic acid and citric acid are also used in some media solutions for their chelating potential, but they are less effective than EDTA.

Silicon is present in the cell walls of many divisions of algae, especially the diatoms, and is an essential nutrient for their growth and production (Healy, 1973). Si limitation often occurs and could also lead to the accumulation of secondary metabolites.

For laboratory grown cultures the water used for making up the growth media is either single or double glass-distilled water, membrane filtered water, or de-ionised water. Often glass-distilled water is also de-ionised because volatile and other compounds may be imparted especially if the stills are not cleaned regularly. The use of distilled, filtered or de-ionised water is not practicable for large-scale production purposes. In such cases either natural (surface or groundwater) or the available domestic waters are used. Thorough chemical analyses are required, and alien algae, if present, should be removed. Residual chlorine may be present in domestic waters and this would necessitate an aging step before the water is used. Aging of the water is achieved by allowing the water to stand in open tanks for about two days.

6.3 Recipes for algal growth nutrient media

Shown in Table 6.2 is the composition of some nutrient media commonly used for growing a few selected algae. Many recipes exist and often special formulations are used in mass production of microalgae, which are not reported as these are made up from commercial fertilisers, using natural waters including ground- and seawater. Also commercial production undertakings view the recipes used as their competitive advantage and they will seldom report exact formulations. The formulations shown in Table 6.2, reflect the variability in culture solutions indicating that many compounds are used in the recipes.

BG-11 is a growth medium that has been used extensively for freshwater green algae and cyanobacteria. The Modified Allen's medium is used for many cyanobacteria. The Bold's Basal nutrient solution is used for many algae and is often supplemented with soil extract. The culture media of Sorokin and Krauss is especially formulated for *Chlorella* cultivation (Sorokin & Krauss, 1956). *Spirulina* requires a bicarbonate-rich growth medium and the most commonly used is the formulation of Zarrouck (1966). Several formulations using seawater as a basis have been used, as well as brackish inland waters. The halotolerant alga *Dunaliella* requires a saline medium and as a basis, the formulation of Ben-Amotz & Avron (1989) is given in Table 6.2.

6.4 Uptake of N and P

N, P and C are often limiting and the oversupply is also no solution to the problem as this may lead to stress and reduced growth. When growth rates are plotted as a function of the nutrient concentrations, essentially four zones are recognised, i.e.:

- (i) a deficient zone with low nutrient concentrations, growth increasing dramatically when nutrients are supplied,
- (ii) a transition zone where the critical concentration is found and in this zone growth is little affected by the addition of more nutrients (often referred to as the zone of the optimal concentration),
- (iii) an adequate zone where no increase in growth is found with an increase in the supply of nutrients (luxury storage takes place at these concentrations), and
- (iv) a toxic zone where an increase in the concentration of nutrients leads to reduced growth.

The zone of adequate supply is fairly wide for macronutrients, but much narrower for micronutrients. Growth is retarded in the deficient zone, and can lead to alien species becoming dominant, increased infections as caused by bacteria, fungi and viruses resulting finally in total collapse of the cultures. In a batch cultivation system (Říčica, 1966) the typical growth response over time versus nutrient content can be idealised as shown in Fig. 6.3. Algae are adapted to scavenge their environments for resources, be it through structural changes, storage or increased resource utilisation efficiency. Internal adjustments involve biochemical and physiological acclimation, whilst externally they can excrete a variety of compounds to, amongst others, render nutrients available or limit the growth of competitors. As mentioned before they are

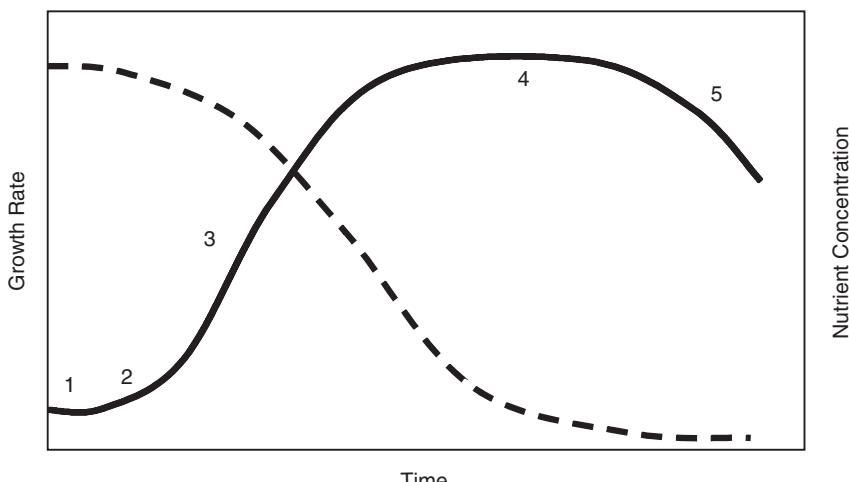


Fig. 6.3. Schematic growth curve in a batch culture (solid line) where the different growth phases can be recognised, i.e. 1 = lag phase, 2 = exponential phase representing the maximum growth rate under the specific conditions, 3 = phase of linear growth, 4 = stationary growth phase, and 5 = decline or death phase. The nutrients (dashed line) follows the opposite pattern indicating depletion of the nutrients during the stationary phase and onwards. In autotrophic systems light for photosynthesis usually becomes the limiting factor.

also capable of *luxury uptake* which is clearly distinct from Michaelis-Menten or Monod nutrient uptake kinetics (Monod, 1950). These models are based on external nutrient resource concentrations, and Epply and Strickland already concluded that the growth rate of algae is more dependant on the internal cellular concentrations than on the external quantities (Epply & Strickland, 1968). Droop developed a model for algal growth that is dependant on the cell quota of a nutrient and the growth rate of an alga, and in its generalised form it can be written as (Droop, 1968, 1983):

$$\mu = \mu_{\max} \left(1 - \frac{k_q}{Q}\right) \quad (6.1)$$

where μ = specific growth rate, μ_{\max} = maximum specific growth rate, k_q = minimum cell quota for the limiting resource or nutrient (subsistence quota), and Q = cell quota for the limiting resource. This model has been tested with a number of species and nutrients such as, N (NO_3^- , NH_4^{2-} , urea), P, Si, vitamin B₁₂ and Fe (Droop, 1983). In some cases the model, however, did not work, notably when NH_4^{2-} was limiting. The cellular nutrient quota, necessary for maintenance and growth, is the storage capacity of an organism for a specific nutrient. The ratio k_q/Q_{\max} has been defined as the luxury storage coefficient, where Q_{\max} is the maximum cell quota. The greater the difference between k_q and Q_{\max} , the greater is the organisms' quota flexibility and therefore, the potential to adapt to nutrient limitation (Fig. 6.4). In batch cultures where the nutrient content decreases as

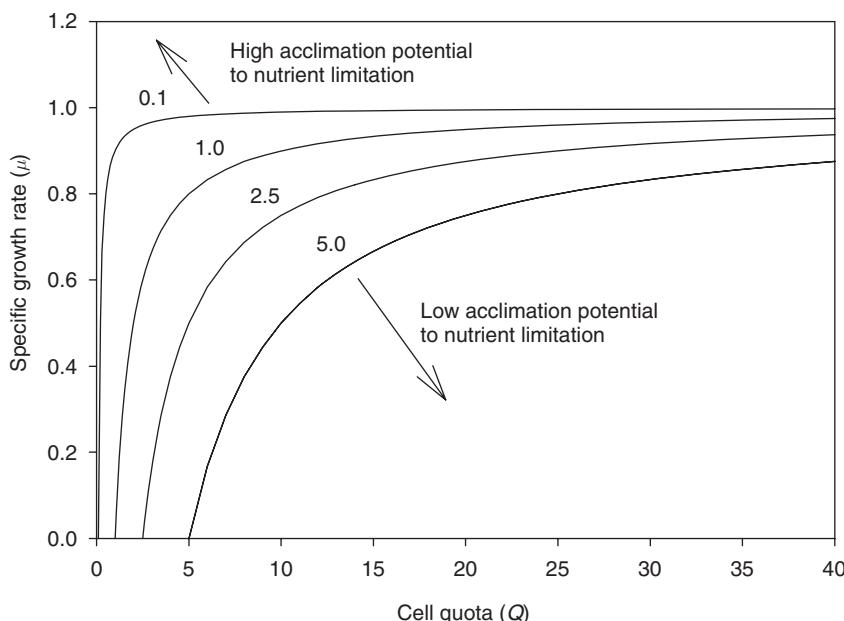


Fig. 6.4. Specific growth rate (μ) of algae against the cell quota (Q), as calculated from the Droop (1983) equation (6.1), assuming a $\mu_{\max} = 1$ and values for k_q of 0.1, 1.0, 2.5 and 5.0. Both the high and low acclimation potential to nutrient limitation are shown for this hypothetical example.

the growth proceeds (Fig. 6.3), the growth rate of an organism with a constant cell quota will decrease accordingly (Grobbelaar & House, 1995). If an organism can decrease its cellular requirements for a nutrient, it will be able to offset much of the decrease in the availability of the nutrient (i.e. high quota flexibility), thus minimising the effects of the limitation on growth.

The hyperbolic relationships between the quota flexibility (Q) and the specific growth rates at different minimum cell quotas (k_q) are shown in Fig. 6.4. This clearly shows that the lower the minimum cell quota for a nutrient the steeper the initial slope of the specific growth rate (μ) becomes and vice versa.

For steady-state nutrient assimilation equation (6.1) can be written as (Droop, 1968):

$$\mu = \frac{\mu_{\max}[S]}{K_s + [S]} \quad (6.2)$$

where $[S]$ = the steady-state substrate concentration and K_s = the half-saturation constant for steady-state nutrient uptake.

The slope of the specific growth rate as shown in Fig. 6.4, has a direct relation to the half-saturation constants (K_s), being high for low quota flexibility and low for high quota flexibility. As shown in Fig. 6.5 where specific growth rates are plotted against substrate concentrations at different half-saturation constants using equation (6.2), the marked differences in the

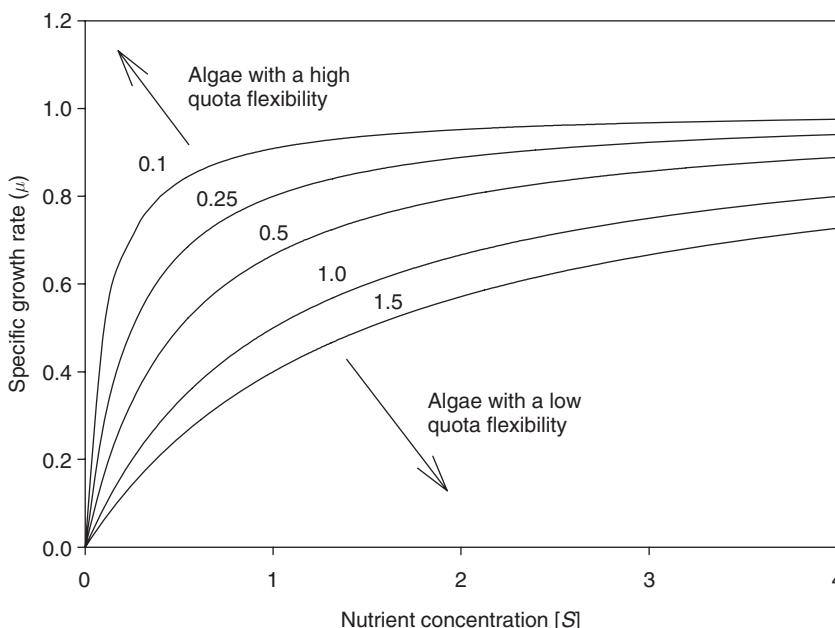


Fig. 6.5. A hypothetical example showing the specific growth rate (μ) of an alga against substrate concentration (S) and the concept of algal quota flexibility acclimation. The Monod kinetics as shown in equation (6.2) was used at K_s values of 0.1, 0.25, 0.5, 1.0, and 1.5.

responses are clear. Algae with a high quota flexibility will easily acclimate to nutrient variations as found in batch cultures, whereas those with a low quota flexibility will be more suited for cultivation in continuous culture systems. Algae that can adapt their quota flexibility will also be able to outcompete alien algae, especially under nutrient limited conditions, as those that will be found in batch cultures towards the end of the production cycle.

Low half-saturation constants are typical of nutrients such as N and P (Gilbert *et al.*, 1982), whereas high half-saturation constants are common for dissolved inorganic carbon (Turpin *et al.*, 1985). When an alga can acclimate its quota flexibility by lowering its half-saturation constant, this means a higher initial slope of the growth rate versus the nutrient concentration curve. A higher initial slope implies a high growth rate at low resource concentrations, which is a competitive advantage for the particular organism (Fig. 6.5).

6.5 Competition for limiting resources (nutrients)

Resource limitation refers to the yield attainable from a nutrient that is limiting, usually the primary limiting nutrient, or the rate at which the final yield is reached. For example, when P is limiting, its concentration would determine the final yield, but the available light energy for photosynthesis would determine the rate at which this final yield is attained, being low under low light and vice versa. These are important considerations for mass algal cultures, especially in high cell-density cultures which are highly light limited.

Several possibilities could manifest in mass algal cultures, especially in extensive and open cultures. Such cultures are prone to invasion by alien algae and other organisms. The following three examples suffice to indicate how different maximum specific growth rates (μ_{\max}) and nutrient half-saturation constants (K_s) can lead to one species outcompeting another.

Example 1

Should the μ_{\max} 's of the two competing algae be the same, but K_s of the invading species is lower than the K_s of the candidate species, then neither will dominate in the culture should the nutrient supply be in excess. As soon as the nutrients become depleted then the invading species will become the dominant one.

Example 2

If both the candidate and invading species have the same half-saturation constants (K_s), but the μ_{\max} of the invading species be greater than that of the candidate species, then the invading species will become dominant irrespective of the nutrient availability. The more nutrient stressed the culture the more the invading species will become dominant.

Example 3

If both the μ_{\max} and K_s of the invading species are greater than those of the candidate species, then under adequate nutrient supply conditions the candidate species will dominate, but as soon as the nutrients become limiting, the invading species will become dominant.

These three examples are rather simplistic, since many other factors such as light, temperature, pH, turbulence, excreted products, autoinhibition, etc. can play a role. However, they serve to illustrate the complexity of culture maintenance and management especially where invasion by alien algae is possible, such as in large open raceway ponds.

Both the Monod and Droop models state that the growth rate of an organism may be limited by a single resource only (Droop, 1974), once its availability becomes very low. In practice, however, co- and multiple limitations (stresses) are usual. Crucial for high yields is the supply of N, P and C, and in particular their optimum ratios. The optimum nutrient ratio is that at which a transition from one nutrient limitation to another occurs (thus both could be limiting at this transition) or where the cellular ratio of the resources required is such that the resource is not in short supply relative to another (Rhee & Gotham, 1980). Since the internal nutrient content is important in determining the uptake rates, it is possible to determine both the limiting resource concentration and the uptake rate at the transition point where limitation occurs. For example, if the optimal N:P ratios for two algal species are 20 and 10 respectively, both will be P limited when the N:P > 20. The second species will, however, be more P limited than the first. If they have similar μ_{\max} values, then the first species will eliminate the second at N:P > 20. Since a limiting nutrient can be defined as the one with the smallest $Q:k_q$ ratio (Droop, 1974), the transition between N and P limitation (same holds for other transitions) occurs when:

$$\frac{Q_N}{k_{qN}} = \frac{Q_P}{k_{qP}} \quad (6.3)$$

According to Rhee & Gotham (1980), the relationship between $Q_N:Q_P$ and the optimum ratio holds true only when $\mu_{\max N} = \mu_{\max P}$. Therefore, the optimum ratio of N:P is the ratio $k_{qN}:k_{qP}$ where $\mu = 0$, or the ratio $Q_N:Q_P$ at very low growth rates. Another important aspect is that the maximum growth rates for different nutrients are not necessarily equal. This is because storage pools differ and also because of differences in the $Q:k_q$ ratios (Goldman & McCarthy, 1978). This causes the $Q_N:Q_P$ ratio to deviate significantly from the optimum ratio, especially at high growth rates. Rearranging equation (6.1) for Q , it can be written as:

$$Q = \frac{k_q}{1 - \mu/\mu_{\max}} \quad (6.4)$$

For the optimum ratio of N:P, as determined by the $Q_N:Q_P$ ratio on the relative growth rates (Turpin, 1988), equation 6.3 can be written as:

$$\frac{Q_N}{Q_P} = \frac{k_{qN}/(1 - \mu/\mu_{\max N})}{k_{qP}/(1 - \mu/\mu_{\max P})} \quad (6.5)$$

Shown in Fig. 6.6 are the specific growth rates for a hypothetical alga at optimal N:P ratios. On either side of the curve, either N or P limits growth.

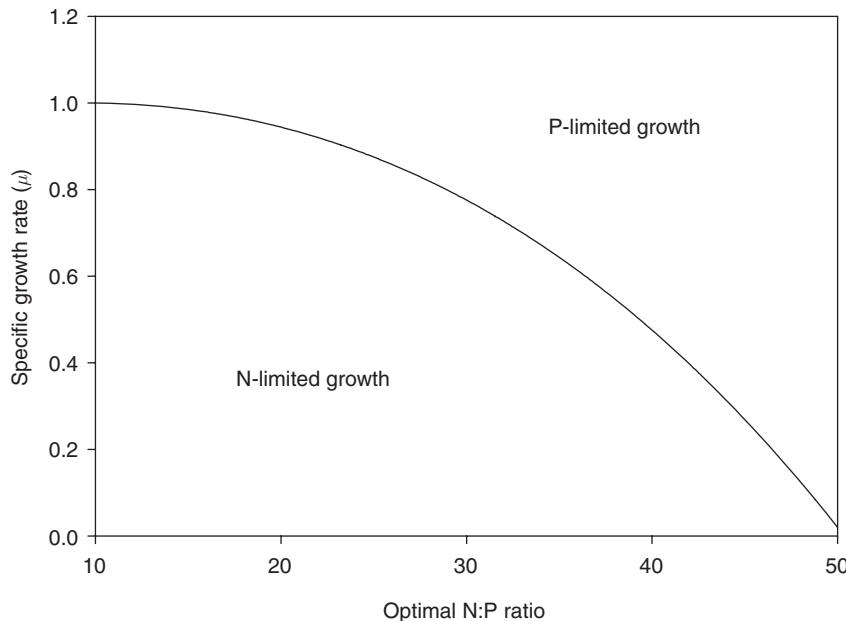


Fig. 6.6. The specific growth rate dependence for a hypothetical alga for the optimal N:P ratio with a $\mu_{\max} = 1$, a $k_{qN} = 5 \text{ mg l}^{-1}$ and a $k_{qP} = 0.5 \text{ mg l}^{-1}$.

From this it is clear that at higher growth rates, *pro rata* more N is required and vice versa. This is important in mass algal cultures especially where maximal productivities are important. Ahlgren (1985) showed that algae were able to adapt to different N:P ratios at lower growth rates and that the ratio becomes more fixed at higher growth rates.

6.6 Nutrient ratios

From the above it is clear, that complex interactions exist between the chemical constituents, their availability and uptake by algae in mass algal cultures. The situation is, furthermore, complicated due to complexities related to physical and biological variance. The ability of algae to acclimate should not be underestimated, but important in the acclimation, is that threshold concentrations in nutrients not be exceeded. For this, the Redfield ratio of 106C:16N:1P is widely used, as a point of departure, to quantify possible nutrient limitation.

It is noteworthy that both BG-11 and the Modified Allen's nutrient solutions have N:P ratios of 45:1 (Table 6.2). From the relationship for optimal N:P ratios as shown in Fig. 6.6, it is clear that the algae would be severely P-limited at high growth rates and that generally relatively low growth rates would be found when these nutrient solutions are used. The Bold's Basal and the Zarrouck solutions have N:P ratios of 4:1 and 6:1, which according to Fig. 6.6 suggests that these nutrient solutions are N-limited.

6.7 Physical factors influencing nutrient uptake

Nutrient uptake depends on all factors that influence algal growth such as light, temperature, and turbulence. The uptake rate of nutrients will track the light dependant growth rate as empirically established for the photosynthetic-irradiance (P/I) relationship (Harris, 1978). During the light dependant photosynthesis, nutrient uptake will depend on the available light energy, while at saturating light intensities, the nutrient uptake will be constant.

Temperature influences the so-called temperature coefficient (Q_{10}) where enzymatic reaction approximately doubles with a 10°C temperature rise (Goldman & Carpenter, 1974). For the mass cultivation of algae this would mean an approximate doubling in the uptake of nutrients with every 10°C increase in culture temperature. Predictive models that have been developed for algal growth in large outdoor cultures use both the influence of light availability and temperature on algal productivity (Grobbelaar *et al.*, 1990). This implies a synergism between temperature and light energy, which again will affect nutrient uptake.

Fauchot *et al.* (2000) found that UV-B radiation had an influence on the nitrogen utilisation by phytoplankton. They found that exclusion of UV-B generally increased the NO_3^- , NH_4^+ and urea uptake rates. Particularly urea was very sensitive to UV-B radiation where its progressive exclusion increased the urea uptake from 17 to 130%.

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7 Algal Nutrition

Heterotrophic Carbon Nutrition*

Yuan-Kun Lee

Some microalgae assimilate and thus utilise organic carbon and energy sources for growth in the dark (examples are listed in Table 7.1). The heterotrophic culture mode circumvents technical and physiological difficulties associated with the supply and distribution of light and carbon dioxide involved in the photosynthetic mode of cultivation. It offers the possibility of increasing cell concentration and productivity. The operating cell concentration of about 0.5 g l^{-1} is widely reported in open pond photosynthetic cultures (Richmond *et al.*, 1990), whereas cell concentrations of above 45 g l^{-1} (Gladue & Maxey, 1994) have been reported in heterotrophic cultures of microalgae.

7.1 Uptake of organic C-substrates

Hexoses are sterically incapable of diffusing across plasma membrane passively (Fiechter *et al.*, 1981). *Chlorella* cells possess an inducible active transport system of glucose, which could be monitored by the preferential synthesis of cytoplasmic membrane-bound protein and a significant increase in glucose uptake activity (Tanner, 1969; Haass & Tanner, 1974; Fenzl *et al.*, 1977). With glucose as inducer, the minimum time needed to induce the synthesis of glucose transport protein is 15 min and the process is energy dependent (Haass & Tanner, 1974). Thus, an energy starved cell could not be induced to take up glucose in the dark despite the presence of glucose in the culture medium. The transport protein is in the 30–40 kDa range (Sauer, 1986). Uptake of glucose is accompanied by a proton uptake (proton/hexose symport) in a 1:1 stoichiometry and a depolarisation of the membrane potential (Komor & Tanner, 1978).

Amino acids, such as arginine (Berger & Happel, 1974), are actively transported across cell membranes involving periplasmic binding proteins. The small molecular size of organic acids (acetic acid, lactic acid) and alcohol (ethanol) allow their diffusion into the cells. The free acids are more lipid soluble and less negatively charged than the ionised form, hence reduction in pH will favour penetration of the acids into the cell.

*See also Chapter 31.

Table 7.1. Commercially important microalgal species that may be cultured heterotrophically.

Microalga	Organic carbon substrate	References
<i>Brachiomonas submarina</i>	Acetate	Tsavalos & Day (1994)
<i>Chlamydomonas reinhardtii</i>	Acetate	Chen & Johns (1996)
<i>Chlorella pyrenoidosa</i>	Acetate, glucose, lactate, glutamate	(Gladue & Maxey, 1994; Running <i>et al.</i> , 1994)
<i>C. regularis</i>	Acetate, glucose, ethanol	Endo <i>et al.</i> (1977)
<i>C. saccharophila</i>	Glucose	Tan & Johns (1991)
<i>C. sorokiniana</i>	Glucose	Chen & Johns (1991)
<i>C. vulgaris</i>	Acetate, glucose, lactate, glutamate	Gladue & Maxey (1994)
<i>Cryptothecodium cohnii</i>	Glucose	Kyle & Gladue (1991)
<i>Dunaliella salina</i>	Acetate, lactate, glucose, glutamate	Gladue & Maxey (1994)
<i>D. tertiolecta</i>	Glucose	Gladue & Maxey (1994)
<i>Euglena gracilis</i>	Acetate, glucose, alanine, aspartate, asparagine, glutamine, ethanol	(Cook & Heinrich, 1965; Ogbonna <i>et al.</i> , 1998)
<i>Haematococcus pluvialis</i>	Acetate, asparagine	Kobayashi <i>et al.</i> (1997)
<i>Nannochloropsis oculata</i>	Glucose	Gladue & Maxey (1994)
<i>Nitzschia alba</i>	Acetate, glucose, glutamate	(Lewin & Lewin, 1967; Barclay <i>et al.</i> , 1994)
<i>Poteriochromonas malhamensis</i>	Glucose, yeast extract	Gladue (1991)
<i>Scenedesmus acutus</i>	Glucose	Ogawa & Aiba (1981)
<i>Tetraselmis chuii</i>	Glucose	Gladue & Maxey (1994)
<i>T. suecica</i>	Acetate, glucose, glutamate, lactate	(Gladue & Maxey, 1994; Day <i>et al.</i> , 1991)
<i>T. tetrathele</i>	Acetate, glucose, glutamate, lactate	Gladue & Maxey (1994)
<i>T. verrucosa</i>	Glucose	Gladue & Maxey (1994)

7.2 Growth and productivity

It is widely observed that the maximum specific growth rate of algae cultured heterotrophically on simple sugars and organic acids is lower than that typical of photosynthetic cultures (Table 7.2). Most cyanobacteria are not able to assimilate, or assimilate at very slow rates, organic carbon substrates in the dark (Smith *et al.*, 1976). In the case of *Spirulina platensis*, the maximum specific growth rate of heterotrophic (glucose-fed) culture was a third of that measured in photosynthetic cultures. This implies that to achieve the same product output rate, cell concentration of a heterotrophic *Spirulina* culture must be at least three times higher than that of a photosynthetic culture. A reason for the slower maximum specific growth rate in heterotrophic algal cultures concerns the low affinity for these organic carbon substrates. High organic substrate concentrations, however, would result in substrate inhibition of growth.

Table 7.2. Comparison of the maximum specific growth rate of some of the commercially important microalgae cultured photosynthetically, heterotrophically and mixotrophically.

Species	Maximum specific growth rate (h^{-1})			References
	Photosynthetic	Heterotrophic	Mixotrophic	
<i>Chlorella pyrenoidosa</i>	0.082	0.038 (glucose)	–	Droop (1974)
<i>C. vulgaris</i>	0.110	0.098 (glucose)	0.198 (glucose)	Ogawa & Aiba (1981)
	0.081	0.050 (glucose)	0.131 (glucose)	Martinez & Orus (1991)
<i>Haematococcus pluvialis</i>	–	0.014 (acetate)	0.037 (acetate)	Droop (1955)
	0.013	0.009 (acetate)	0.024 (acetate)	Kobayashi <i>et al.</i> (1992)
<i>Scenedesmus acutus</i>	0.061	0.040 (glucose)	0.048 (glucose)	Ogawa & Aiba (1981)
<i>Spirulina platensis</i>	0.028	0.008 (glucose)	0.026 (glucose)	Chen <i>et al.</i> (1996) and Marquez <i>et al.</i> (1995)

The heterotrophic mode of cultivation may not be appropriate for most microalgae maintained in culture collection centres. These algae were usually isolated and maintained under photosynthetic conditions, and may not possess the facility to assimilate and metabolise organic carbon substrates efficiently. It is necessary to screen and isolate microalgae in the presence of organic carbon substrates and from sources which are rich in organic materials (Martinez & Orus, 1991; Barclay *et al.*, 1994). *Chlorella vulgaris* is one of the exceptions, in that the photosynthetic and heterotrophic maximum specific growth rates are comparable.

7.3 Culture systems and cost of production

Commercial cultivation of *Chlorella* heterotrophically in conventional stirred tank fermentors is common (Lee, 1997). Cell concentration of 45 g l^{-1} and volumetric productivity of $20 \text{ g l}^{-1} \text{ d}^{-1}$ were achieved in *Nitzschia alba* in a conventional stirred tank fermentor (Gladue & Maxey, 1994). The cost was estimated at $\text{US\$12 kg}^{-1}$ dry biomass, due to the high cost of equipment and a relatively low productivity compared with yeast cultivation, where biomass productivity could be maintained at $450\text{--}680 \text{ g l}^{-1} \text{ d}^{-1}$ (Atkinson & Mavituna, 1983). The cost of production for baker's yeast is about $\text{US\$1 kg}^{-1}$ dry weight.

In heterotrophic cultures, techniques which facilitate high cell concentrations such as the fed-batch, chemostat and perfusion methods can be applied to achieve a higher concentration of biomass or to overcome the inhibitory effect of substrate or products. Growth of *Chlamydomonas reinhardtii* was inhibited when acetate exceeded 0.4 g l^{-1} , but the inhibitory effect could be overcome by a fed-batch strategy in which the residual acetate concentration in the culture was maintained at below the inhibitory concentration, resulting in a higher cell concentration (Chen & Johns, 1996).

7.4 Mixotrophy

Mixotrophy is broadly defined as a growth regime in which CO_2 and organic carbon are simultaneously assimilated, both respiratory and photosynthetic

Table 7.3. Commercially important microalgae which could be cultured mixotrophically.

Microalgae	Organic carbon substrate	References
<i>Anabaena variabilis</i>	Fructose, glucose	Pearce & Carr (1969) and Valiente <i>et al.</i> (1992)
<i>Brachiomonas submarina</i>	Acetate	Tsavalos & Day (1994)
<i>Chlorella minutissima</i>	Methanol	Kotzabasis <i>et al.</i> (1999)
<i>C. regularis</i>	Acetate	Endo <i>et al.</i> (1977)
<i>C. sorokiniana</i>	Glucose	Lee <i>et al.</i> (1996)
<i>C. vulgaris</i>	Glucose	Ogawa & Aiba (1981)
<i>Chlorococcum</i> sp.	Acetate, fructose, glucose, maltose, manose	Non-published data
<i>Cyclotella cryptica</i>	Glycerol, glucose	Wood <i>et al.</i> (1999)
<i>Euglena gracilis</i>	Glucose, glycerol, galactose, ethanol	Tanim & Tsumura (1989)
<i>Haematococcus pluvialis</i>	Acetate	Kobayashi <i>et al.</i> (1992)
<i>Nannochloropsis CCAP879/5</i>	Glycerol, glucose	Wood <i>et al.</i> (1999)
<i>Nannochloropsis CCAP211/78</i>	Glycerol	Wood <i>et al.</i> (1999)
<i>Navicula saprophila</i>	Acetate	Kitano <i>et al.</i> (1997)
<i>Nitzschia</i> sp.	Acetate	Kitano <i>et al.</i> (1997)
<i>Phaeodactylum tricornutum</i>	Glycerol	Garcia <i>et al.</i> (2000)
<i>Rhodomonas reticulata</i>	Glycerol, glucose	Wood <i>et al.</i> (1999)
<i>R. salina</i>	Acetate	Kitano <i>et al.</i> (1997)
<i>Scenedesmus acutus</i>	Glucose	Ogawa & Aiba (1981)
<i>S. obliquus</i>	Methanol	Kotzabasis <i>et al.</i> (1999)
<i>Spirulina platensis</i>	Glucose	Chen & Zhang (1997) and Marquez <i>et al.</i> (1993)

metabolism operating concurrently. The specific growth rate of the mixotrophic culture is, therefore, approximately the sum of the specific growth rates of cells grown under photoautotrophic and heterotrophic conditions (Table 7.3). Advantages of heterotrophy, such as high cell concentration and productivity, are applicable to mixotrophy. Microalgae expend energy and resources in synthesis of the photosynthetic apparatus, hence the clear advantage of using this unique property of algae and using light as an energy source. Moreover, light is the inducer and regulator for the production of some algal products such as pigments (Chen & Zhang, 1997; Lee & Zhang, 1999) and fatty acids (Cohen, 1999; Sukenik, 1999). Examples of microalgal species that could be cultured mixotrophically and the suitable organic C-substrates are listed in Table 7.3.

7.4.1 Effect of light on organic C-substrate metabolism

In heterotrophically grown *Scenedesmus acutus* and *Spirulina platensis*, growth is slow on glucose alone. In the presence of light, however, the algae were able to utilise glucose more efficiently (Ogawa & Aiba, 1981; Marquez *et al.*, 1995; Chen *et al.*, 1996), perhaps light facilitating improved assimilation of the sugar. Martinez & Orus (1991) reported that the specific growth rate of mixotrophic *Chlorella* increased with increasing light intensity. A similar effect was not observed in photoautotrophic cultures exposed to

the same light intensities. This was explained by the stimulatory effect of light on metabolism of sugar by mixotrophic cells.

The growth yield and productivity of mixotrophic *Chlorella* sp. grown in glucose was found to be greatest when compared to heterotrophic and photoautotrophic cells (Lalucat *et al.*, 1984; Lee *et al.*, 1989, 1996). Lalucat and his team did an extensive bioenergetic analysis and found that there was a significant decrease in the fraction of light energy used for CO₂-fixation by mixotrophic cells. Most of the light energy was instead used as an energy source for carbon assimilation. They concluded that mixotrophy resulted in high energetic efficiency since the amount of energy dissipated was minimal. The energy–carbon ratio of *Chlorella* biomass is about 138 kcal mol⁻¹ C (Pirt *et al.*, 1980), whereas the energy–carbon ratio of glucose is 114.3 kcal mol⁻¹ C. There is not enough energy in the organic C-substrates to support total conversion of carbon molecules in the substrate to algal biomass. Utilisation of light energy for the metabolism of glucose or re-fixation of CO₂ into glucose through photosynthesis using light energy has also been observed in outdoor mixotrophic *Chlorella* cultures (Lee *et al.*, 1996). It was concluded that mixotrophic growth utilising both organic C-substrate and light is the more efficient process for producing algal biomass.

7.4.2 Photosynthesis and respiration

Since mixotrophy represents the use of light as energy coupled with a simultaneous assimilation of CO₂ and organic carbon as carbon sources, a natural question is how would photosynthesis and respiration be affected by mixotrophy? In a mixotrophic culture, both Martinez & Orus (1991) and Valiente *et al.* (1992) reported a decrease in cell affinity for the inorganic carbon substrates in *Chlorella* cultures. Though mixotrophy decreased the affinity for CO₂, photosynthetic efficiency was increased by almost sevenfold (Lalucat *et al.*, 1984). As it was also reported that exogenous organic carbon enhanced respiration both in light and dark (Martinez & Orus, 1991; Valiente *et al.*, 1992), one would anticipate enhanced respiration to result in a large amount of CO₂ evolutions, the CO₂ evolution from mixotrophic cells was found, however, to be low. The anomalous situation of low affinity for CO₂, high photosynthetic and respiratory activities and low CO₂ evolution could probably be explained by the photosynthetic re-utilisation of respiratory CO₂ in *Chlorella* cultures. Exogenous CO₂ appeared to stimulate photosynthesis, CO₂ acting as an additional source of inorganic carbon for fixation. Re-utilisation of CO₂ was also observed in blue-green algae where CO₂ evolved by respiration was immediately refixed in light without being liberated (Scherer & Boger, 1982).

7.4.3 Effect of light on glucose uptake

Glucose uptake in mixotrophic cultures of some strains of *Chlorella vulgaris* was inhibited by light (Haass & Tanner, 1974; Lalucat *et al.*, 1984; Kamiya & Kowallik, 1987). Unlike other photosynthesising eukaryotes, however, photoinhibition did not affect endogenous respiration. Light intensity as

low as 5 W m^{-2} inhibits the synthesis of glucose transporter at the translation level and causes a rapid degradation of the glucose transporter on the cell membrane. This light-mediated regulatory mechanism ensures that *Chlorella* cells can perform photosynthesis only in the presence of light. Other strains of *Chlorella* and other microalgal species, however, are able to undergo mixotrophic growth (Table 7.2). Photoinhibition of the glucose uptake system appears to be strain-dependent. Only those algal strains which are not sensitive to photoinhibition of organic C-substrate-uptake are suitable for mixotrophic cultivation.

7.4.4 Culture system and cost of production

Any sterilisable photobioreactor could be used for mixotrophic cultivation of microalgae. In an outdoor horizontal tubular photobioreactor (10 l volume, land surface area of 1.0 m^2), area productivity of a *Chlorella sorokiniana* culture was $127 \text{ g-biomass m}^{-2} \text{ d}^{-1}$ during the day (mixotrophically using glucose and CO_2) and $79 \text{ g-biomass m}^{-2} \text{ d}^{-1}$ during the night (heterotrophic growing on glucose). The biomass productivity of photosynthetic *Chlorella* culture operating at a comparable cell concentration ($4\text{--}5 \text{ g l}^{-1}$) was about $40 \text{ g-biomass m}^{-2} \text{ d}^{-1}$ (Lee & Low, 1992) and there was no biomass production at night. Assuming that the cost for photosynthetic production of *Chlorella* biomass is $\text{US\$15 kg}^{-1}$, the cost for the production of additional $166 \text{ g-biomass m}^{-2} \text{ d}^{-1}$ ($127 + 79 - 40 = 166 \text{ g-biomass m}^{-2} \text{ d}^{-1}$) is the cost of the culture medium, as there are no additional capital investment, operational and harvesting costs. The biomass growth yield of glucose for heterotrophic *Chlorella* culture is $0.35 \text{ g-biomass g}^{-1}$ glucose, and $0.56 \text{ g-biomass g}^{-1}$ for mixotrophic culture. Assuming that the cost of glucose and associated mineral nutrients is $\text{US\$0.5 kg}^{-1}$ of glucose, the production cost for the additional 166 g-biomass is $\text{US\$0.15}$ or $\text{US\$0.89 kg}^{-1}$ biomass. Accordingly, the overall cost for mixotrophic production of *Chlorella* biomass in a tubular photobioreactor would be ca. $\text{US\$3.6 kg}^{-1}$ biomass.

In reality, it is difficult to sterilise a photobioreactor of a large surface area to volume ratio (Lee, 2001). A compromise is necessary to have a bioreactor which has a sufficiently large illuminated surface to support optimal mixotrophic growth and could be effectively thermo-sterilised by steam.

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8 Biological Principles of Mass Cultivation

Amos Richmond

8.1 Light: the major factor in growth and productivity

The central issue involved in mass cultivation of photoautotrophic microalgae concerns effective use of strong light for photosynthetic productivity of cell mass and secondary metabolites. This is particularly true for mass cultivation of microalgae outdoors, in which effective use of solar energy is a foundation stone on which the prospects for this biotechnology rest.

Light energy received by photoautotrophic microorganisms is a function of the photon flux density (PFD) reaching culture surface. The cells absorb only a fraction of the photon flux, the actual size of which is governed by several factors, including cell density, the optical properties of the cells, length of the optical path of the reactor and rate of culture mixing. Photons which are not absorbed by the cells' photosynthetic reaction centers dissipate mostly as heat or may be reflected. As a rule, microalgal mass cultures reflect only a small or very small fraction of the photons impinging on a culture surface, light reflectance of a *Spirulina* cell suspension in the range of 400–670 nm was reported to be less than 2% (Iehana, 1987).

Since essentially all photons of a high flux density may be captured by high cell density cultures, cell density will continue to increase exponentially until all photosynthetically available photons are absorbed. Once this cell density is reached, cell mass accumulates at a constant, linear rate until light per cell or some substrate in the culture medium becomes overly low, or alternatively, some inhibitory activity or conditions arrest cell growth.

In the light-limited linear growth phase, the relationship between biomass output rate and light energy absorbed by the culture (I_oA) can be expressed as follows (Pirt *et al.*, 1980):

$$I_oA = \mu X V / Y$$

where A = irradiated culture area, μ = specific growth rate, X = biomass density, V = total culture volume, Y = bioenergetic growth yield (see Chapter 2).

This relationship implies that the biomass output rate in continuous cultures (μX) is determined by area-volume relationships (A/V), and in order to obtain high cell densities it is mandatory to use a reactor of high A/V ratio. Finally, if the value of Y for a particular microalga is a constant, the specific

growth rate (μ) can be altered by adjusting X without changing any other culture parameters (Lee & Tan, 1988). It may be desirable to regulate μ to encourage formation of desirable products (see Chapter 5) e.g. *Porphyridium cruentum* produces more arachidonic acid and eicosapentaenoic acid (EPA) at high growth rates, while it accumulates more polysaccharides at low growth rates (Lee & Richmond, 1998).

Maximal culture productivity may be obtained only when culture nutritional requirements are satisfied and temperature is about optimal. There exists, indeed, a strong interaction between light and temperature, well illustrated in the study of Collins & Boylen (1982) who investigated the physiological response of *Anabaena variabilis* (Cyanobacteria) to instantaneous exposure to various combinations of light intensity and temperature. As observed for *Chlorella* (Sorokin & Krauss, 1962) as well as for other algae, increasing temperature affected an increase in saturating light intensity for photosynthesis. For each temperature there was a specific light intensity at which the maximum photosynthetic rate (P_{\max}) was reached. At the lowest light intensity tested ($42 \mu\text{m}^{-2} \text{s}^{-1}$) for example, P_{\max} was achieved at 15°C . At this low light level, high temperatures drastically decreased photosynthetic rate. At higher light intensities the photosynthetic rate increased with an increase in temperature and at high, ca. optimal temperature, the photosynthetic rate increased with increasing light intensities. An elementary aspect of the interaction of light and temperature thus revealed is that the optimal temperature for photosynthesis increases with increasing light intensities.

As a rule, growth of phototrophic mass cultures should be limited by light only. Efficient utilization of strong light by the individual cells in the culture is associated, however, with many constraints: One difficulty rests with the fact that the photosynthetic photon flux density (PPFD) required to saturate the photosynthetic units in the cell is usually 1/5 or 1/10 the PPFD impinging on the culture at midday. Even relatively short exposure of the photosynthetic unit (PSU) or photosynthetic reaction center, to a light dose much above saturation may impair the photosynthetic complex and may reduce productivity. The kinetic response of an algal cell to light intensity is shown in a generalized shape of the curve relating algal growth to the intensity of the light source (Fig. 8.1), provided the light source is strictly the sole limiting factor for growth and cell development. As elucidated by Goldman (1980), the main features of this curve are as follows: At some very low light intensity, the resulting low growth-rate is balanced by decay and the net growth is zero (compensation point). As light becomes more intense, growth is accelerated, the initial slope of the curve representing maximal efficiency of growth in response to light. With a further increase in light intensity, the light saturation function is reached, at which point the growth rate is the maximal attainable, a further increase in light intensity above this point would not result in further increase in growth rate, but may become injurious, manifested initially by decreased growth rate, culminating in photo-damage and in extreme cases, in culture death (see Chapter 2).

If all growth conditions are optimal and the culture is optically thin (i.e. of low cell density), the intensity of the light source is indeed the sole determinant of light availability to the cells and hence the sole factor which controls

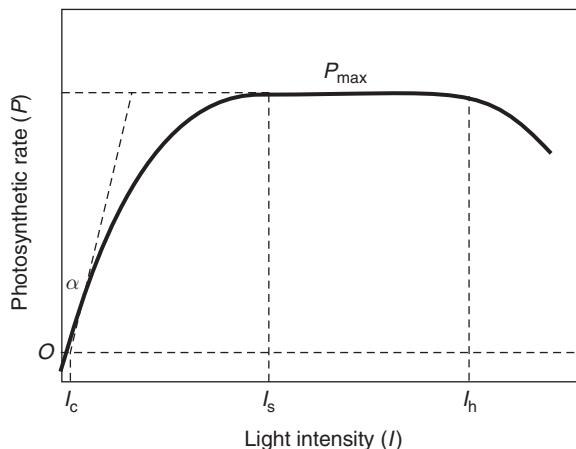


Fig. 8.1. Light-response curve of photosynthesis (P-curve). The intercept on the vertical axis is the measure of O_2 uptake due to dark respiration. I_c , light compensation point; I_s , light saturation intensity; I_h , light intensity value at which photoinhibition occurs.

growth. Under these conditions, the effect of the intensity of the light source on algal photosynthesis and growth is faithfully portrayed by the *light response curve* shown in Fig. 8.1. It is thus essential to note at this point that much misunderstanding concerning the complex effect of light on culture growth has originated from irrelevant application of the *light curve* for interpretation of the growth response of mass cultures, in which cell density, as a rule, is such that *mutual shading* (Tamiya, 1957) may greatly modify light availability for the individual cells. This internal shading (clearly visible in that light does not pass through the culture's optical path, being essentially fully absorbed in the outer surfaces), results in cells receiving light intermittently, a phenomenon augmented by the fact that light energy attenuates exponentially in passing through the culture column. The higher the cell density, the shorter the depth light penetrates into the culture. Two light zones are thereby established in the culture: the outer illuminated volume, in which light is sufficient to support photosynthesis (i.e. the photic zone); and the dark volume, in which net photosynthetic productivity cannot take place, since light intensity is below the compensation point (Fig. 8.1). The higher the population density (and the longer the optical path), the more complex it becomes to address the basic requirements for efficient utilization of strong light, i.e. an even distribution of the available light to all cells in the culture, at an optimal dose per cell (to be elucidated somewhat later).

Clearly then, when mutual shading prevails, cells are not exposed to continuous illumination but rather to cycles of light and darkness (L-D cycle), which may take scores of milliseconds to a few seconds to complete, depending on the optical path and the extent of turbulence in the culture. The endless combinations of light intermittency expressed in L-D cycles to which the individual cells are exposed at a given instant, relate to two basic parameters: first, the ratio between the light and the dark period in the cycle and second, the frequency of the cycle. As shall be elucidated, the higher the

frequency of the L-D cycle, the more efficient strong light may be used for photosynthesis.

It can be readily seen therefore, that the effect of light on photosynthetic productivity (i.e. cell mass produced per illuminated area per time) as depicted in Fig. 8.1 may be misleading or altogether irrelevant, ignoring, in effect, the decisive impact on productivity exerted by other factors which concern the photon flux to which the cells are exposed, i.e. cell density, the length of the optical path and the extent of culture turbulence, all of which represent major determinants effecting phototrophic productivity, not less important than the intensity of the light source. In what follows, these determinants, all having decisive effects on photosynthetic productivity of mass cultures, will be elucidated.

8.2 Cell concentration: a prominent factor of the light-regime of cells in the culture

8.2.1 Areal- and population-densities

In a series of experiments concerning mass cultures of algae, Myers & Graham (1958) discovered that photosynthetic productivity was sensitive to cell concentration (Table 8.1). Manipulating cell concentration of *Chlorella* from 70 to 390 mg l⁻¹, maximal cell mass yield was obtained at a cell concentration of 155 mg l⁻¹, which generated the highest photosynthetic efficiency obtained at an average irradiance per cell of 4700 (measured μ watts cm⁻²), a light dose which was ca. half as high as that available for cells in the smallest cell concentration cultures and ca. twice as high as that available for cells in the highest cell concentration cultures (Table 8.1). It became evident that for a given light source, a certain optimal cell density existed, in which the individual cells were exposed to the optimal irradiance regime. This optimal cell density (OCD) exhibited the highest photosynthetic

Table 8.1. The effect of cell concentration on basic culture parameters (data from Myers & Graham, 1959).

Exp No.	Irradiance k cal day ⁻¹	Cell concentration mg l ⁻¹	Yield mg day ⁻¹	Efficiency* %	Specific** growth rate d ⁻¹	Rate respiration $\mu\text{l O}_2 \text{mg}^{-1} \text{h}^{-1}$	Chlorophyll Content %	Ic*** $\mu\text{watts cm}^{-2}$
1	15.2	71	126	4.5	1.75	9.6	1.7	8800
2	14.4	155	140	5.3	0.90	6.2	3.4	4700
3	14.1	226	101	3.9	0.43	3.3	3.8	3100
4	14.5	390	87	3.2	0.22	2.1	4.4	2000

* Yield $\times 0.0054/\text{Irradiance}$; heat of combustion of cells produced taken as 5.4 cal mg⁻¹.

** Yield/10.1 \times cell concentration.

*** Average irradiance per cell.

efficiency, as reflected in the highest net yield of cell mass per illuminated area. In their experiments, the specific growth rate of continuous cultures (see Chapter 3) at OCD was ca. one half the maximal and four times the minimal growth rates measured in the lowest and highest cell concentrations, respectively. Cell chlorophyll content was also very sensitive to cell concentration. Respiration rate per unit cell mass decreased with increasing cell concentration, but when calculated on the basis of culture volume, respiration rate was rather similar in all cultures, irrespective of cell concentration.

Some 20 years later, very much the same general relationships were reported for *Spirulina platensis* (Richmond & Vonshak, 1978, Fig. 8.2), highlighting two significant aspects related to mass cultures: In contrast with what may be erroneously understood from the *light curve*, any specific growth rate, essentially, may be manifested in response to a given light intensity, depending on cell density. Second, the major parameter in continuous mass cultures at steady state (see Chapter 3), i.e. the yield of cell mass or some specific product per unit reactor volume or reactor area, is not solely a function of the specific growth rate but also of cell concentration. Optically very thin, or in contrast extremely dense cultures, both yield below maximal output rates because, as in any biological phenomenon related to optimal exploitation of resources per unit area, there is a certain optimal stand which results in the highest areal harvest. This *optimal cell density* (OCD) facilitates the most efficient exploitation of the irradiance reaching the culture. In cultures exposed to strong light, the growth rate is highest when mutual shading and thus light-limitation are at the permissible minimum, i.e. sufficient cell mass is available to exert some mutual shading, protecting from photoinhibition (see Chapter 5) that would take place if cell concentration is overly low and cell exposure to light being thus excessive.

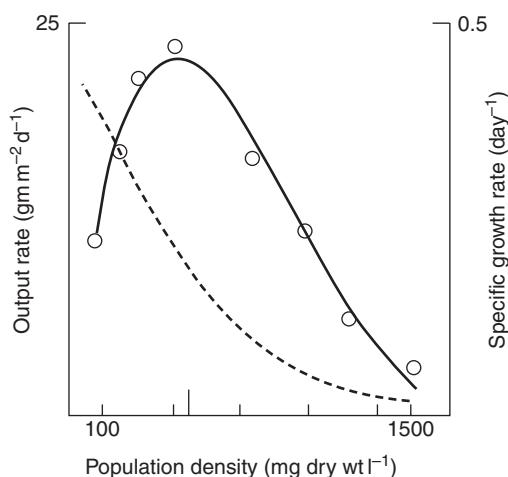
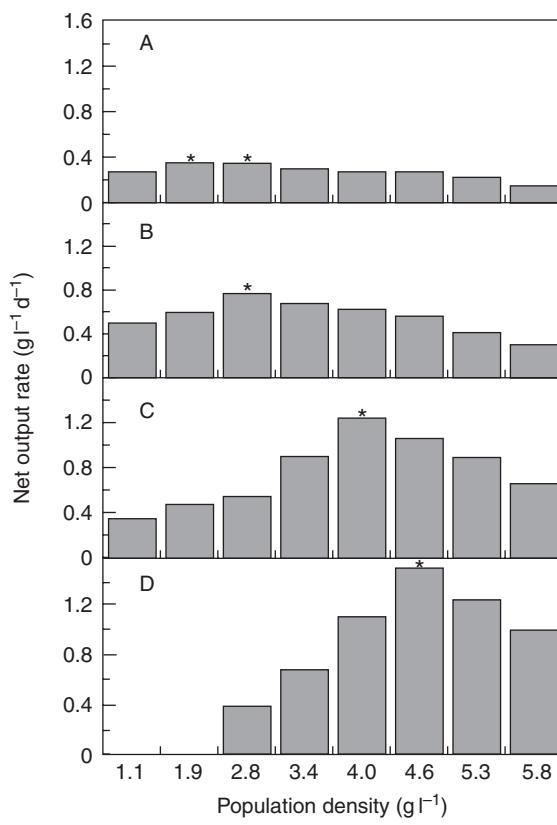


Fig. 8.2. The effect of population density on the specific growth rate (dashed line) and the output rate (continuous line) in outdoor *Spirulina* cultures. Nutrients and temperature are not growth-limiting. Peak solar irradiance – above 2000 $\mu\text{E m}^{-2} \text{ sec}^{-1}$ (after Richmond, 1988a).

Conversely, the growth rate in cultures of extremely high cell densities (e.g. three or four times the OCD) would be much reduced, even in a highly illuminated culture outdoors, due to a severe shortage of light available to the individual cells. The OCD falls somewhere between these extremes, the specific growth rate (Chapter 3) at this point being about one half the maximal attainable.

The output rate (in continuous cultures of *Isochrysis galbana*), as a function of the interaction between the intensity of the light source and the population density, is shown in Fig. 8.3. With increasing light intensity, the OCD shifts from a low of 1.9 g l^{-1} at 10% of full sunshine (A) to the maximal of 4.6 g l^{-1} in full sunlight (D), which yields the highest productivity (Hu & Richmond, 1994). The higher the light intensity to which the culture was exposed, the more pronounced became the dependence of the output rate on the population density, sub-optimal densities resulting in reduced output rates and low densities, culminating in a total loss of the culture within a few hours after transfer to the outdoors (Fig. 8.3D). This is due to the inability to photo adapt quickly enough to excessive light irradiance per cell



*Optimal population density

Fig. 8.3. Interrelationships between incident PFD, optimal population density and net output rate. A = 90% shade; B = 60% shade; C = 30% shade; D = no shade, full sunlight (from Hu & Richmond, 1994). Reprinted with permission from Kluwer Academic Publishers (*J. Appl. Phycol.*).

(see *light-shade adaptation*, Chapter 4). Shading the *Isochrysis* culture provided protection from excessive light, but reduced the productivity, in contrast to Vonshak & Guy (1992) who reported productivity of *Spirulina* being higher for cultures grown outdoors under 15–30% shade. As seen in Fig. 8.3, however, no shading was necessary once the culture became fully photoadapted and the population density of *I. galbana* was optimal. All the evidence obtained from this author's lab indicated full exposure to sunlight always yielding the highest output rate of cell mass, provided the population density was carefully optimized and photoadaptation had taken place. This same effect may be seen by increasing the intensity of the light source and adjusting (increasing) the population density to each added increment of irradiance. Investigating the growth physiology of *Chlorococcum littorale*, Hu *et al.* (1998c) showed the *Optimal Population Density* (OPD) (arrows in Fig. 8.4) rose steadily in response to increasing light intensity, from 120 up to 2000 $\mu\text{mole m}^{-2}\text{s}^{-1}$, yielding at the high photon flux as high as 380 mg dry cell mass $\text{l}^{-1}\text{h}^{-1}$. The direct linear relationships shown between the OCD at increasing light intensities indicated light-use efficiency was constant up to as high as 2000 $\mu\text{mole photons m}^{-2}\text{s}^{-1}$, as long as cell density was elevated to match the increasing intensity of the light source, and not less important, growth-inhibition conditions were removed or corrected daily (e.g. by replacing the growth medium with fresh medium).

The very great sensitivity of the output rate to the cell concentration (mg l^{-1}) is clearly demonstrated in Fig. 8.5 (Hu *et al.*, 1998b). With time, the population density in a 1 cm optical path reactor rose to an extremely high concentration, over 80 gm dry wt l^{-1} , yet as soon as cell density rises

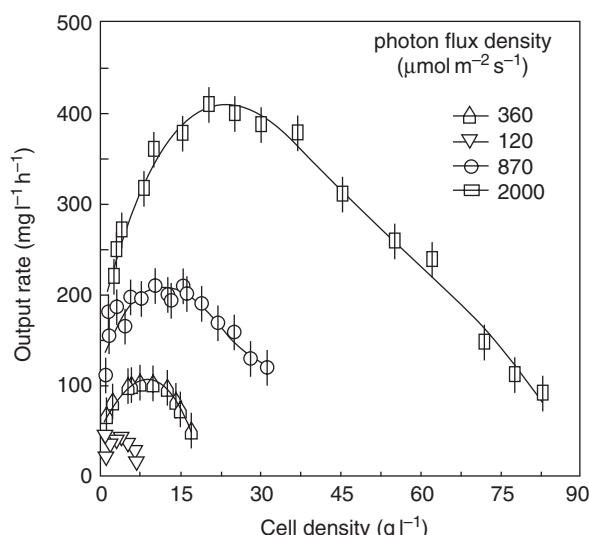


Fig. 8.4. Optimal cell density and maximal productivity of biomass as affected by photon flux density. The cells were grown in 1 cm plate reactor and different rates of incident photon flux density were applied to each side of the reactor. Cell density was adjusted by daily harvesting. Arrows indicate optimal cell densities (from Hu *et al.*, 1998b). Reprinted with permission from Springer-Verlag (*Appl. J. Microbiol. & Biotechnol.*).

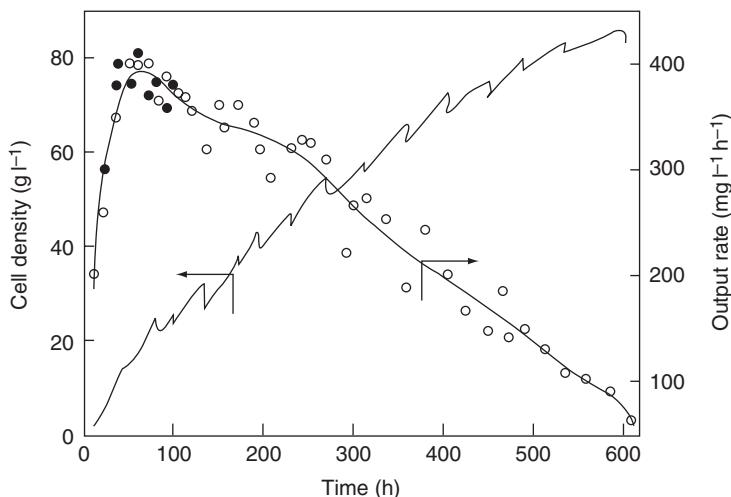


Fig. 8.5. Time courses of cell density (—) and output rate of biomass (○) of *Chlorococcum littorale*, in semi-continuous culture in a 1 cm-light-path plate reactor. The culture vessel was exposed to $2000 \mu\text{mol m}^{-2}\text{s}^{-1}$ light. The output rate obtained under 20% CO_2 concentration (from Hu et al., 1998c). Reprinted with permission from Springer-Verlag (*Appl. J. Microbiol. & Biotechnol.*).

above the optimal of 18 g l^{-1} (or ca. 1/4 of the maximal attainable), the output rate recedes at a linear rate, becoming almost nil at the maximal population density which is so far from the optimal. The fallacy to equate *growth rate* with *productivity* in assessing performance of mass cultures may be readily seen. It is well to note that in some works outdoors, no clear OCD was found, reflecting the fact that the intensity of the light source was not growth limiting. Indeed, any factor affecting growth, e.g. temperature, light per cell, pH, nutrient balance, rate of mixing or cell density, would limit culture growth as soon as it falls too far from its optimal. If therefore, a well-defined OCD is not observed, and the culture is not light limited, growth conditions are not optimal and culture performance falls short of its full potential.

The population density controls the depth light penetrates into the culture (Fig. 8.6). The higher the concentration of cell mass (and chlorophyll), the more energy is trapped and dissipated in the relatively small culture volume closest to the culture surface, impeding light penetration. The photic volume which is thereby demarcated, may be calculated as a relative photic volume, i.e. the ratio of the photic volume over the entire culture volume.

8.2.2 Light penetration depth

A plot of light penetration depth into the culture as a function of both the wavelength and cell density highlights two important aspects involved in mass cultivation (Fig. 8.6). One relates to the spectra of the energy penetrating into the culture. Three ranges of wavelengths are involved: (a) 400–500 nm, the blue region, 440 nm being absorbed maximally by carotenoids and chlorophylls; (b) 500–600 nm, the green light region, which is poorly absorbed by the chlorophyll and carotenoids; and (c) 600–700 nm the red region, which

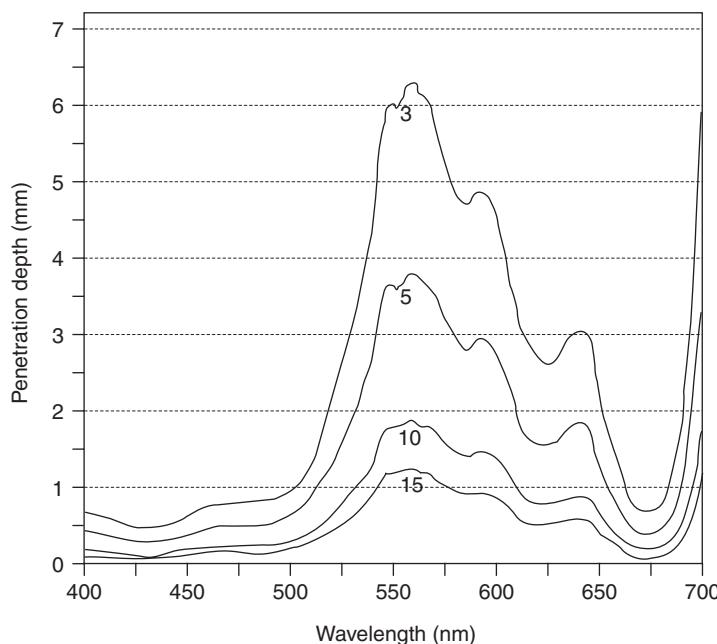


Fig. 8.6. Penetration depth* spectra in *Nannochloropsis* sp. as a function of cell density.

*Penetration depth was defined as the depth in which downwelling irradiance decreased ten-fold numbers (3, 5, 10 & 15) indicate g L^{-1} (from Richmond & Cheng-Wu, 2001). Reprinted with permission from Elsevier Science Ltd (*J. Biotechnol.*).

at 678 nm represents chlorophyll absorption. Clearly, light penetration depth at the blue and red region is very small compared with the poorly absorbed green light, which thus penetrates ca. 20 times deeper into the culture than blue or red light. Green light, therefore, may have an important role in dense algal cultures, in which the cells are strongly light-limited, expanding thereby the photic volume in the reactor (Gitelson *et al.*, 1996, 2000). The other aspect concerns the overall photic volume at optimal cell density, which comprises ca. 5–10% of the overall reactor volume. At any given instant, therefore, most of the cells in continuous mass cultures are out of the photic volume, residing in the dark volume of the reactor. This carries far-reaching ramifications for efficient use of strong light. The *green photic volume* which makes exact demarcation of the photic volume rather complex, is not generally considered in calculations concerning the duration and frequency of the light-dark (L-D) cycle, to which the cells in well-mixed, optically thick cultures are exposed. The quantitative contribution of green light to photosynthetic productivity in light-limited microalgal cultures awaits more research.

8.2.3 Effect of cell density on cellular ultrastructure and composition

This aspect of cell density has not been much researched. One detailed example of this effect is provided by Hu *et al.* (1998c) working on *C. littorale*.

At a cell density of 2–3 g dry cell mass l⁻¹, cell wall was thin ($\sim 0.11\mu\text{m}$), and the chloroplast consisted of a few thylakoid lamellae arranged in single or double rows, with a number of large starch grains. A spherical pyrenoid matrix was separated by single thylakoid lamellae surrounded by two thick starch sheaths. As cell density increased, becoming 10–20 g dry weight l⁻¹, a thicker cell wall was observed and the chloroplast contained many more thylakoid membranes with a high degree of stacking lamellae per chloroplast section. Starch sheaths of the pyrenoid became, in contrast, much thinner, and both the size and the number of starch grains between the lamellae were greatly reduced. Cells maintained at an ultrahigh density of 60–84 gl⁻¹ had even thicker cell walls, the chloroplast being occupied by a large number of expanded starch grains, surrounded with compressed thylakoid membrane lamellae.

Cell composition also varied greatly over a wide range of cell densities. Chlorophylls *a* and *b*, proteins and lipids increased with increasing cell density, reaching maximum at a concentration of ca. 15–20 gl⁻¹, which was the OCD. As cell density increased above OCD, the contents of these components gradually decreased. A high content of carbohydrates was evident in cultures maintained at relatively low cell densities due to accumulation of starch grains in the chloroplast. Carbohydrates decreased to a minimum of 46% dry cell mass as cell density increased to 6 gl⁻¹. A further increase in cell density, however, resulted in carbohydrate accumulation reaching 70% of dry cell mass at the maximal cell density of 84 gl⁻¹ (Hu *et al.*, 1998c). A novel aspect of the relationship between the population density and cell chlorophyll was shown by Hu *et al.* (1996a): The well-known effect of increased cell-chlorophyll associated with increasing cell density was maintained only to a density of 10 gl⁻¹, above which level cell-chlorophyll declined significantly.

8.3 Mixing photoautotrophic cultures

As early as 1954, Phillips & Myers concluded, on the basis of studies concerning growth rate of *Chlorella pyrenoidosa*, that ‘a dense culture growing under sunlight will experience a significant increase in growth if cells are moved in and out of the high light intensity of the front surface at such a rate as to give flash times between 1 and 100 ms’. Some 50 years of experimentation which ensued, bore this observation out. Also, it was clear to these workers that the cultures should be dense enough to ensure that most of the irradiance will be absorbed in the outer 10% of the culture volume, making the dark time to which the cells are exposed about ten times as long as the exposure period to light. A valuable insight into the effect of mixing was demonstrated by Märkl (1980), in which the photosynthetic activity of *Chlorella* cultures of different cell densities was measured as a function of the stirring speed (Fig. 8.7). At the low population density of 0.17 gl⁻¹, essentially no light gradients existed in the culture and mixing had no effect on the photosynthetic rate. As cell densities became higher, however, increasing stirring speed resulted in significantly enhanced photosynthesis. At the highest cell density experimented with, 2.33 gl⁻¹, which produced the

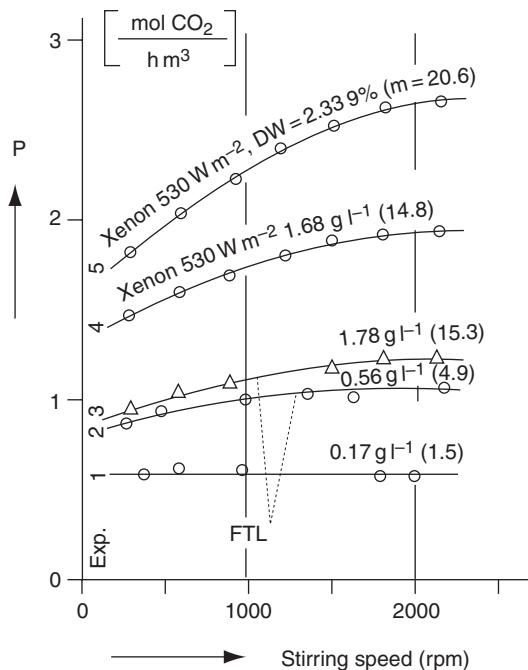


Fig. 8.7. Net photosynthetic reaction rate of *Chlorella vulgaris* at optimal conditions, as influenced by mixing rates (from Märkl, 1980). Reprinted with permission from Elsevier Science Ltd (*Algal Biomass*, Shelef & Soeder (eds) 1980).

highest light gradient in the culture vessel, stirring increased the photosynthetic rate by close to 50% (Fig. 8.7).

The marked effect stirring exerted on areal productivity ($\text{g m}^{-2} \text{ d}^{-1}$) was demonstrated by Richmond & Vonshak (1978) in outdoor *Spirulina* sp. cultures. Three aspects were elucidated: (a) the population density exerted a strong effect on the output rate ($\text{g m}^{-2} \text{ d}^{-1}$); (b) doubling the flow rate in the culture resulted in a 50% increase in productivity and (c) enhancing the flow speed shifted the OCD to a higher level, i.e. from an OCD of 0.22 OD in slow speed to an OCD of 0.28 OD as paddle speed was doubled. This stirring-induced shift in OCD illuminates one aspect of the mode of action involved in stirring-enhanced photosynthetic productivity: Since the output rate of continuous cultures at steady state is a function of both the specific growth rate and cell density [productivity at steady state = cell density \times specific growth rate (Pirt, 1975)], an increase in OCD under given conditions brings about, and in fact reflects, an increase in the output rate. The positive effect that stirring exerts on the output of *Spirulina* sp. cell mass is accentuated as the population density and the extent of light limitation increase. Richmond & Grobbelaar (1986) showed that a relatively slow stirring rate in an open raceway, affecting a flow rate considerably less than 30 cm sec^{-1} , plays havoc on the output rate of cell mass in *Spirulina* cultures as the population density increases far above optimal (Fig. 8.8), a situation which may readily occur in large-scale industrial reactors.

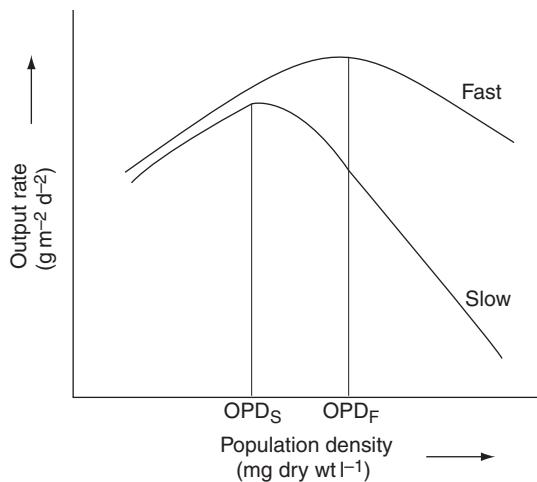


Fig. 8.8. A schematic view of the effect of the stirring rate on culture productivity in relation to the population density (after Richmond & Grobbelaar, 1986).

A basic principle thus unfolds: the higher the intensity of the light source, the higher (potentially) becomes the optimal population density and the more significant the degree to which the extent of mixing may exert on the output rate of cell mass. This has been elucidated in the works of Hu *et al.* (1996a,b) using *Spirulina* cultures in flat plate reactors (Fig. 8.9). The optimal density established at steady state at a PFD of $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ was less than 3 g l^{-1} . As the rate of mixing was increased to $2.11 \text{ air per 1 culture per min}$ (11^{-1} min^{-1}), the OCD shifted up to ca. 5 g l^{-1} and as appropriate, the output rate increased significantly from 70 (at the minimal mixing rate) to $100 \text{ mg dry cell mass l}^{-1} \text{ h}^{-1}$ at the optimal mixing rate. Two aspects concerning the relationship between the output rate and cell density at a low light

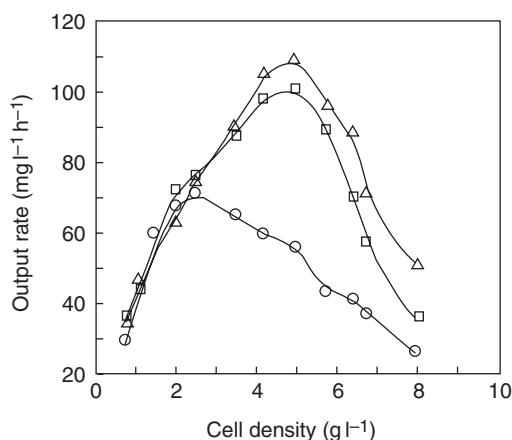


Fig. 8.9. The output rate at PFD of $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ as affected by cell density and rate of mixing in *Spirulina platensis*. Rate of mixing ($\text{L l}^{-1} \text{ min}^{-1}$): $\circ = 0.6$; $\square = 2.1$; $\Delta = 4.2$ (from Hu & Richmond, 1996). Reprinted with permission from Kluwer Academic Publishers (*J. Appl. Phycol.*).

intensity deserve attention: When cell density was relatively low (below 2 g l^{-1}), there was no difference (in output rate) in response to a wide range of mixing rates (i.e. $0.6\text{--}4.2\text{l l}^{-1}\text{ min}^{-1}$) and, increasing the mixing rate above 2.1l l^{-1} had no significant effect on the output rate. Clearly, the magnitude of the effect exerted by mixing was strongly dependent on the PFD as it affected cell density. The highest PFD used, i.e. $1800\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ (Fig. 8.10), is not far from the light flux existing outdoors at noon. The output rates of cell mass obtained at this energy flux indicated a sensitive response to the rate of mixing, an increase in mixing rate from the minimal 0.6 to $4.2\text{l l}^{-1}\text{ min}^{-1}$ resulted in doubling the output rate.

A further increase, to $6.3\text{l l}^{-1}\text{ min}^{-1}$ was harmful, albeit to a lesser extent when compared with the effect of over-mixing in cultures of lower cell density. At the minimally low rate of mixing ($0.6\text{l l}^{-1}\text{ min}^{-1}$), the photosynthetic efficiency (PE) decreased with increasing incident light intensity, i.e. from 9.6 to 7.7 . As the rate of mixing increased up to the optimal (4.2l l^{-1}) however, PE increased concomitantly and, at optimal cell density and mixing rates, a similar PE was obtained despite the fourfold increase in light intensity. Hence indication that when the system is fully optimized, an increase in PFD is not necessarily accompanied with a decline in PE. This is in sharp contrast with the convention, evidenced in many documented observations, denoting that, as a rule, increased irradiance is accompanied with decreased PE (see Chapter 2). The new insight offered here is that this decline in PE would not necessarily take place provided cell density is increased as irradiance increases. It is well to consider, at this point, that these detailed studies concerning the interrelationships between the rate of mixing and culture productivity were carried out with *Spirulina* sp., a long filamentous algae which would readily precipitate if not stirred. Small cells such as *Nannochloropsis* sp. showed a much smaller effect of mixing on productivity. Nevertheless, even with this latter species, very high cell densities (e.g. $20\text{--}30\text{ g l}^{-1}$ and above) could not be maintained without vigorous mixing.

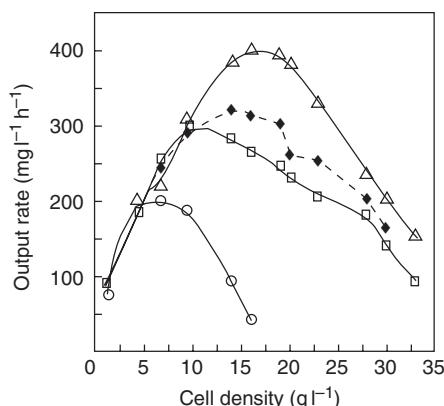


Fig. 8.10. The output rate at PFD of $1800\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ as affected by cell density and rate of mixing in *Spirulina platensis*. Rate of mixing ($\text{L l}^{-1}\text{ min}^{-1}$): $\circ = 0.6$; $\square = 2.1$; $\Delta = 4.2$; $\blacklozenge = 6.3$ (from Hu & Richmond, 1996). Reprinted with permission from Kluwer Academic Publishers (*J. Appl. Phycol.*).

If the forces applied for stirring are too large, hydrodynamic stress is created and reduction in productivity follows (Fig. 8.10), a phenomenon thoroughly investigated by Gudin & Chaumont (1991): Working on mass production of *Porphyridicum cruentum* and other microalgal species, they observed that the rather high rates of mixing necessary to affect a desirable turbulent flow (i.e. Reynolds number larger than 3500), may cause, in certain species, cell damage resulting in greatly reduced output rates. An essential finding was that by changing the method by which to induce flow in their tubular reactor, i.e. from a centrifugal pump to a volumetric pump (e.g. a diaphragm pump), culture productivity was doubled, highlighting the importance of selecting the mode of stirring a culture with careful reference to the physiological state and anatomical characteristics of the cultured species. With such microalgae as e.g. *Dunaliella* sp., which has no rigid cell wall, mixing (involved as a rule with a certain hydrodynamic stress), should be applied with particular caution (Gudin & Chaumont, 1991).

The many reasons mixing of photoautotrophic cultures is of great importance in ensuring efficient use of light and nutrients for photosynthetic productivity may be summarized as follows: A basic requirement for mixing concerns prevention of the microalgal cells from settling, keeping the cells well dispersed in the culture medium. Cell precipitation may be extensive in high-density cultures in which culture streaming is too slow. Settling will be particularly severe in reactor loci in which turbulence is much reduced (e.g. corners or bends). Accumulation of organic matter in *dead* areas of the reactor will affect cell deterioration and anaerobic decomposition, which may bring about culture collapse. Also, fast streaming of the cells interferes with formation of wall growth, which bars light penetration as well as being a harmful source of organic debris. A basic necessity for maintaining high turbulence concerns the nutritional and gaseous gradients formed around the algal cells in the course of their metabolic activity, the restrictions imposed thereby on cell growth are alleviated in high turbulence. Also, high density of actively photosynthesizing cells creates high concentrations of dissolved O₂, which may reach at midday over 400% saturation in slow streaming open raceways and over 600% in enclosed tubular reactors. Vigorous mixing sharply decreases the O₂ tension in actively photosynthesizing open cultures, particularly when mixing is affected by properly designed means.

The major objective for creating a turbulent flow, particularly in high-density cultures, relates to light, which attenuates rapidly in the culture, resulting in a relatively small photic zone in which the cells receive sufficient illumination for photosynthesis. Turbulent flow increases the frequency of shifting cells to and from the photic and dark zones in the reactor. The controversy as to what constitutes the major effect of mixing in a dense photoautotrophic cultures was very much settled by Grobbelaar (1994) who brought experimental evidence to support the thesis that turbulence exerts two major, and complementing effects on phototrophic cell growth: First, facilitating fluctuating, photosynthetically efficient, light regimes and second, decreasing the cells' boundary layers thereby increasing nutrient and gaseous transfer rates between the growth medium and the cultured organism.

8.4 Light–Dark (L–D) cycle frequencies

The fact that photoautotrophic cells may utilize strong light only if exposed to such light intermittently has long been recognized (Burlew, 1953). Two basic approaches for exposing the cells to intermittent illumination are discernable: One method (used in experimentation) entails use of a light source or a system which provides illumination intermittently. This approach may be useful only for low cell densities, in which mutual shading is essentially absent, being useless for mass cultures and whenever high productivity is sought. The second possibility is the only practical one, i.e. use a continuous light source (in the lab or outdoors) and have the cells move at a high frequency, in and out of the illuminated volume. The illuminated cells, which are replaced by *dark* cells, are shifted to the dark volume while these former *dark cells* are in their turn, illuminated. In this fashion more cells (in dense cultures) are exposed to light flashes per unit time. Applied intermittently to the individual cells in the culture, strong light (higher by an order of magnitude than saturating light), is in effect *diluted* by being available in smaller doses to more cells along a given time span, being thus used more effectively, compared with light-use of cells illuminated continuously in low density or poorly stirred cultures. Märkl (1980) provided a detailed, accurate demonstration that an increased mixing rate resulted in higher yields of cell mass. He did not, however, attribute the results to the effect of increased L–D cycle frequencies, which is a form of *light dilution*. The explanation why a higher cell density requires a higher Reynolds number (an indication of the extent of turbulence), in order to obtain maximal productivity is rooted in L–D cycle frequencies: as cell density is increased, efficient use of strong light requires that the frequency of L–D cycles is also increased, which in turn facilitates a higher optimal population density. The increased light yield reflects a more efficient use of light as originally realized by Kok (1953), who summarized his classic experiments on the effects of flashing light on photosynthesis in *Chlorella*, correctly predicting that it should be possible to grow high yields of algae in full sunlight, provided the turbulence and the density of the culture are adjusted to produce the proper pattern of intermittence in illuminating the individual algal cells. In turbulent motion, however, cells are moved at random toward and away from the illuminated volume. As a result, the L–D cycle length and the fractional flash time (i.e. the time fraction in light relevant to the time of the entire cycle) are not unique, but have each a distribution of values. Kok (1953) pointed out that it would be advantageous to avoid this situation and expose all the cells to favorable flash patterns, i.e. fixed at some optimal L–D cycle frequency and some optimal fractional flash time, rather than the random distributions of these quantities when naturally generated in a turbulent flow. Laws *et al.* (1983) adapted this concept and introduced foils into an open raceway to induce orderly patterns of flow, conducive to uniform L–D cycles. The foils generated a more favorable frequency of L–D cycles, and increased the yields of cell mass. Whether, however, the cause for increased productivity was the mere order induced in cell motion, or the shorter L–D cycles that were generated due to the presence of the foils, remains an open question.

8.5 The optical path, a decisive parameter in growth and productivity of photoautotrophic cultures

The most powerful means by which to increase L–D cycle frequency is by reducing the optical path (OP), i.e. reducing the diameter of the tube or the gap between the walls of a flat plate reactor or the height of the water column in open ponds. The effect of the optical path in augmenting photosynthetic productivity was first shown by Hu *et al.* (1996a), who discovered that a radical reduction of the optical path of a flat plate reactor affected a significant increase in areal ($\text{g m}^{-2} \text{ d}^{-1}$) productivity of *Spirulina* grown outdoors (reflecting a more effective use of strong light for photosynthetic productivity), providing the culture medium was replaced daily with fresh growth medium to remove inhibitory substances or correct inhibitory conditions associated with dense cultures (Fig. 8.11).

Reduction of the OP in inclined flat plate reactors facing the sun resulted, as expected, in an increase in the OPD (Table 8.2) and thus in the volumetric yield ($\text{g l}^{-1} \text{ d}^{-1}$). The very significant rise of some 50% in areal yield ($\text{g m}^{-2} \text{ d}^{-1}$), however, obtained by decreasing the OP from 10.4 to 1.3 cm, was surprising, such an effect not having been previously reported (Table 8.2).

Also surprising was the finding that reduction in OP, which was accompanied with ca. tenfold increase in OCD, resulted in an increase (of some 50%) in the specific growth rate, rather than the expected decrease due to increased cell concentration which is associated with lesser light penetration and a greater extent of mutual shading. It was clear that since the culture in the 1.3 cm reactor received the same light-dose as did the 10.4 cm culture, the observed increase in photosynthetic productivity resulted from a significant improvement in the light regime to which the cells were exposed, due to the increased L–D cycle frequency accompanying the extreme shortening of the optical path. Grobbelaar *et al.* (1996) provided strong evidence

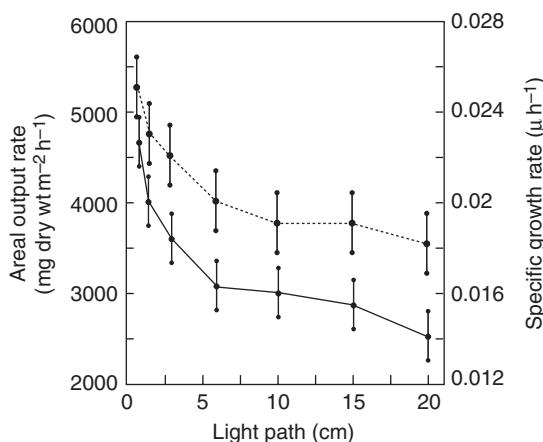


Fig. 8.11. The effect of the length of the light-path on the areal output rate (dashed line) and the specific growth rate (continuous line). Bars represent ± 1 SD in *Spirulina* sp. (from Hu *et al.*, 1998b). Reprinted with permission from Cambridge University Press (*Europ. J. Phycol.*).

Table 8.2. The effect of reducing the optical path from 10.4 to 1.3 cm, on areal productivity* of *Spirulina plantesis* (data from Hu et al., 1996a).

	Reactor volume	Cell density	Specific growth rate				
Light-path	V (L)	X (g l ⁻¹)	μ (h ⁻¹)			Productivity (m ⁻² h ⁻¹)	
10.4 cm	104	×	1.7	×	0.014	=	2.5 g
1.3 cm	12	×	15.8	×	0.021	=	4.0 g

* At steady state, productivity = $V \times X \times \mu$.

to support, in effect, this interpretation, showing the photosynthetic rate (O_2 evolution) in *Scenedesmus* cultures, to increase exponentially with increasing L-D cycle frequency: Low L-D frequency exerted the effect of low light conditions, high L-D frequency exerting the effect of high light. Accordingly, boosting the L-D cycle frequency would reduce the extent of light limitation in dense photoautotrophic cultures, thereby increasing photosynthetic productivity.

A detailed study under controlled lab conditions of the productivity response to a significant reduction of the OP revealed that reducing the OP from 20 to 0.75 cm resulted in essentially the same surge in areal productivity observed outdoors (Table 8.2). The specific growth rate increased hand in hand with the decrease in OP, in spite of the 40-fold increase in cell density. Unlike the conventional observations in which a reduction in the specific growth rate is affected in response to an increase in cell density under a given light source, the very large increase in cell density which follows an extreme reduction in the optical path was accompanied, in contrast, by an increased growth rate, signifying that light utilization, in the strictly light-limited culture, became more efficient as the optical path was much reduced. In what is soon to follow, this effect will be elucidated.

8.6 Ultrahigh cell density cultures

Attention should be paid to a salient feature of the culture-protocol employed with these *Spirulina* cultures: the entire growth medium of each experimented culture was removed daily (by filtering the cell suspension), and replaced with fresh growth medium. This procedure was adapted following preliminary experiments revealing that as the OCD greatly increased accompanying the reduction in OP, such frequent replacement of the growth medium was mandatory to maintain high cell density cultures and obtain maximal growth rates and productivity.

Likewise, a kinetic study of *Nannochloropsis* sp. growth in a 2 cm OP plate reactor revealed that a few days from the start, growth of the culture (in which the growth medium was replaced every 24 h) displayed linear growth for an additional period of 22 days, reaching a cell concentration of 44 g l⁻¹. In contrast, if the growth medium was not replaced and only nutrients added frequently, the culture reached after 16 days a maximal cell concentration of

only 18 g l^{-1} , at which state net growth ceased altogether (Fig. 8.12) (Zou & Richmond, unpublished).

The high cell concentrations maintained with cultures grown in small OP reactors were termed by Hu *et al.* (1996b) *ultrahigh cell densities* (UHCD), arbitrarily defined as cultures of cell densities higher than $10 \text{ g dry cell mass}$, or $150 \text{ mg chlorophyll l}^{-1}$. The prerequisites for establishment of UHCD cultures have been identified as follows:

1. A narrow optical path (i.e. ca. 1–2 cm).
2. A strong light source, i.e. $>2000 \mu\text{mole photons m}^{-2} \text{ s}^{-1}$.
3. Conditions arresting cell growth must be corrected and growth inhibitory substances barred from building up in the culture (this may be addressed by a frequent replacement of the growth medium).
4. Turbulent mixing adjusted to the optimal (i.e. conducive to highest productivity) rate.

The potential of UHCD may be seen in the work of Hu *et al.* (1998a). Aiming to approach the highest photosynthetic productivity possible per irradiated culture area, they used a *Spirulina* sp. culture in flat glass plate reactors, which was gradually exposed to increasing rates of irradiance. At the maximal irradiance used, i.e. $4000 \mu\text{mole photons m}^{-2} \text{ s}^{-1}$ from each side of a 1.4 cm optical path reactor, the optimal population density was ca. $33 \text{ g dry cell l}^{-1}$ (over $300 \text{ mg chlorophyll l}^{-1}$) and the output rate was over $8 \text{ g dry cell mass m}^{-2} \text{ h}^{-1}$. Since the reactor was irradiated from both sides, the irradiation dose was $8000 \mu\text{mole photons per 141}$ (the areal volume per 2 m^2 reactor panels, the sides and top being covered), yielding at this photon flux $1200 \text{ mg dry wt, l}^{-1} \text{ h}^{-1}$ or $8 \text{ g m}^{-2} \text{ h}^{-1}$.

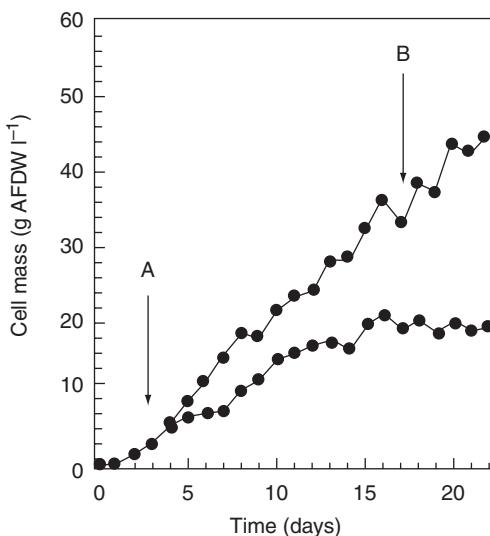


Fig. 8.12. Growth kinetics of *Nannochloropsis* culture as affected by growth-inhibition (from Zou & Richmond, 1999b). Reprinted with permission from Kluwer Academic Publishers (*J. Appl. Phycol.*).

8.6.1 Growth inhibitory substances and conditions

The decisive effect of growth inhibition which unfolds in UHCD is demonstrated in a recent study of Zhang *et al.* (unpublished), who compared the areal productivity of *Nannochloropsis* sp. grown in 1, 3 and 9 cm OP glass plate reactors, in which the entire growth medium was either replaced every 24 h or not replaced. In the later case, growth medium nutrients were added to the culture every 72 h (Table 8.3). If the medium was not replaced and only replenished with nutrients, highest productivity was obtained in the 9 cm OP reactor, lowest productivity being obtained in the 1 cm reactor. If the growth medium was, however, replaced every 24 h, highest areal productivity was obtained in the 1 cm OP reactor, being higher by close to 45% compared with the 9 cm reactor under identical conditions, or higher by over 100% above the productivity obtained in the 9 cm OP, in which growth inhibition was not removed or corrected (Table 8.3). Frequent replacement of the entire growth medium was also mandatory in UHDC of *Chlorococcum litoralle*, cultured in 1 cm OP plate reactors (Fig 8.5), which reached record high cell concentrations of over 80 dry cell mass L^{-1} (Hu *et al.*, 1998c). Similarly, Javanmaradian & Palsson (1991) used an online ultrafiltration unit to exchange spent with fresh medium, which was mandatory to achieve very high cell densities, i.e. up to 10^9 of *Chlorella vulgaris* cells ml^{-1} . What is the nature of the growth inhibitory substances or growth inhibitory conditions, without the removal or correction of which the very large surge in the areal output of cell mass taking place in narrow (e.g. 1 cm) OP reactors could not be expressed?

The presence of algae-inhibitors in culture filtrates of several algal species has been rather extensively reported (Pratt, 1942; Leving, 1945; VonDennffer, 1948; Rice, 1954; Steeman Nielsen, 1955; Jørgensen, 1956; Proctor, 1957a; Lefevre, 1964; Harris, 1971, 1975; Fogg, 1971; Keeting, 1978). Other studies provided evidence for the existence of algal antibiosis *in situ* (Vance, 1965; Proctor, 1957a; Keeting, 1977). Excreted algal metabolites were reported to inhibit their own species' growth, as well as other species': Pratt & Fong (1940) observed growth of *Chlorella vulgaris* depressed by its own product excreted into the culture medium, naming the active substance *chlorellin*. Likewise, Curl & McLeod (1961) reported that dense cultures of

Table 8.3. Effect of removing growth-inhibition in *Nannochloropsis* sp. culture on output rate of cell mass* (from Richmond *et al.* (2003)).

Optical path (OP)cm	Output Rate of Cell-mass ($\text{mg m}^{-2} \text{h}^{-1}$)		
	Growth inhibition (GI)		
1.0	504	2184	+333
3.0	798	1764	+121
9.0	924	1512	+64

* Cultures exposed to a total of $2000 \mu\text{mole photons m}^{-2} \text{s}^{-1}$ from both sides of the reactors. Temperature and pH are optimal. Reprinted with permission from Blackwell Scientific Publications.

Skeletonema costatum may inhibit their own growth. Proctor (1957b) demonstrated a substance produced by *Chlamydomonas reinhardtii* toxic to *Haematococcus pluvialis*, a condensate from a boiling acidified *Chlamydomonas* medium also inhibiting growth of *Haematococcus*. McCracken *et al.* (1980) assayed antialgal substances in the culture medium of *Chlamydomonas reinhardtii*, and identified the active substances as linoleic and linolenic acids. Kakisawa *et al.* purified a substance with antialgal activity from the brown alga *Cladophora okamuranus* and identified as 6Z, 9Z, 12Z, 15Z-octadecatetraenoic acid (ODTAC C_{18:4}). Imada *et al.* (1991, 1992) identified an inhibitor, 15(s)-hydroxyeicosapentaenoic acid, probably an oxygenated metabolite of EPA, in cultures of *Skeletonema costatum*.

An essential feature of growth inhibition was that growth inhibitory activity was most pronounced in cultures of high cell densities (Lee & Palsson, 1994; Richmond, 2000; Zou *et al.*, 2000). In order to achieve a high density highly productive algal culture, the inhibitory activity has to be continuously removed by ultrafiltration of the growth medium or by a complete, daily change of culture medium with fresh growth medium. Quantitative effects of the inhibitory activity were determined by Zhang, C.-W. & Richmond, A. (unpublished) who developed a bio-assay for testing the supernatant of 20-day-old stationary phase culture of *Nannochloropsis* (Richmond *et al.*, 2003). Bio-assayed filtrates from this culture revealed considerable inhibitory activity (Fig. 8.13), which increased sharply as cell concentration of *Nannochloropsis* sp. culture rose to 3×10^9 cells ml⁻¹, reaching maximal inhibition as cell concentration began to decline at the late stationary phase (Fig. 8.14) (Zhang, C.-W. & Richmond, A., unpublished).

Despite the substantial evidence supporting the existence of autoinhibitors being formed in high cell density cultures of photoautotrophic microalgae, this phenomenon is far from being well understood. The possibility that artifacts are often involved or that the inhibitory activity results from unusual critical conditions associated with high cell densities rather than occurring commonly in ultrahigh cell density cultures, cannot be ruled out: Scutt (1964) concluded that growth of *Chlorella* was not inhibited by the production of an autoinhibitor and that despite the many reports to the contrary, inhibitor

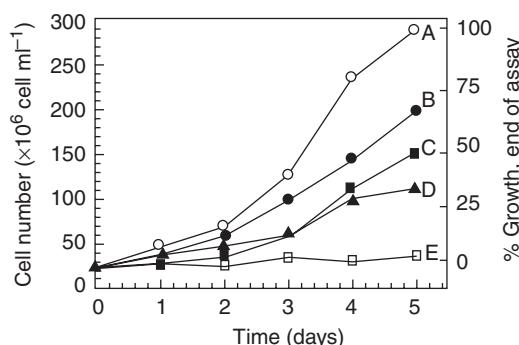


Fig. 8.13. Bioassay of the inhibitory activity in filtrates of *Nannochloropsis* cultures at the advanced stationary phase (Zhang, C.-W. & Richmond, A., unpublished).

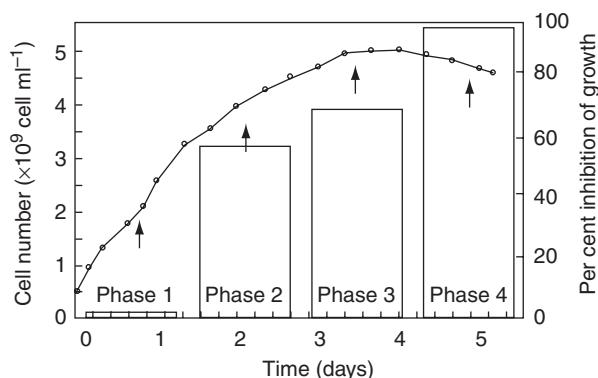


Fig. 8.14. Relative inhibitory activity in *Nannochloropsis* culture filtrates along four phases of the growth curve (Zhang, C.-W. & Richmond, A., unpublished).

production is not a general phenomenon with *Chlorella*. Similarly, Blanchemain *et al.* (1994) suggested that the degeneration of *S. costatum* cultures, observed by Imada *et al.* (1991) who identified the growth inhibitor as hydroxy EPA, could have been an artifact, associated with the Tris buffer. Nevertheless, Blanchemain *et al.* (1994) concluded there was an accumulation of a termolabile inhibitory material in their *S. costatum* culture. Javanmaradian & Palsson (1991) first reported the appearance of a factor blocking cell division, which had to be continuously removed for ultrahigh (i.e. $2 \times 10^9 \text{ cells ml}^{-1}$) culture densities of *Chlorella vulgaris* to be established and sustained. In a later work, however, Mandalam & Palsson (1995) dismissed the existence of a specific autoinhibitor and in a later study, Mandalam & Palsson (1998) suggested the inhibition of cell division in high cell density cultures was due to an imbalance of nutrients: N-8 medium, commonly used for culturing *C. vulgaris* in their experiments was evaluated for its capacity to support high density cultures based on the elemental stoichiometric composition of *C. vulgaris*. Their analysis indicated that N-8 medium becomes deficient in iron, magnesium, sulfur, and nitrogen, in high cell density cultures, arresting growth as cell density reaches a certain high-level. The medium was redesigned (M-9) to contain stoichiometrically balanced quantities of the four deficient elements to support a high biomass concentration of 2% (v/v). Replacing N-8 medium with the M-9 medium resulted in up to three- to fivefold increase in total chlorophyll content per culture volume. Addition of each of the four elements separately to the N-8 medium did not improve culture performance; the balanced supplementation of all four deficient elements was mandatory to yield the enhanced performance. Long-term (24 d) *C. vulgaris* culture in M-9 medium showed continuous increase in chlorophyll content and biomass throughout the period of cultivation, whereas the increase in chlorophyll content and biomass in N-8 medium under the same conditions ceased after 7–12 days. This work naturally raises the possibility that the performance of high cell density cultures can be significantly enhanced by proper design of the growth medium required for sustaining very high cell densities. Nevertheless, Zou & Richmond

(1999a) could not erase the growth-inhibitory activity of *old growth medium* by adding the full nutrient formula into the culture. Likewise, Hu *et al.* (1998b) reported *Spirulina platensis* cultures grown in a narrow (i.e. 1–2 cm) optical path and exposed to $4000 \mu\text{E m}^{-2} \text{s}^{-1}$ would reach well over 50 g dry weight per liter culture only if the entire culture medium was replaced daily, simply adding the full nutrient medium formula without replacing the growth medium exerted no significant effect on growth.

In summary, considerable evidence indicates that sustaining ultrahigh density cultures (UHDC) and harvesting the high photosynthetic productivity obtainable in such cultures requires relief from growth inhibitory substances and/or provide carefully balanced nutrient media necessary for sustaining cultures of very high cell densities. To become economically feasible, therefore, UHDC would require practical protocols with which to prevent the buildup of inhibitory activity or conditions, which arrest cell growth.

8.6.2 Areal density in relation to the optical path (OP)

The areal cell density (cells m^{-2}) played an important role in affecting culture response to reduction of the OP: In clear contrast with *Spirulina* culture, in which the highest yields were obtained at the shortest OP (providing daily replacement of the growth medium with fresh medium took place), in several outdoor experiments using flat plate reactors with microalgal species other than *Spirulina* (e.g. *Nannochloropsis* sp., *Porphyridium* sp.), in which culture medium was not replaced along a continuous culture period of several days, the optimal optical path (yielding the highest output rate of cell mass outdoors) fell in the range of 20 cm. In contrast with the *Spirulina* experiments, shorter OP reactors always yielded significantly lower outputs (Fig. 8.15). In Table 8.4, this relationship is further illuminated. Increasing the optical path from 5 to 20 or 30 cm, results in decreasing population density (due to increased areal volumes, i.e. 1m^{-2}), but the areal density steadily increases, resulting in increasing areal productivity. This is an indication that in this system the increase in population density accompanying reduction in the

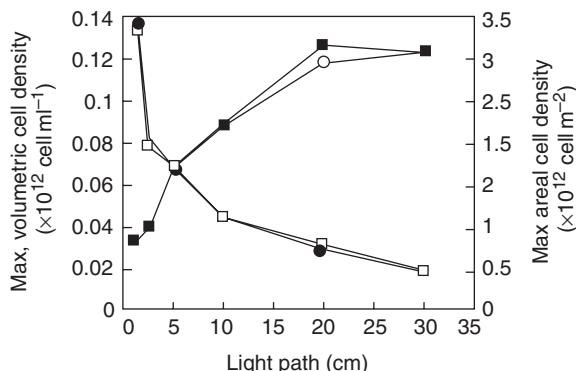


Fig. 8.15. Effect of light path length on maximal volumetric and areal cell density. Winter, volumetric (●); summer, volumetric (○); winter, areal (■); summer, areal (□). Reprinted with permission from Kluwer Academic Publishers (*J. Appl. Phycol.*).

Table 8.4. The effect of the optical path on light cell $^{-1}$ in *Isochrysis galbana* grown in vertical plate reactors, in summer (data from Zhang, C.-W. & Richmond, A., unpublished).

The OP: light path length (cm)	5	10	20	30
Areal volume (l m $^{-2}$)	25	50	100	150
Optimal cell density ($\times 10^{-9}$) (cells m $^{-2}$)	44.3	36.5	27.8	19.3
Optimal areal* cell density ($\times 10^{-12}$) (cells m $^{-2}$)	1.11	1.83	2.78	2.90
Light cell $^{-1}$ (10^{-6})* ^{**} (cal cell $^{-1}$ d $^{-1}$)	3.49	2.11	1.39	1.33

* Total area (front and back panels) of illuminated surfaces.

** Assuming a daily average of 4.5 kWh m $^{-2}$ d $^{-1}$ (3870 k cal m $^{-2}$ d $^{-1}$), falling on the overall illuminated surfaces of the reactors (Prof. David Faiman, personal communication).

optical path is associated with growth inhibition which, not having been removed or controlled, results in decreased areal densities and areal productivity. How can these differences in cultures' response to a reduction in the OP be resolved? Table 8.5 describes three distinct culture types: Types A and C, in which there is no growth inhibition either because cell density is relatively low, thus without inhibitory conditions (C), or because growth inhibition was removed (A). From the other type culture (B), conditions or materials which arrest cell growth, have not been removed or corrected. In Type B culture, reduction of the OP was accompanied with a significant reduction of areal density (cells m $^{-2}$ reactor area), resulting in increased average irradiance or irradiance per cell (l cell $^{-1}$, Table 8.4). This indicated the existence of inhibitory factors or conditions, which must have evolved as the population density (cells l $^{-1}$) became high in response to the reduction in OP. The failure to maintain the areal density as the OP was reduced resulted in impairment of the light regime for the average cell, in that cells were exposed to excess irradiance which was either damaging or simply used ineffectively, and hence the reduction in areal output rates of cell mass. When no inhibitory conditions existed in the culture (Type A), reduction in the OP did not involve reduction in areal density which remained constant. As found for *Spirulina*, the significant surge in areal output rate obtained as the OP was shortened and inhibitory conditions removed may be manifested only when the OP is reduced to an extreme, i.e. ca. 1.0 cm OP. Indeed, reduction of the OP from 100 to 10 cm (e.g. *Isochrysis galbana*, Table 8.5) was not accompanied by an increase in photosynthetic productivity. This phenomenon will be elucidated in Table 8.5.

8.7 Reaction timescales in photosynthesis, in relation to the effect of the optical path on culture productivity

The discovery of the enhancing effect, a very narrow (ca. 1.0 cm) optical path exerts on photosynthetic productivity in strong light (providing growth inhibition is controlled), illuminates some basic features involved in effective use of strong light, on which the foundation of mass cultivation of photo-autotrophic microorganisms rests. A number of parameters are involved, e.g. photosynthetic timescales, cell travel (in the reactor) timescales, long as well as short optical paths and finally the average irradiance in the reactor, which

Table 8.5. The effect of a drastic reduction in the optical path (OP) on the output rate of cell mass (g m^{-2}) in relation to the areal density and growth inhibition.

Algal species (Laboratory) (Outdoors)	Culture type	Inhibitory activity or conditions	Optical path (OP) (cm)	Optimal population density (OPD) (g l^{-1}) or ($\text{cells} \times 10^{10} \text{l}^{-1}$)	Optimal areal density (g m^{-2}) or ($\text{cells} \times 10^{12} \text{m}^{-2}$)	Areal output rate ($\text{g m}^{-2} \text{h}^{-1}$) or ($\text{g m}^{-2} \text{d}^{-1}$)	References
<i>Nannochloropsis</i> (laboratory)	A	Continuously removed	9.0 1.0	3.9 (g l^{-1}) 34.5 1.7 (g l^{-1}) 15.8	351.0 (g m^{-2}) 345.0	1.42 ($\text{g m}^{-2} \text{h}^{-1}$) 2.15	Richmond et al. (2003)
<i>Spirulina</i> (outdoors)	A	Continuously removed	10.4 1.3		177.0 (g m^{-2}) 205.0	33.0 ($\text{g m}^{-2} \text{d}^{-1}$) 51.3	Hu et al. (1996a)
<i>Nannochloropsis</i> (laboratory)	B	Present, not removed	9.0 1.0	2.4 (g l^{-1}) 12.1	216.0 (g m^{-2}) 121.0	0.9 ($\text{g l}^{-1} \text{h}^{-1}$) 0.5	Richmond et al. (2003)
<i>Isochrysis</i> (outdoors)	B	Present, not removed	20.0 5.0	2.8 ($\times 10^{10}$) 4.4	2.78 ($\times 10^{12}$) 1.11	13.0 ($\text{g m}^{-2} \text{d}^{-1}$) 4.0	Zhang & Richmond (2003)
<i>Isochrysis galbana</i> (outdoors)	C	Not present (low cell density)	100.0 10.0	0.05 (g l^{-1}) 0.5	~ 50.0 (g m^{-2}) ~ 45.0 (g m^{-2})	9.5 g carbon ($\text{g m}^{-2} \text{d}^{-1}$) 9.5 g carbon ($\text{g m}^{-2} \text{d}^{-1}$)	Sukenik et al. (1991)

A = Areal density not changing (or becoming higher), whereas areal productivity greatly increasing when OP is reduced to ca. 1 cm.

B = Areal density and productivity falling drastically when OP is greatly reduced.

C = Areal density and productivity not changing when OP is greatly reduced.

is proportional to irradiance per cell ($I \text{ cell}^{-1}$). These parameters shall be now elucidated, Prof Yair Zarmi having contributed the mathematical aspects and the conceptual analysis involved in the following relationships.

8.7.1 Reaction timescales in photosynthesis

Two timescales are involved in the photosynthetic reaction, the light reaction time, τ_1 (of the order of ns to μs), and the dark reaction time, τ_d (of the order of 1–15 ms). The first timescale is so short that the light reaction may be viewed as instantaneous. Hence, the photosynthetic unit (PSU), or photosynthetic reaction center turnover time (Dubinsky *et al.*, 1986; Dubinsky, 1992) is, for all practical purposes, equal to the dark reaction time.

8.7.2 Cell travel-times between the lit and dark volumes in the reactor

The cell travel-time is the average time required for cells to move back and forth between the lit and dark parts of the reactor. Cell motion in the reactor stirred by air is a consequence of the turbulent motion of the fluid, which is induced by the passage of air bubbles. In the absence of information regarding the hydrodynamical parameters characterizing this turbulent flow, it is not possible to estimate the average cell travel-time. A possible estimate is provided by considering two extreme scenarios for cell motion, the actual mode expected to lie between these two extremes. One extreme scenario, which, if prevailing, solely, would yield the shortest possible cell travel-times, is that of regular motion; cells moving back and forth with a lateral velocity, v , of the order of the bubble velocity. Travel-time would then simply be (L/v) , L being the optical path.

The other extreme scenario for cell motion is that of random, diffusion-like motion of the cells along with the turbulent eddies generated by the air bubbles, corresponding to cell travel-times that are much greater than in regular motion. The average cell travel-time will then be given by $(L^2/(2D))$, where D (in units of $\text{cm}^2 \text{ s}^{-1}$) is the diffusion coefficient (Berg, 1972). For turbulent fluid motion, D is given by $(1/2)lu_0$, where l is the characteristic eddy size and u_0 is its typical velocity (Okubo, 1980). Assuming $l = 0.2 \text{ cm}$ (typical size of air bubbles) and u_0 of the order of the velocity of the air bubbles, one finds that $D = 3\text{--}5 \text{ cm}^2 \text{ s}^{-1}$. Expecting the actual cell motion to be some combined effect of regular and random motion, these rough estimates indicate that the real L–D cycle time for an optical path of 6 cm lies between 200 and 6000 ms, for the orderly and random cell motion, respectively. For an OP of 1 cm, L–D cycle times range between 33 and 167 ms for orderly and random motion, respectively. As the optical path is reduced to an extreme of 0.375 cm, L–D cycle times of orderly and random motion would converge, being 13 and 23 ms, respectively. This set of data, which is based on an assumed PSU turnover time of 10 ms, a photic volume of 5% and a fluid velocity of 30 cm s^{-1} , indicates that cell travel-time begins to represent a relevant parameter to photosynthetic productivity when the optical path is reduced to ca. 1.0 cm or below.

8.7.3 Long optical paths

In reactors with long optical paths (above 4–5 cm), cell travel-times are appreciably greater than the timescales of the complete photosynthetic reaction or the PSU turnover. In this case, timescales should be altogether ignored, because the time spans cells spend in both the photic and dark zone of the reactor along an L–D cycle are orders of magnitude longer than photosynthetic reaction time. Culture productivity in this case depends on the intensity of the light source, as well as on the exponential decline of radiation intensity (modulated by cell density) across the optical path. A simple model that takes these factors into account shows that under these circumstances, the volumetric productivity (e.g. $\text{gL}^{-1} \text{h}^{-1}$) is inversely proportional to the light path, L :

$$\rho_{\text{opt}} \propto \frac{1}{L}$$

As a result, the areal density, which equals ρL , is constant, namely, should not change as the length of the optical path is manipulated. This has indeed been observed in experiments in which the optical path was not reduced to less than 5 cm (see Table 8.5).

8.7.4 Short optical paths

When the optical path is appreciably shorter than ca. 5 cm, travel times begin to approach the turnover of the photosynthetic unit (Dubinsky, 1992). At this increasing proximity, obtainable in reactors with short optical paths and high cell density, only a small fraction of the cells at any given instant is exposed to irradiance sufficient for photosynthesis. This fraction comprises 5–7% of a reactor's volume, if radiation is applied on one side of the reactor. If all sides are equally illuminated, the illuminated volume fraction is of the order of 10–15%, rest of the cells residing in the dark zone (or volume), exposed to radiation levels that are close to, or below, the compensation point. Ideally, cells in the photic zone should be optimally exposed to light for the extremely short duration required for the light reaction, then be removed into the dark zone, replaced by cells from the dark zone, receptive to incoming photons. (Turbulent streaming is mandatory to this mode of events.) Upon being moved into the dark zone, the cells should optimally be returned into the illuminated zone when the dark reaction is complete and the photosynthetic units being, once again, receptive to photons. It is, however, practically impossible to move cells mechanically over a distance of the order of a few nm (the photic zone for a reactor with a 1 cm optical path is ca. 0.1 cm), within a few ns to a few μs . Thus, cells in mass cultures are always exposed to wasteful dark periods, and it is therefore that the length of the dark period in the L–D cycle may be practically targeted for improving the light regime for increased photosynthetic productivity. It is because the timescale of the PSU-photosynthetic reaction center-turnover rate (equal to the dark reaction time) and the

timescale of cell travel across the reactor walls (the L–D cycle) assume values which approach the same order of magnitude in high cell-density short optical path reactors, that the photosynthetic efficiency of the culture is augmented as observed (Hu *et al.*, 1998a,b). In other words, the significant increase in photosynthetic productivity observed in 1.0 and 0.75 cm reactors holding UHDC from which growth-inhibitory factors have been removed (Fig. 8.11), reflects a reduction in the wasteful residence time cells spend in the dark volume of the reactor. Indeed, since the mixing rate cannot be increased without limit, reduction of the optical path represents the only practical mode by which to make the travel time through the dark zone as close as possible to τ_d . The shorter the optical path, the closer becomes the synchronization between the travel-time in the dark zone and the PSU turnover time. This is portrayed in Fig. 8.16, showing that not before the OP is reduced to ca. 1 cm, may a marked reduction in the wasteful dark fraction of the L–D cycle take place, and clarifying why a reduction of OP in the range of long optical paths, e.g. 20–10 or even to 5 cm (Fig. 8.11) is not accompanied with a meaningful decrease in the wasteful dark residence, as well as increase in areal production.

To get an idea of the lateral velocity with which the cells ought to move so that travel time is equal to, or close to τ_d (1–15 ms), consider a reactor with a 1 cm optical path, in which a typical thickness of the photic zone is of the order of 1 mm. On average, a cell would have to move 9–10 mm in about 10 ms. This corresponds to a lateral velocity of about $90\text{--}100\text{ cm s}^{-1}$. Thus the velocities presently used in photobioreactors are, at best, about 1/3–1/2 of that required to significantly reduce wasteful residence time in the dark volume, once the OP has been sufficiently shortened. Too high velocities, it should be remembered, are harmful to many species. In summary: providing inhibitory factors or conditions are controlled or eliminated and cell density and mixing rates are carefully optimized for the intensity of the light source and the OP, areal productivity is expected to greatly increase as the optical path is reduced below ca. 2 cm.

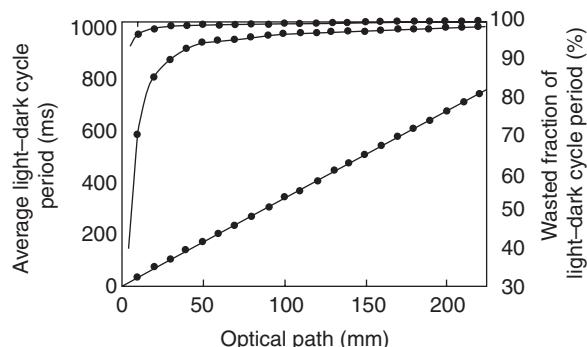


Fig. 8.16. Calculated average light-dark cycle period (ms), assuming cell-flow speed of 30 cm/s. Percentage of wasted dark time is computed for PSU turnover rate of 1 ms – uppermost curve and 10 ms – lower curve.

8.7.5 Radiation dependence of the PSU – photosynthetic reaction center – turnover time

Experiments in which the cells were allowed to get acclimated to a given radiation intensity showed the dark reaction time to vary with radiation intensity: it was of the order of 1 ms for high radiation intensities and up to about 15 ms for low intensities (Dubinsky, 1992). In mass cultures, radiation intensity falls off exponentially along the optical path and since proper mixing ensures that all cells move back and forth across the optical path, all the cells are exposed to radiation levels which vary much more rapidly than the time required for acclimation. Despite the variability in radiation intensity across the optical path, all the cells have the same dark reaction time corresponding to acclimation under exposure to the average radiation intensity in the culture (proportional to the light dose available per cell; $I \text{cell}^{-1}$). The latter parameter is the most basic component of the light regime essential for analysis of culture productivity, as shall be elucidated in the following sections.

8.8 The average radiation intensity

The parameter describing the average light intensity inside a reactor, from which the average irradiance available for the individual cells in the culture may be estimated, was elucidated by Molina-Grima *et al.* (1995, 1999) and Acién-Fernández *et al.* (1997). This is a most basic and useful concept for understanding the effect of light on microalgae grown in photobioreactors: Since light penetration is greatly restricted due to high cell density, only the average irradiance, which is proportional to light cell^{-1} (rather than the irradiance falling on the illuminated culture area), provides a meaningful quantity by which to interpret culture response to high irradiance.

The radiation intensity falls off exponentially across the optical path and if the radiation intensity at the edge of the illuminated side of the reactor is denoted by I_0 , then the intensity at a distance x from the illuminated side of flat plate reactors is given by

$$I(x) = I_0 e^{-\mu x}$$

Here μ is the absorption coefficient (if x is measured in cm, then μ has units of cm^{-1}). As shown by Zarmi (2002), the average radiation is given by averaging the value of $I(x)$ over the whole optical path ($0 \leq x \leq L$):

$$I_{\text{av}} = \frac{1}{L} \int_0^L I(x) dx = \frac{I_0}{L} \int_0^L e^{-\mu x} dx = I_0 \frac{1 - e^{-\mu L}}{\mu L}$$

For very low densities, the absorption coefficient is small, hence $\mu L \ll 1$, and the last expression may be approximated by

$$I_{\text{av}} \cong I_0 \left(1 - \frac{1}{2} \mu L\right) \quad (\text{for low cell densities})$$

For very high densities the absorption coefficient is large, hence $\mu L \gg 1$, and the exponential in the expression for the average radiation intensity is so small, that it may be neglected, yielding

$$I_{av} \cong \frac{I_0}{\mu L} \quad (\text{for high cell densities})$$

In a crudest approximation, the absorption coefficient varies linearly with culture density:

$$\mu = \alpha \rho$$

where ρ is the culture density (measured, say, in g L^{-1}), and α is a proportionality coefficient, which has to be determined empirically. In very high culture densities, μ may begin to have a nonlinear dependence on density:

$$\mu = \alpha \rho + \beta \rho^2$$

Here β is another parameter, which has also to be determined empirically.

To obtain a rough idea of how the average radiation varies with culture density, we use the linear approximation ($\mu = \alpha \rho$). The expression for the average radiation intensity is then

$$I_{av} = I_0 \frac{1 - e^{-\alpha \rho L}}{\alpha \rho L}$$

Note that in the last expression, I_{av} depends on the areal density (ρL).

As long as the average radiation remains fairly constant as irradiance increases, the culture is responding well to increased irradiance. As soon as $I \text{ cell}^{-1}$ rises sharply, however, the cells are exposed to excessive irradiance. It is well to note that even at relatively low areal densities, the average radiation intensity, I_{av} , is only a small fraction of the incoming intensity, I_0 (Gitelson *et al.*, 2000). Consider, for instance, an areal density of 10 g m^{-2} . For a reactor with (optical path) $L = 1 \text{ cm}$, this corresponds to a volumetric density of 1 g L^{-1} . If the incoming irradiance is $2000 \mu\text{mole m}^{-2} \text{ s}^{-1}$, the value of ($I_0 = I_{av}/I_0$) is low, about 0.083 and the dark reaction time (i.e. PSU turnover rate) is expected accordingly to be rather long (estimated to be of the order of 10 ms).

In conclusion: two basic factors must be satisfied for obtaining maximal PE and productivity in strong photon irradiance:

1. Areal cell density must be both as high as possible and optimal (cell growth inhibition having been checked), insuring the average photon irradiance available per cell is falling at the end of the linear phase of the curve-relating rate of photosynthesis to photon irradiance per cell (PI_{av} curve).
2. The dark period, to which the PSU is exposed in the course of the random travel of cells between the dark and the light volumes along the optical

path, should be as short as practically feasible, as close as possible to the PSU turnover rate.

This is obtainable by greatly reducing the optical path and augmenting cell density.

8.9 Effective use of sunlight and high irradiance for photosynthetic productivity

The biotechnology for production of photoautotrophic microalgae is based on the premise that strong light (e.g. sunlight) may be used effectively for photosynthetic productivity. Indeed, one reason this biotechnology has not been progressing quickly is rooted in the lack of success in achieving this basic goal. To be cost-effective, culturing microalgae outdoors should, as a rule, reap high rates of photosynthetic productivity, which should well compare with the most productive higher plants. Since solar energy, however, strikes the earth at very different angles and intensities along the diurnal as well as annual cycles, effective use of solar irradiance, which involves great many considerations, represents admittedly a formidable technological challenge.

Goldman (1979) summarized the relevant characteristics of sunlight: Solar energy reaches the outer atmosphere at a rather constant intensity of $1.94 \text{ cal cm}^{-2} \text{ min}^{-1}$, which dissipates considerably by the time it reaches the earth's surface due to various factors, e.g. water vapor, CO_2 , dust particles and cloud cover. The angle at which the sun's rays strike the earth influences the magnitude of energy losses and determines the radiant energy incident to the ground surface. On a monthly basis, peak total sunlight intensities vary from $700 \text{ cal cm}^{-2} \text{ d}^{-1}$ during the spring and fall equinoxes at the equator, to $780 \text{ cal cm}^{-2} \text{ d}^{-1}$ at latitude 50, in June (Fig. 8.17).

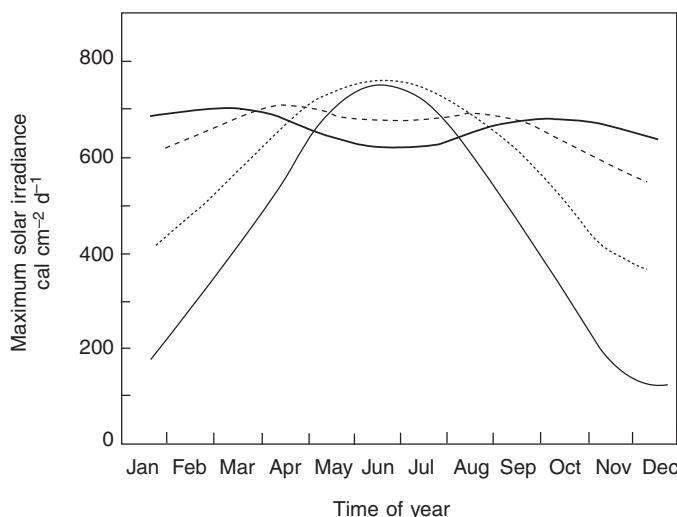


Fig. 8.17. Maximum expected total solar irradiance as a function of latitude and month of year. Data originally compiled from US Weather Bureau records. Reprinted with permission from Elsevier Science Ltd (*J. Biotechnol.*).

When a photoautotrophic culture is exposed, under controlled conditions, to a given irradiance and the population density is kept constant, the culture will yield constant productivity (cell mass per unit area per time). This ensues since the size and capacity of the light absorbing mechanism, the antenna complexes, have adapted to the PFD by modifying the antenna pigment content and size (see Chapter 2). The pattern of response to the changing irradiance outdoors, however, is much more complex.

8.9.1 Response to changing irradiance outdoors

Photosynthetic cells in outdoor cultures are subjected to diurnal illumination of varying intensity from dawn to dusk and then to complete darkness at night. The photon flux density during the day increases rapidly from almost complete darkness to about $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$ in about 6 h, decreasing thereafter to complete darkness during the latter part of the day, i.e. the photon flux density varies by more than $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ in an hour, along most of the day. The fact that the intensity of solar irradiance during mid day may be ca. one order of magnitude higher than the energy required to saturate the photosynthetic units in the cells of most species, is of particular significance for mass production of phototrophic microalgae outdoors. As already explained, the only practical mode by which to cope with this difficulty is for the cells to be illuminated intermittently by manipulating cell density and thereby mutual shading. In many production facilities, however, the high solar flux at high noon often results in photoinhibition (see Chapters 2 and 4). Indeed, dissolved oxygen may often decline at noon, as has been reported for many species, reflecting inability of the culture to respond effectively to high irradiance as the cells become photoinhibited due to excess light (Vonshak *et al.*, 1988). The work of the Hu *et al.* (1996b) highlights the role of the population density in modifying the ill effects of strong noon irradiance on photodamage and photosynthetic productivity: Three population densities of *Spirulina* sp. were selected for a study designed to test interrelationships between cell density and the extent of photoinhibition along the day: (a) 1.8 g l^{-1} , was much below the optimal cell density (OCD); (b) 8.5 g l^{-1} , which was the OCD and (c) 15.2 g l^{-1} , well above the OCD (Fig. 8.18). The low cell density culture exhibited greatly reduced PS II efficiency in midday, whereas at optimal density only a relatively small reduction in Fv/Fm took place in midday. Cultures in which the population density was ca. twice the OCD, no photo damage was observed. Certainly, if 1 cell^{-1} is too high, damage caused to PS II may result in reduction in culture productivity (Vonshak *et al.*, 1988). High cell concentrations in contrast, which by mutual shading diminish the light dose available per cell (I cell^{-1}), provide effective, albeit not full, protection against the photodamaging potential imbued in strong midday irradiance. This phenomenon was in effect addressed by Acién-Fernández *et al.* (1998) who studied the growth response of a *Porphyridium cruentum* culture in tubular reactors of 3 and 6 cm optical paths. The reactors were run each in three different dilution rates (DR), and therefore differed in population densities, which were inversely related to the dilution rate (Fig. 8.19). The average irradiance (proportional to I cell^{-1}) prevailing inside the reactors

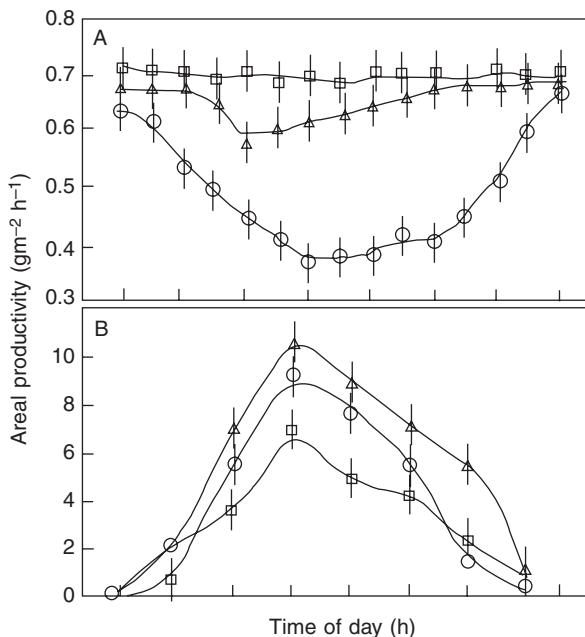


Fig. 8.18. Effect of culture density on (A) F_v/F_m and (B) output rate of biomass in outdoor cultures of *S. platensis* in the flat plate bioreactor during June–July. Cultures were operated on semi-continuous modes. Population density ($g l^{-1}$): $\square = 1.8$; $\Delta = 8.5$; $\circ = 15.2$ (from Hu *et al.*, 1996b). Reprinted with permission from Kluwer Academic Publishers (*J. Phycol.*).

was not affected by the optical path, as long as the population density increased proportionally with the increase in irradiance, the areal density in both the 3 and 6 cm reactors remained identical. In the somewhat faster DR (middle curve) resulting in lower cell density cultures, a clear increase in I_{cell}^{-1} seen in the somewhat sharper slope of the curve, takes place due to the decrease in areal density as irradiance is steadily increased. Nevertheless, both these cultures respond well to increasing irradiance in both reactor diameters, as evidenced in that the areal cell density remains essentially constant. In response to the still fastest DR, cell density has become too low, average irradiance and I_{cell}^{-1} has thus become too high, resulting in slowing down cell growth. Thus as irradiance was further increased in this DR, I_{cell}^{-1} increased, rapidly reaching values four- to fivefold higher than the original present at low light. If these conditions were prolonged, photooxidation would have soon ensued. To save the culture, the DR must be sharply reduced, thereby affecting an increase in cell density which would be accompanied by a reduction in I_{cell}^{-1} . Cell growth would soon be resumed returning to the pattern of a linear response to increasing PFD (Fig. 8.19).

A useful insight concerning the I_{av} was provided in Fuentes *et al.* (1999), who offered quantitative analysis of the daily cyclic variations in culture parameters of *Porphyridium cruentum*: A linear relationship between external irradiance (i.e. sunlight) and the average irradiance I_{av} was evident (Fig. 8.20A). Oxygen generation rate was high at the morning light, the rate

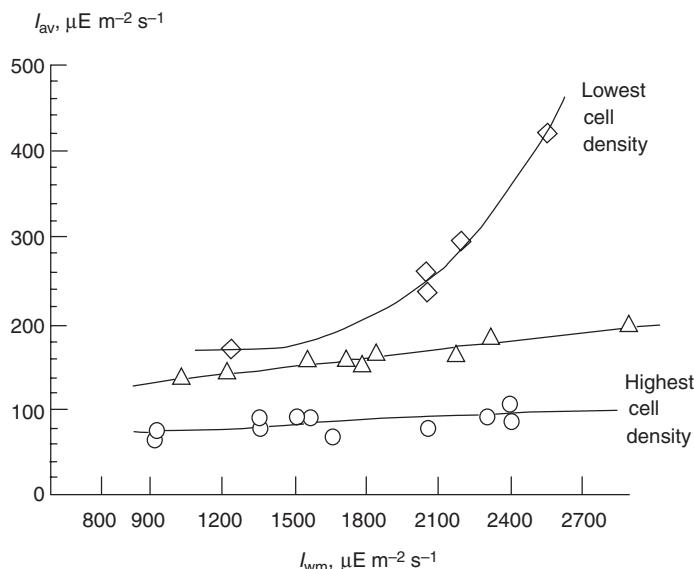


Fig. 8.19. Variation of average irradiance, I_{av} , in the culture with daily mean photosynthetic irradiance inside the pond, I_{wm} , for the various dilution rates and the photobioreactors used.

○	$\phi_c = 0.03 \text{ m}, D = 0.025 \text{ h}^{-1}$
△	$\phi_c = 0.03 \text{ m}, D = 0.040 \text{ h}^{-1}$
◇	$\phi_c = 0.03 \text{ m}, D = 0.050 \text{ h}^{-1}$

(From Fernandez *et al.*, 1998.) Reprinted with permission from John Wiley & Sons Inc. (*Biotechnol. & Bioengin.*).

decreasing as I_{av} becoming higher along the day. Nevertheless, O_2 generation kept increasing steadily with the rising I_{av} (Fig. 8.20C). The information compiled in Fig. 8.20 reveals an important aspect for interpreting the response of outdoor cultures (maintained at optimal cell density) exposed to the strong noon irradiance: Although the photosynthetic rate decreases from its morning high, photosynthetic productivity, as evidenced in Fig. 8.20B, is steadily increasing, responding positively to the strong light. It is possible that at noon, many of the cells of the *Porphyridium cruentum* culture investigated have been photoinhibited (i.e. exhibiting a significant reduction in their photosynthetic potential). This probable reduced potential, however, was more than compensated by the increase in available irradiance. Thus even at noon, when the cells in the culture may have been photo-damaged and lost some of the capacity for photosynthesis, the culture as a whole remained nevertheless light-limited by definition, having responded to increased irradiance with increased carbon fixation. Clearly, a reduction in light-use efficiency by the cells does not necessarily imply a total loss of culture productivity, which in this case continued to grow at midday, albeit at a lower rate. A phenomenon which is often seen is here clearly portrayed: although a high proportion of cells (having been exposed to excess light) may

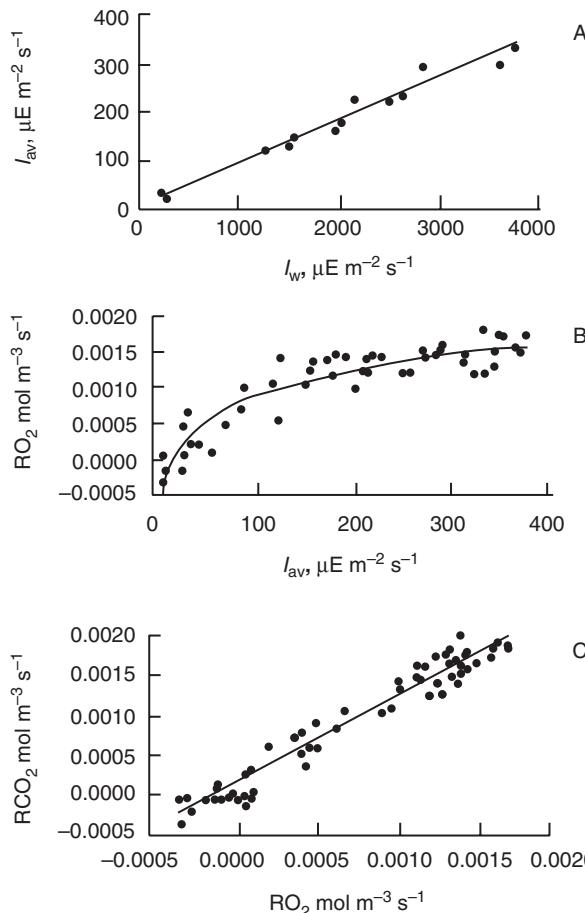


Fig. 8.20. Correlations between major culture parameters along the daily cycle; *P. cruentum* (adapted from Fuentes Rebelloso *et al.*, 1999). (A) Correlation between the average irradiance inside the culture, I_{av} and the external irradiance on the culture surface, I_w during the quasi steady state reached. (B) Estimated variation of oxygen generation rate with the average irradiance inside the culture. (C) Correlation between carbon consumption rate and oxygen generate rate during the quasi steady state.

have been photoinhibited, the culture was nevertheless light-limited, in that it responded to increased I with increased P . The view that these circumstances make the culture both light limited and photoinhibited, as may be interpreted, is incorrect: A culture may be either light-limited, in which case increased irradiance, by definition, would increase productivity or a culture could be photoinhibited, in which case increased irradiance would affect a further decline in photosynthetic productivity. If a culture is exposed to strong light and is yet light-limited, cells may exhibit characteristics of photoinhibition (Chapter 4) which indicate some damage to PS II affecting thereby a decline in the photosynthetic potential. When the latter, however, is not rate limiting, photosynthetic productivity will not be affected. Nevertheless,

outdoor cultures exposed to strong irradiance (e.g. $2500 \mu\text{E m}^{-2} \text{s}^{-1}$) may be readily severely photoinhibited at noon, being light-limited in the morning and on the afternoon. To ease this unfavorable situation, the population density should be maintained at the highest point of the optimal cell concentration range. Under these circumstances therefore, harvesting the culture should be confined to late afternoon or evening.

Indeed, purely from a biological standpoint, it would be correct to modify cell concentration in a reactor along the day, maintaining high cell density, above the daily optimal, along midday hours while maintaining lower cell densities in the morning and afternoon, when irradiance is significantly lower than at midday. There is evidence that the productivity of many outdoor cultures is primarily limited by the inability of the cells to respond swiftly to the rapid increase in light irradiance along the day. Hence the premise that algal cultures could achieve a higher energy conversion efficiency and productivity after having been exposed to a high initial light irradiance. Accordingly, maintenance protocols and reactor designs which allow cells to experience higher irradiance in the early morning (practically may be brought about by evening harvesting) may improve the overall productivity of outdoor algal cultures (Lee & Low, 1991).

Under controlled lab conditions, it is possible to accurately adjust cell density to the intensity of the light source. As already described, and as shown in Fig. 8.4, providing this adjustment is made and care is taken to eliminate conditions or substances which inhibit cell growth, a *Spirulina* sp. culture responds positively, i.e. with increased output rate of cell mass, to each increment of additional irradiance up to $4000 \mu\text{mole m}^{-2} \text{s}^{-1}$ (applied on each side of a flat plate, 1.4 cm OP reactor). The same effect was obtained with *Chlorococcum littorale* grown in 1 cm OP flat plates. The OCD rose steadily in response to increasing light intensity from 120 to $2000 \mu\text{mole m}^{-2} \text{s}^{-1}$ but without any loss in light-use efficiency: A flux of $360 \mu\text{mole m}^{-2} \text{s}^{-1}$ resulted in ca. $50 \text{ mg l}^{-1} \text{ h}^{-1}$, $2000 \mu\text{mole m}^{-2} \text{s}^{-1}$ yielding $400 \text{ mg l}^{-1} \text{ h}^{-1}$ (Fig. 8.4). In stark contrast, Molina-Grima *et al.* (1997) found in their system a steady decrease in quantum efficiency as the PFD rose from 820 to $3270 \mu\text{E m}^{-2} \text{s}^{-1}$. The inability of that system to utilize successfully high irradiance was seen in that each rise in the intensity of the light source and the dilution rate resulted in increased light cell^{-1} (from ca. 6 to 62) and although areal productivity increased initially in response to increased irradiance (i.e. from 821 to $1620 \mu\text{mole photons m}^{-2} \text{s}^{-1}$), it decreased when the culture was exposed to $3270 \mu\text{mole photons m}^{-2} \text{s}^{-1}$ (Table 8.6). In another study, Molina-Grima *et al.* (1995) reported decreased productivity in a chemostat culture of *Isocrysis galbana* in response to elevating the irradiance dose from 1630 to $3270 \mu\text{E m}^{-2} \text{s}^{-1}$. There is no simple explanation for the disagreement between these results and the works of Hu *et al.* (1998a,b). Perhaps only in a flat plate reactor, in which vigorous mixing is readily applied by using compressed air and in which O_2 does not accumulate, is it possible to increase culture density to the extent required (i.e. $10\text{--}30 \text{ g l}^{-1}$) for effective use of strong light. Also, growth inhibition may have curtailed the capacity of the culture to reach the very high areal cell densities required for efficient use of strong light (see Table 8.5). Finally the cultures may not have had sufficient

Table 8.6. Biomass generation as related to bioenergetic parameters, calculated for steady-state conditions obtained in each experiment (data from Molina-Grima *et al.*, 1997).

($\mu\text{E m}^{-2} \text{s}^{-1}$)	D (h^{-1})	P _b ($\text{g l}^{-1} \text{h}^{-1}$)	F _{ab} ($\mu\text{E g}^{-1} \text{s}^{-1}$)	Ψ (%)
820	0.0049	0.0083	6.76	4.22
820	0.0241	0.0166	12.05	7.62
1620	0.0192	0.0251	11.59	6.53
1620	0.0307	0.0250	20.01	5.68
3270	0.0059	0.0183	16.52	1.93
3270	0.0242	0.0165	62.41	1.48

time to photo adapt as light intensity was doubled. This stark difference in cultures' response to strong light deserves a thorough study.

8.9.2 Tilting reactor surfaces in adjustment to the solar angle

Failures of outdoor cultures to respond positively to strong light at midday only accentuate the basic dogma and challenge involved in outdoor cultures. Solar irradiance should be ever accentuated, directed towards the cultures and not evaded, once photoadaptation has taken place. Indeed, for maximal productivity, outdoor cultures must be so maintained and manipulated as to respond with increased productivity to the highest irradiance available outdoors. With this in mind, Pirt *et al.* (1983) described the advantage of placing the photobioreactor at an appropriate angle to the sun to collect maximal solar energy. Tredici *et al.* (1991) devised and patented a vertical alveolar panel (VAP), one advantage of which was seen in its facility for variable orientation with respect to the sun (Tredici & Chini Zittelli, 1998). Lee & Low (1991) in Singapore (Latitude: 2°) however, who reported in detail the effect (on the productivity of *Chlorella* cultures) of inclining the photobioreactor at different angles to the horizontal, concluded that the overall biomass output rates from tubular reactors set at different inclinations were comparable, regardless of the tilt angle. The effect of reactor orientation, it is worth noting, is expected to become more significant as the geographic latitude becomes higher, where the availability of sunlight is lower than at the equator (see Chapter 9).

Seeking to increase the irradiance available to *Spirulina* cultures in flat plate reactors throughout the year, Hu *et al.* (1998a) tilted flat plate reactors toward the sun at various angles (i.e. 90°, 60°, 30° and 10°), facing south (Fig. 8.21). Total irradiance falling on a tilted surface of slope (b) to the horizontal was computed using this algorithm:

$$I(\beta) = B^* \cos 0 + D^*(1 + \cos \beta)/2 + \rho^* G^*(1 - \cos \beta)/2 \quad (8.1)$$

As explained by Prof. David Faiman (personal communication), 0 in equation (8.1) is the angle of incidence to the receiving surface made by the incoming solar ray, ρ is the reflectivity of the ground and D is the diffuse sky radiation – assumed to be isotropically distributed. Equation (8.1) is applicable to the

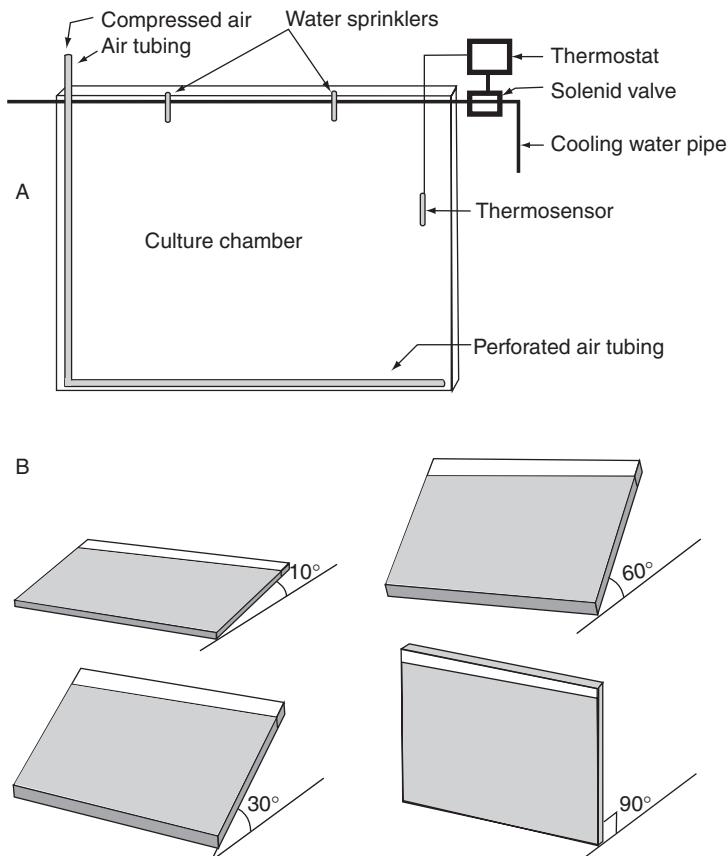


Fig. 8.21. A schematic diagram of the flat plate reactor (A) and its tilt angles (B) (from Hu et al., 1998a). Reprinted with permission from The Society of Bioscience and Bioengineering, Japan (*Optimal Orientation and Inclination of Photobioreactor*).

front surface of the reactors (i.e. for $\beta = 90^\circ$) but not for the rear surface. This is because the ground behind the reactor becomes increasingly shaded from the sun, the closer the reactor is to the horizontal inclination. In order to compensate for this shading, the minus sign in the last term of equation (8.1) was changed to a plus sign for the rear surface calculations. This change makes no difference for a vertical reactor since $\beta = 90^\circ$ but it has the effect of preventing any ground-reflected radiation from entering the rear surface of a reactor lying horizontally on the ground (for which $\beta = 0^\circ$). For the intermediate angles of 60° , 30° and 10° , this sign-change gradually reduces the amount of ground reflected light entering the rear surface of the reactor, according to the increasing amount of rear ground shading.

The reactor tilt angle exerted a significant effect on the optimal population density and thus on the productivity of cell mass of *Spirulina* sp. cultures, owing to its control over the amount of solar radiation entering the reactor. A direct relationship between solar energy and productivity was observed: the

higher the amount of solar energy that was admitted by varying the reactor tilt angle according to season, the higher the productivity that could be sustained in the culture. Small tilt angles of 10°–30° in summer and larger angles, in the vicinity of 60° in winter, resulted in maximal productivities for these seasons (Hu *et al.*, 1998a). An increase of some 35% in productivity was obtained providing the optimal angle for each season was established throughout the year.

8.10 Photosynthetic efficiency in mass cultures (see Chapter 2)

Photosynthetic efficiency concerns the extent and effectiveness by which light is converted into chemical energy, i.e. cell structure and metabolic products as well for maintenance and storage. It may be defined in terms of the energy stored in biomass per unit of light energy impinging on the culture. Down-welling irradiance falling on the surface of the reactor may be utilized in varying efficiencies, a major goal of algal biotechnology, being conversion of high PFD to chemical energy at high efficiency. Just what is the minimum number of mole photons required to convert a mole of CO₂ to cell mass, is controversial. The conventional Z-scheme (see Chapter 2) does not permit less than 8 mole photon for this purpose and according to Raven (1988), 16 mole photons are practically needed for conversion of 1 mole of CO₂. Pirt *et al.* (1980), however, found in *Chlorella* culture a maximal photosynthetic efficiency (PE) of 46.8%, which is not feasible according to the Z-scheme theory which predicts a maximal photosynthetic efficiency of up to 30%. Pirt *et al.* (1983) put forth a theory suggesting that 6 mole photons may be sufficient to convert a mole of CO₂ to cell mass. This suggestion has been controversial, unacceptable to most researchers in the field. It is well to note that Pirt *et al.* (1980) attained the reported high PE by using rather low intensity (neon) light for their chemostat cultures, highlighting the requirement to *dilute* strong sunlight maximally in outdoor cultures in order to attain maximal efficiency and productivity per ground area.

Assuming incident photon flux density (400–700 nm, vectorial) 2000 µmole photons m⁻² s⁻¹ for 12 h d⁻¹, complete light absorption, and C = 0.5 of the cell organic dry weight, Raven (1988) predicted maximum yields of 130–346 g organic weight m⁻² d⁻¹, covering the 16 to 6 range of mole photons requirement for assimilation of one mole of CO₂ into cell material. This prediction, when translated into parameters of agricultural productivity sums up to a huge yield range of 475–1263 ton of dry cell matter of useable product per ha year⁻¹. It is worth noting that in some experiments, conducted by Pulz (1992) on a very small scale of a few square meters, using vertical flat plate reactors arranged closely together (i.e. 20 cm apart), providing thereby ca. 10 m² or more of illuminated culture surfaces per 1 m² ground area, the productivity per ground area, when translated to ha year⁻¹, fell in (the lower end) of this unbelievable productivity range, as follows: A yield of 130 g m⁻² (of ground area) d⁻¹ (attained experimentally in closely set flat plate reactors), represents 1300 kg ha⁻¹ d⁻¹ or 474.5 ton of dry mass ha⁻¹ year⁻¹, higher by more than an order of magnitude over conventional

agriculture production, as well as falling close to the yield predicted as attainable by Pirt *et al.* (1980).

Finally, it should be noted that increased photosynthetic efficiency (PE) may not be associated with increased, or even high, productivity: at low culture irradiance, PE would be as a rule high and productivity relatively low. In light-limited systems, increased productivity results in cultures maintained in optimal cell density in response to increased irradiance, which in itself is associated with a reduction in PE. A good example is seen in the productivity obtained in flat plates outdoors, which were either vertical, or tilted for maximal direct beam exposure (Tredici *et al.*, 1993): In vertical reactors the output rate of *Spirulina* cell mass was rather low i.e. $17.9 \text{ g m}^{-2} \text{ s}^{-1} \text{ d}^{-1}$ but PE was rather high for outdoor cultures i.e. 2.7%. In contrast, the productivity of reactors tilted to induce maximal exposure to the high direct beam irradiance was some 40% higher whereas the PE was only 1.9%, i.e. 42% lower, compared with the less exposed vertical reactors. As already explained, increased productivity associated with increased PE readily takes place on ground area basis as a result of strong light *dilution* i.e. as the ratio between the illuminated culture surfaces and the ground area which the reactors occupy, increases.

8.10.1 Appraising algal productivity and assessment of reactor efficiency (see also Chapter 9)

Productivity or yield of microalgal cell mass or products (the *output rate* in continuous cultures, Chapter 3), may be computed and recorded on several bases. One is volume, i.e. cell mass or metabolic product per unit culture volume per unit time, e.g. $\text{g l}^{-1} \text{ day}^{-1}$ outdoors, or g l h^{-1} under controlled lab conditions. Relating the productivity of a culture per day is correct for outdoor cultures only, and should not be used for artificially illuminated laboratory cultures. The most essential parameter for productivity assessment is obtained by relating the yield to the irradiated area of the culture e.g. $\text{g m}^{-2} \text{ d}^{-1}$. In a flat plate or a tubular reactor (see Chapter 9) the areal yield should be related to the entire irradiated surfaces, whereas in an open-raceway, the areal yield is related to the pond area.

The ground area taken up by the reactor represents another basis, particularly important for computing photosynthetic efficiency outdoors. Since irradiance enters the culture not only by direct beam but often no less or even more so, by diffuse and scattered light, calculating productivity on the basis of the ground area is prone to mistakes: Disregarding peripheral effects in calculating the energy available per ground area for a single reactor unit may greatly affect yield calculations. The correct computation of the ground area a reactor occupies is obtained by including the ground distances in between the plates or tubes (Table 8.7). An important aspect of solar energy utilization rests in the mode (essentially the distances apart), by which plate reactors, or tubes are arranged in the field. This phenomenon was well used by Pulz (1992) and Pulz & Brioneske (1995), who as already mentioned, reported record-high yields on the basis of the ground area, reflecting record photosynthetic efficiencies obtained by placing plate reactors short distances

Table 8.7. Recording microalgal yields (of cell-mass or products).

Volume yield:	$\text{g l}^{-1} \text{ d}^{-1}$ (outdoors) $\text{g l}^{-1} \text{ h}^{-1}$ (laboratory)
Areal yield:	<p>(a) On basis of the entire illuminated reactor area: $\text{g m}^{-2} \text{ d}^{-1}$ (or h^{-1}, in laboratory)</p> <p>(b) Cell mass per ground area including the distance between reactors or tubes, arranged horizontally or vertically: $\text{gm}^{-2} (\text{ground}) \text{ d}^{-1}$.</p> <p>(i) Horizontal system: open raceways, tubular systems to be based on the entire ground area occupied by the reactor*</p> <p>(ii) Vertical systems: <i>biocoil</i>, flat plates, sleeves, a tubular fence: must be based on the entire ground area occupied by a cluster of reactors**</p>

* Small peripheral effects.

** Large peripheral effects.

apart, thus achieving a large dilution of high irradiance. This setting, however, clearly requires relatively large reactor and culture volumes to produce a given yield, thus increasing substantially investment- as well as production-costs. A greatly reduced *reactor volume efficiency* results thereby (Richmond, 1996).

The interrelationships existing between areal and volumetric yields and the implications involved in correctly measuring the areal yield are addressed in Table 8.7 (also, see Chapter 9). Both volume and area yields are important to calculate and record for correct evaluation of culture performance. The areal yield is nevertheless the most important and meaningful parameter from which to evaluate culture performance in general and particularly for assessment of the effectiveness by which the culture responds to strong light. Indeed, photosynthetic productivity is most meaningfully expressed on areal basis – ground- or illuminated-area. A point which must be fully comprehended in this respect concerns the distinction between the photosynthetic rate per unit culture area or volume and the culture growth rate. In an attempt to uproot a common mistake concerning phototrophic productivity, it is here stressed that productivity is not necessarily directly related to the growth rate. It is the case in batch cultures which are often wrongly used to assess productivity by dividing the cumulative yield obtained in the growth period by the number of hours or days in which it was obtained. This method is grossly inaccurate, since the growth rate differs along the growth curve. Only continuous cultures (Chapter 3), in which the optimal cell density may be maintained at steady state, should be used to assess productivity. Using continuous cultures, one may readily observe that highest productivity (P) can never be associated with the highest growth rate (μ) since culture productivity relates to both μ and P : The highest growth rate is obtained when all requirements for cell growth are optimal and light is not limiting growth. Highest productivity, in contrast, is obtained in continuous cultures which are light-limited, thus the growth rate of which being far from maximal.

In evaluating reactor effectiveness, the biological principles elucidated in this treatise should be addressed to photobioreactor design, which must relate to the species' production protocol in a cost effective manner. Indeed, the past decade witnessed great many attempts to decipher the principles and essentials with which to best cope with these goals. The ultimate basic photobioreactor design is yet to emerge. One reason for this is rooted in the complexity involved in optimizing outdoor cultures, which are exposed to an ever-changing environment, diurnally and annually. Also, different species, cultured to obtain specific metabolic products call for specific production protocols, may require very different types of culturing devices. Finally, the choice of materials represents rather complex economic as well as biological issues. Details about great many types of photobioreactors shown in Chapter 9 naturally pose the need to adopt criteria by which to evaluate reactor effectiveness. One important criterion concerns culture volume required to produce a given quantity (e.g. 1 kg) of product or cell mass (Table 8.8). For example: the approximate volume of culture required to produce 1 kg of *Nannochloropsis* cell mass is ca. one order of magnitude larger if grown in an open raceway, as compared with enclosed reactors (tubular or flat plates), in which the optimal cell density is higher by close to one and up to two, orders of magnitude. A given volume of enclosed reactors, however, is usually much more costly than a similar volume in an open system.

Obviously, the smaller the culture volume and the higher the cell concentration, the less in general become the overall costs of culture maintenance as well as downstream processing. Another relevant question in comparing different designs of photobioreactors concerns the relative effectiveness of the irradiated areas in producing the desired cell mass or products. A high areal productivity ($\text{g m}^{-2} \text{ d}^{-1}$) obtainable in strong light, indicates the effectiveness of the reactor for strong light-use, the basic parameters underlying mass production of photo-autotrophic microorganisms. As an example, an areal volume of 50 L m^{-2}

Table 8.8. Parameters which concern evaluation of reactor efficiency for *Nannochloropsis* sp. (data from Zou & Richmond, 1999a).

Treatment No.	Areal* volume (L m^{-2})	Optical path (cm)	Optimal cell concentration (g l^{-1})	Total illuminated reactor surfaces required to produce 1 kg product d^{-1} (m^{-2})	Volume required to produce 1 kg product d^{-1} (L)	Volumetric productivity (AFDW) (mg l d^{-1})
1	6.5	1.3	8.5	182	1182	846
2	13	2.6	5.6	138	1792	558
3	26	5.2	3.6	108	2814	355
4**	50	10.0	2.3	89	4444	225
5	85	17.0	1.2	99	8474	118
6 (Open raceway)	300	30.0	0.35	83	25000	35

* Based on the total illuminated reactor surfaces in plate reactors.

** Optimal optical-path.

(Table 8.8) was found to be most effective for *Nannochloropsis*, grown in flat plate reactors (Chapter 9), requiring the lowest illuminated area to produce a given quantity of cell mass.

The requirements associated with effective use of photobioreactors should receive careful consideration, particularly (a) cooling requirements which become most demanding in certain geographical areas as humidity and irradiance increase; (b) ease of regular cleaning of the reactors to ensure homogeneous conditions of growth, i.e. checking growth of contaminating organisms and preventing light obstruction by wall-growth; and finally, (c) effectiveness of mixing to ensure turbulent streaming devoid of cell-damage, evermore essential as cell density increases. Naturally, the most important parameter is finally the investment cost of a given reactor volume in relation to the product value this volume would yield per given time period. Volume costs of open raceways and tanks, which in many cases (e.g. in aquaculture) represent the correct economic alternative, should also be analyzed.

There have been attempts to experimentally compare the performance of different reactors, exposed to the same growth conditions (Tredici & Chini Zittelli, 1998). Such experiments could yield valuable information and are much needed, providing two basic conditions are met: First, each of the systems to be compared must be maintained at its optimal, i.e. at its maximal output rate under the given experimental circumstances, a condition which requires intensive preliminary research. Second, peripheral effects, which could have a very large affect on the areal outputs, must be greatly reduced and most essential – should have a similar, quantitative affect, on the output rate of the systems to be compared. It is incorrect, e.g. to compare performance of a small reactor to that of a much larger reactor. Indeed, the larger the reactors, the more meaningful the comparison.

All these considerations find their economic expression in the truly ultimate goal of creating a durable and sustainable, cost-effective production system. Clearly, this goal requires continuous experimentation aimed at optimizing the complex parameters involved in photoautotrophic production outdoors. Commercially available photobioreactors for outdoor mass cultures have so far not fared well in this respect, being far too expensive.

8.11 Maintenance of mass cultures

Successful maintenance of microalgal cultures aimed at obtaining cost-effective productivity requires continuous information for online assessment of culture performance. Due to the dynamic nature of algaculture, it is important to facilitate a quick and reliable evaluation of the physiological state of the culture. Negative developments that, if left unattended could culminate in significant losses, must be detected early. In essence, the basic requisite for correct maintenance concerns consistent evaluation of some basic parameters from which to infer the relative culture performance in reference to the maximal performance known by experience to be possible under the given environmental circumstances (Richmond, 1986). In practical terms, the aim

for management of mass cultures is to sustain the optimal state, i.e. in which growth and metabolic activity leading to growth and product synthesis and accumulation are carried out at their genetically inherent maximal, limited only by irradiance.

8.11.1 Online monitoring of photosynthetic activity

8.11.1.1 Measuring dissolved oxygen (DO)

The DO, which may be readily measured with an oxygen electrode, is recognized as a reliable and sensitive indicator of the state of the culture, in relation to growth and productivity. Very high concentrations of DO may build up in actively growing cultures of photoautotrophic microorganisms. Concentrations of $35\text{--}45 \text{ mg O}_2 \text{l}^{-1}$, representing maximal supersaturation values (with respect to air) of up to 500%, have been observed in *Spirulina* cultures grown in large (e.g. 1000 m^{-2}), open raceways in which mixing was insufficient. Higher oxygen tensions have been measured in enclosed tubular systems, in which the DO concentration builds up rapidly (particularly in summer), up to 80 mg l^{-1} in *Spirulina* cultures grown in tubular reactors (Vonshak *et al.*, 1994). The full array of effects which very high concentrations of DO exert on commercially grown algal species has not been sufficiently studied. Excessive DO in the culture results in decreased yields of cell mass as well as in pigment content in *Spirulina* (Tredici & Chini Zittelli, 1997) and would promote, under suitable conditions, photoinhibition and photooxidation resulting in quick culture death (Abeliovich & Shilo, 1972; Richmond, 1986). Finally, high O_2 pressures in the medium promote photorespiration-inhibiting assimilation of CO_2 (see Chapter 2). In *Spirulina*, one effect of DO concentration relates to the protein content: A treatment of 45% O_2 in the gas phase applied to a *Spirulina* culture greatly reduced protein content, from 48% (of dry weight) to 22% (Torzillo *et al.*, 1984). In an open raceway, vigorous stirring effects a significant reduction in DO, bestowing an additional advantage for maintaining a strong turbulent flow in the culture, particularly in relatively high cell densities.

Early detection of an inexplicable decrease in DO or an obvious decline in the normal rate of the daily increase under given environmental conditions, serves as a reliable warning signal that the culture is stressed and may quickly deteriorate if corrective measures are not taken (Richmond, 1986). Experience shows that as a quick *first aid* remedy, a significant volume of the culture under such circumstances should be immediately replaced with fresh growth medium.

8.11.1.2 In situ monitoring of chlorophyll fluorescence

The chlorophyll fluorescence technique (see Chapters 2 and 4) is becoming a valuable tool by which to evaluate the photosynthetic activity of the culture and its relative performance under given environmental conditions, manifested

by the quantum yield of PS II which relates the radiation absorbed by the photosynthetic apparatus to the CO₂ assimilated. A decline in the quantum yield may indicate stress conditions, on which basis Torzillo *et al.* (1996) suggested that measurements of chlorophyll fluorescence should be useful in evaluating environmental effects on the physiological state of the culture. Measurements of chlorophyll fluorescence can well complement DO measurements, facilitating a rapid and accurate assessment of the relative *well being* of the culture.

8.11.2 Measurement of cell growth and culture productivity

Net growth may be estimated quickly by measuring changes in the overall turbidity of the culture. This, however, provides only a rough estimation of growth and should be followed routinely with other measurements such as cell count, dry weight or total organic carbon (TOC). Cell chlorophyll and protein may be suitable for expression of growth in algae, but should be used with caution, particularly in outdoor cultures, being strongly affected by environmental conditions. Chromatic adaptation or even a slight nitrogen deficiency may, within days, effect considerable changes in the cells' chlorophyll and protein content, which do not necessarily correspond with changes in cell mass. Indeed, cell protein content may change greatly during the diurnal cycle (Torzillo *et al.*, 1984). Therefore, if the effect of cell density on culture productivity would be calculated on the basis of the reactor illuminated area (areal productivity), the response of different cell densities to increasing irradiance would yield a set of results rather different from that obtainable if the productivity were to be calculated on the basis of cell chlorophyll which in itself is significantly affected by cell density.

8.11.3 Night biomass loss

It is worth noting that the productivity recorded for mass cultures outdoors represents a net figure, the overall gross productivity having been reduced due to decay processes, most prominent of which is the loss of cell mass and products, taking place mostly during the night. Factors which control night biomass loss (NBL) have been identified in part, including: night temperature, the extent to which temperature deviates from optimal during the day, the type of cell products produced during the day, and the population density. In experiments carried out in Florence (latitude 43.8N) with *Spirulina platensis* cultures in tubular photobioreactors (Torzillo *et al.*, 1991), the rates of night biomass loss were higher in a culture grown at 25°C (averaging 7.6% of total dry weight) than in the one grown at 35°C (averaging 5%). Night biomass loss depended on the temperature and light irradiance during the day, since these factors influenced biomass composition. A net increase in carbohydrate synthesis occurred when the culture was grown at a low biomass concentration under high light irradiance or at the suboptimal temperature of 25°. Excess carbohydrate synthesized

during the day was only partially utilized for night protein synthesis (Torzillo *et al.*, 1991). Hu *et al.* (1996b) investigated the effect of the population density in UHDC of *Spirulina* and found (in contrast with some other reports) that the extent of biomass loss at night, mainly due to dark respiration, was found to be relatively small when cell density was optimal, exerting only a minor effect on overall net productivity. Measurements of oxygen consumption at night revealed low rates of respiration, perhaps explainable by the low volumetric mass transfer coefficient (K_L) of oxygen. Hence, reduced oxygen tension may play a role in preventing full expression of the respiratory potential of ultrahigh cell density cultures. NBL, however, is certainly species-specific, and may be different in species other than that observed with *Spirulina* sp.

8.11.4 Maintaining optimal population density (OPD)

The population density represents a major parameter in the production of photoautotrophic mass, exerting far-reaching effects on the general performance and productivity of the culture. Optimal population density (OPD) or optimal cell density (OCD) is defined as that cell mass or concentration in continuous cultures, which results in the highest output rate of biomass and/or desired products. Since the culture is most stable when the population density is optimal, continuous cultures should always, as a rule, be maintained at that density representing the preferable mode of microalgal production. The OPD is determined empirically, guided by a rule of thumb that the optimal cell density in an outdoor culture is established when the specific growth rate (see Chapter 3) is approximately between one quarter to one half its maximal. Table 8.9 illustrates the effect of cell density, established by controlling the extent of the daily harvest, on the output rate of EPA, as well as on the productivity of cell mass (Richmond & Cheng-Wu, 2001). A 10% daily harvest yielded some 50% more EPA than a daily harvest of 20% or 5%, at which harvesting rates cell density was below or above optimal, respectively.

Table 8.9. Effect of the harvesting regime on steady-state EPA productivity of *Nannochloropsis oculata* in outdoor cultures of 500 l (440 l of culture volume) flat plate (10 cm of light path) glass photobioreactor, during summer.

Daily harvest (%)	Cell density (10^6 cells ml^{-1})	Harvesting volume (l)	EPA content (%)	EPA content (pg cell^{-1})	Volumetric EPA output ($\text{mg l}^{-1} \text{d}^{-1}$)	Areal EPA output ($\text{mg m}^{-2} \text{d}^{-1}$)
0	1050		3.66			
5	879	22	4.15	0.174	7.63	798
10	691*	44	4.71	0.185	12.76	1333
15	455	66	4.52	0.168	11.43	1195
20	249	88	4.45	0.163	8.10	846

* Optimal Cell Density (OCD).

8.11.5 Preventing nutritional deficiencies

Correct maintenance of continuous cultures requires routine tests to check any possible development of a deficiency in mineral nutrients. Nutrient elements in the growth medium are being constantly depleted in continuous cultures, having been absorbed by the cells. In addition, the concentration of some elements, relative to others in the nutrient formula, may be grossly altered. One practical method to supplement the nutritional status in the culture is to monitor the concentration of nitrogen, using it as a guideline for adding, in proportional amounts, the entire nutrient formula. Carbon and phosphorous however, should be monitored specifically and added to the culture separately. No doubt such a protocol is prone to error since the concentration of some elements (particularly minor), may with time either buildup or become completely depleted, irrespective of the extent of algal growth and nitrogen utilization. It may be thus advisable, in continuous cultures, to replace, from time to time, a considerable volume (e.g. 50%) to secure nutritional sufficiency and nutrient balance.

Culture pH tends to rise continuously during the day, as a result of HCO_3^- depletion through photosynthesis. The daily rise in pH could be rather small (0.1 or 0.2 units) in highly buffered (e.g. 0.2 M bicarbonate) *Spirulina* medium, or as high as a few pH units in lightly buffered media, typical of natural water bodies or fishponds. The pH may be maintained by an inflow of CO_2 , occasional addition of inorganic acid to a high alkalinity growth medium being also useful. Sodium bicarbonate may be used, in addition to CO_2 , to adjust the pH of species such as *Spirulina*, the pH optimum of which being in the range of 8.7–10.0. Carbon nutrition represents a major component in the operating cost of commercial production of microalgae.

8.11.6 Maintenance of monoalgal cultures and combating contamination

Contaminants (i.e. microorganisms different from the cultured species) often represent a major limitation to the overall productivity of microalgal cultures, particularly in open systems outdoors. Estimation of growth should be accompanied by detailed microscopic observations and measurements, the most important reason for which is tracing possible growth of foreign algal species as well as protozoa or fungi, which may quickly proliferate and cause damage, finally taking over the cultivated species. Buildup in the number of foreign organisms in the culture should be regarded as a warning signal, usually indicating that the cultured species have most probably come under stress: The temperature may have deviated too much from optimal, bestowing a relative advantage on competing species, or it may signal that the concentration of a particular nutrient had declined below the minimal threshold limiting culture growth. In short, appearance of contaminating microorganisms in the culture may indicate the unfolding of several possible growth limitations to the cultured species, which often culminates in loss of the culture.

The ecological niche that in effect is formed in non-sterile large-scale reactors is exposed to pressures of competition and succession. One good example is provided by Goldman & Ryther (1976), who found that competition among five tested species of marine phytoplankton in outdoor cultures, was highly dependent on temperature. The two presently most important commercially grown species, *Spirulina* and *Dunaliella*, thrive in growth media which represent extreme environments for most other species. The high bicarbonate (0.2 M) and pH (up to ca. 10.2) which are optimal for *Spirulina* and the high salinity concentration (2.0–4.0 M of NaCl) in which *Dunaliella* may be cultivated, represent effective barriers against most contaminants. Nevertheless, certain *Chlorella* and *Spirulina* species may play havoc in a *Spirulina platensis* culture (Richmond, 1988b). Algal species that require growth media of a more general nature, such as *Haematococcus*, *Porphyridium* or *Nannochloropsis*, in which a large variety of other species, as well as protozoa, could grow well, are more susceptible to contamination in open systems. In cultures growing vigorously, exposed to the optimal environment for its species, contamination represents, as a rule, little difficulty. Indeed, contamination is usually not prevalent in enclosed reactors although definitely not absent. A decline in temperature, which coincides with an increase in the organic load of the medium, for example, imparts a clear advantage to mixotrophic *Chlorella* spp., which may rapidly take over a culture of the photoautotrophic *Spirulina*. This process is accelerated if the *Spirulina* culture is harvested by screening, since *Chlorella* is ca. 3–5 µm in diameter, its population is steadily enriched in the course of harvesting by screening which specifically removes the long *Spirulina* filaments (Vonshak *et al.*, 1982). In cultures maintained at low population densities (i.e. a high light dose per cell), *Chlorella* was particularly successful in becoming rapidly dominant, whereas in relatively dense *Spirulina* cultures, *Chlorella* contamination has always been less severe (Richmond *et al.*, 1990). This is explained in that cyanobacteria are as a rule sensitive to high light intensity but have low maintenance energy requirements (Mur, 1983). Organisms such as *Chlorella* spp., which require relatively high maintenance energy, are thereby placed at a disadvantage.

Several strategies are available for control of contaminating organisms: In the case of *Spirulina platensis* contaminated by *Chlorella*, high alkalinity and a high pH (10.3 and over) were shown to impede growth of *Chlorella* (Vonshak *et al.*, 1982). Repeated pulses of 1–2 mM NH₃, followed by a 30% dilution of the culture also comprise an effective treatment which is based on the differential sensitivity of *Spirulina* and *Chlorella* cells to NH₃ toxicity.

An increase in the organic load of the culture medium due to cell lyses or leakage of cell constituents (e.g. glycerol in the case of *Dunaliella*) is of particular significance in mass cultures. It is a common cause for the loss of entire cultures of strict photoautotrophs such as *Spirulina* or *Dunaliella*, by bestowing an advantage on bacterial or fungal population which may quickly decimate the host culture.

Grazers, mainly of the amoebae type, may afflict great damage in cultures of unicellular, colonial or filamentous species, and amoebae grazing on *Chlorella* and *Spirulina* were observed in some commercial ponds, which were

improperly maintained. Grobbelaar (1981) described a case in which a culture containing mainly *Chlorella* sp. was infected by a *Stylonichia* sp. Within five days, *Scenedesmus* sp. became dominant in the culture, since its colonies were too large to be taken in by the *Stylonichia*. Addition of ammonia (2 mM) arrested the development of these grazers, evidenced when ammonia was used as the main nitrogen source (Lincoln *et al.*, 1983). In one work the use of rotifers (*Brachionus plicatilis*), efficient unicellular grazers, has been suggested for keeping *Spirulina* cultures free of unicellular contaminants such as *Morularaphidium minutum* and *Chlorella vulgaris* (Mitchell & Richmond, 1987).

Fungi may also proliferate in algal cultures under specific conditions. A recent example is a parasitic fungi *Rhizophydioides* sp. (Chytridiomycota) (Hoffman *et al.*, 2002), found to destroy cells of *Haematococcus pluvialis* as culture temperature increased in summer. The zoospore attaches itself on the algal cell wall forming a cyst, which grows into a monocentric sporangium in which zoospores are formed (Hoffman, 1999). Such contaminations amplify the need to have all the inputs going into the culture i.e. water, CO₂, air, and nutrients free of microorganisms. Certain types of zooplankton have been known to decimate, within days, cultures of *Haematococcus* and *Nannochloropsis*. In each case, treatment must be *tailor fit* to the specific case, considering the unique characteristics of the algal species. A good example is served in the use of hyperchloride in *Nannochloropsis* sp. cultures to control protozoa, which in effect, facilitated mass production of this species in open raceways outdoors (see Chapter 20). With enclosed systems, it is of utmost importance to keep the reactor clean by preventing cells and debris from accumulating on the reactor walls (*wall growth*) or in corners where, due to reduced turbulence, cell debris tend to accumulate. Cleaning enclosed reactors periodically is thus mandatory, in spite of the burden on the cost of production, and the relative ease by which a reactor may be cleaned represents, therefore, a most important feature in photobioreactor evaluation for a cost-effective production of cell mass.

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9 Mass Production of Microalgae: Photobioreactors

Mario R. Tredici

9.1 Introduction

Photobioreactors (PBR) are reactors in which phototrophs (microbial, algal or plant cells) are grown or used to carry out a photobiological reaction. In a broad sense, the open shallow basins widely used for microalgae cultivation could also be viewed as photobioreactors (Tredici, 1999). In this chapter, however, the term *photobioreactor* is used only for closed systems.

At present, commercial production of phototrophic microbial biomass is limited to a few microalgal species that are cultivated in open ponds by means of a selective environment or a high growth rate. Most microalgae cannot be maintained long enough in outdoor open systems because of the risk of contamination by fungi, bacteria and protozoa, and competition by other microalgae that tend to dominate regardless of the original species used as inoculum (Richmond, 1999). PBR offer a closed culture environment, which is protected from direct fallout, relatively safe from invasion by competing microorganisms, and where conditions are better controlled ensuring dominance of the desired species. Thus, PBR allow the exploitation of the potential of the more than 50 000 microalgal species known, many of which may be interesting sources of high value compounds.

Although the main topic of this chapter is PBR, an overview of open ponds is also presented. Open ponds have been well described by Dodd (1986), Richmond & Becker (1986), Oswald (1988) and Becker (1994). The topic of PBR, has been also reviewed by Lee (1986), Chaumont (1993), Prokop & Erickson (1995), Torzillo (1997), Tredici & Chini Zittelli (1997), and more recently by Pulz & Scheibenbogen (1998) and Tredici (1999).

9.2 Open ponds

Cultivation of microalgae for commercial purposes is presently carried out, with only a few exceptions, in open systems (Richmond, 1999). The main

reason for this is that large (commercial) open ponds are easier and less expensive to build and operate, and more durable than large closed reactors. A number of types of ponds have been designed and experimented with for microalgae cultivation. They vary in size, shape, material used for construction, type of agitation and inclination. Often the construction design is essentially dictated by local conditions and available materials (Becker, 1994).

The materials used to build the walls and bottom, and for lining determine pond performance, cost and durability (Richmond, 1990). Materials for construction of the side walls and bottom vary from simple sand or clay, to brick or cement, and to expensive plastics like PVC, glass fibre or polyurethane (Becker, 1994). For lining, most commercial plants use long-life plastic membranes (e.g. 1–2-mm thick UV-resistant PVC or polyethylene sheets), whose cost can vary from €10 to more than €25 m⁻². To reduce costs the use of unlined ponds has been suggested. Unlined ponds, however, suffer from silt suspension, percolation and heavy contamination, and their use is limited to a few algal species and to particular soil and environmental conditions (see *natural ponds* below).

Despite the many different kinds of ponds proposed, only three major designs have been developed and operated at a relatively large scale: (1) inclined systems where mixing is achieved through pumping and gravity flow; (2) circular ponds with agitation provided by a rotating arm; (3) raceway ponds constructed as an endless loop, in which the culture is circulated by paddle wheels. Only the last two, together with natural ponds, are used for commercial production of microalgae.

9.2.1 Lakes and natural ponds

When microalgae find suitable climatic conditions and sufficient nutrients, they grow profusely. If the chemical characteristics of the water are selective, for example, due to high pH or high salinity, the bloom is almost monospecific. There are many examples of eutrophic lakes or small natural basins exploited for microalgae production. Along the northeast border of lake Chad, numerous temporary or permanent lakes can be found where the chemical composition of the aquifer and strong evaporation create conditions suitable for *Arthrospira* growth in almost a monospecific culture. These lakes are highly productive natural systems and some of them are exploited by the Kanembu people who harvest the bloomed biomass (Fig. 9.1a) to use it as food (Abdulqader *et al.*, 2000).

In Central Burma (now Myanmar) *Arthrospira* grows as a monoculture throughout the year in four old volcanic craters filled with alkaline waters (Min Thein, 1993). During the blooming season, the thick suspension of the cyanobacterium is collected from boats (Fig. 9.1b); during the rest of the year it is pumped and concentrated on polyester filters. The slurry is further dehydrated using cloth bags which are pressed or squeezed by hands (Becker, 1994). The paste is then air dried and powdered before being punched into tablets which are sold on the local market. Current production is around 30 t year⁻¹ (Lee, 1997).

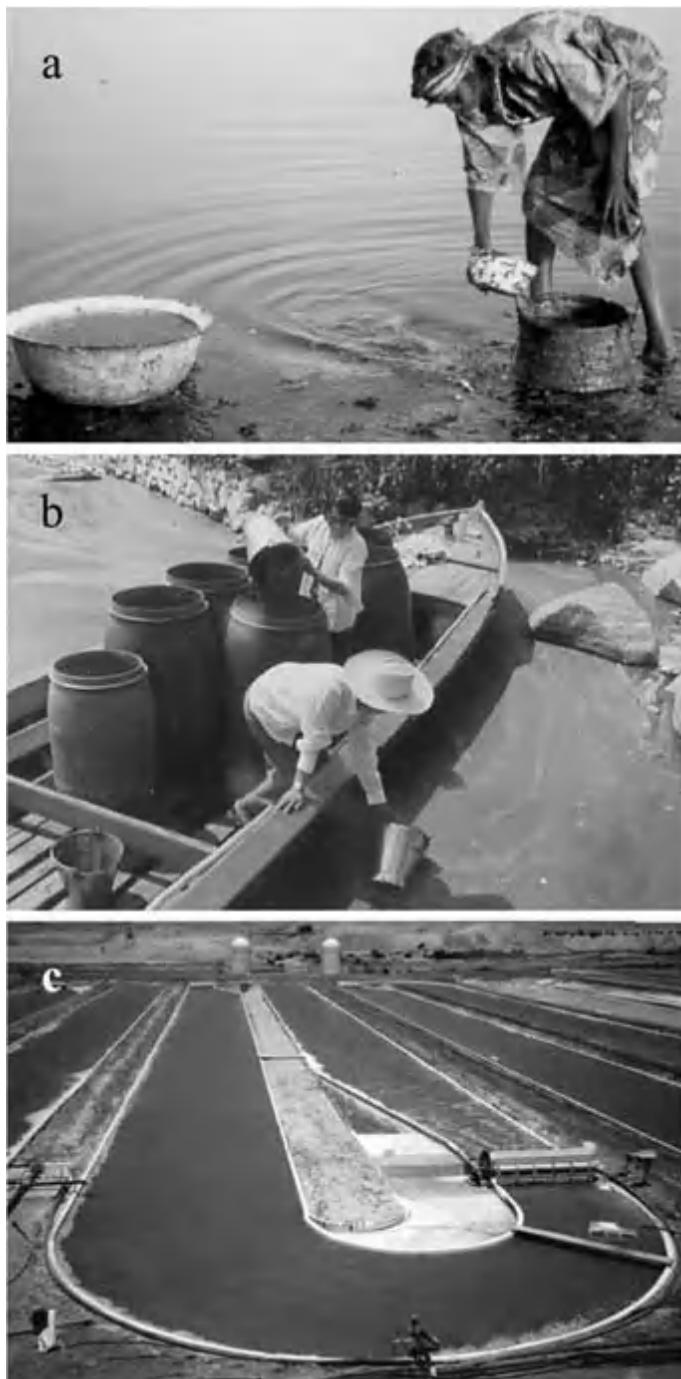


Fig. 9.1. (a) A Kanembu woman harvesting *Arthrospira platensis* from lake Kossorom, a permanent alkaline lake at the north-east fringe of lake Chad (Chad). (Reprinted with permission from Kluwer Academic Publishers (*J. Appl. Phycol.*)); (b) Harvesting of *Arthrospira* from an old volcanic crater filled with alkaline water in Myanmar (photograph courtesy of A. Vonshak); (c) A *Dunaliella* pond at Nature Beta Technologies Ltd (Eilat, Israel) (photograph by the author).

Extensive cultivation systems using no mixing and with minimal control of the environment, which still require, though, a certain amount of work for building and maintenance are here referred to as natural ponds. The best examples of natural ponds are the *caracol* in Mexico and the shallow lagoons used to cultivate *Dunaliella salina* in Australia.

The *caracol* (Spanish word for snail) is a 900 ha artificial solar evaporator of spiral shape from where Sosa Texcoco Co. extracted soda until some years ago. In the 1970s and 1980s Sosa Texcoco Co. cultivated *A. maxima* in the external ponds (about 40 ha) of the caracol (Becker, 1994), producing about 300 t of spirulina biomass per year, before closure in the mid-1990s due to a labour dispute. Average productivity was reported to be about $10 \text{ g m}^{-2} \text{ d}^{-1}$.

The largest natural ponds used for commercial production of microalgae are *Dunaliella* lagoons in Australia. Western Biotechnology Ltd operates 250 ha of ponds (semi-intensive cultivation) at Hutt Lagoon (Western Australia), producing about 6 t of β -carotene per year. Betatene Ltd, now a division of Henkel Co. (Germany), uses 460 ha of large unmixed ponds (extensive cultivation) at Whyalla (South Australia) to produce 7–10 t of β -carotene per year (Lee, 1997). These are very large ponds, up to 50-cm deep and 250 ha in area, which are unmixed other than by wind and convection (Borowitzka, 1999). Productivity does not exceed $1 \text{ g m}^{-2} \text{ d}^{-1}$ and, given the very low cell concentration, a very efficient system must be utilised to harvest the biomass.

Unmixed ponds may be cost-effective culture systems when suitable climatic conditions allow almost year-round cultivation.

9.2.2 Inclined systems

In inclined systems, turbulence is created by gravity, the culture suspension flowing from the top to the bottom of a sloping surface. Inclined systems should have received more attention (Richmond, 1999), because they obtain highly turbulent flow and allow adoption of very thin culture layers (less than 1 cm), facilitating higher cell concentrations (up to 10 g l^{-1}) and a higher surface-to-volume ratio (s/v) compared to raceway ponds. These systems are, however, limited by several problems, among which sedimentation of the cells at points of lower turbulence, strong evaporative losses, high rates of CO₂ desorption, and considerable requirement of energy for continuously pumping the culture to the head of the inclined surface. The sloping surface concept, patented in 1999 (Doucha & Livansky, 1999), has been largely applied and developed by the group of Trebon (Czech Republic) since the 1960s. In a 2600 m² plant of this type installed in Roupite (Bulgaria), areal productivities of 18 and $25 \text{ g m}^{-2} \text{ d}^{-1}$ were attained with *Arthrospira* and *Scenedesmus*, respectively (Fournadzhieva & Pillarsky, 1993). A 0.5-ha sloping pond was used to produce *Chlorella* in Western Australia (Borowitzka, 1999). This system was rather successful and, before being closed for technical reasons, it attained an average productivity of $25 \text{ g m}^{-2} \text{ d}^{-1}$ operating semi-continuously for a whole year. In the long run, however, inclined surfaces do not achieve areal productivities significantly higher than those

achieved in raceway ponds, and commercial systems of this type are not in operation at present.

9.2.3 Circular ponds

Circular ponds are not favoured in commercial plants since they require expensive concrete construction and high energy input for mixing. Circular ponds are nevertheless widely used in Japan, Taiwan and Indonesia for *Chlorella* biomass production (Lee, 2001).

9.2.4 Raceway ponds

Raceway culture ponds are used in most commercial plants for production of *Arthrospira* biomass. The major producers of *Arthrospira* in the USA, Cyanotech in Hawaii and Earthrise Farms in California (with pond areas of 75 000 and 150 000 m², respectively) (Lee, 1997), adopted large raceway ponds (from 1000 to 5000 m²) with stirring accomplished by one large paddle wheel per pond. Raceway ponds are also used for intensive cultivation of *Dunaliella salina* (e.g. by Nature Beta Technologies Ltd in Israel) (Fig. 9.1c).

The most simple example of raceway ponds consists of a shallow ditch dug into the ground and covered with plastic sheets draped up the sloping earth embankments. This construction is relatively inexpensive, but its cost is strongly influenced by the characteristics of the ground. The lining must be fixed very carefully to the ground to avoid displacement by winds. Bubble formation due to gas or water accumulation below the liner is another problem frequently encountered. In a different design, used in several commercial plants in Asia, the walls of the pond are erected on the ground with concrete blocks, bricks or even adobe (sun-dried clay) blocks which are covered with a plastic membrane that covers also the pond bottom (Becker, 1994). The principles for design and construction of large paddle stirred raceway ponds were reviewed by Dodd (1986) and Oswald (1988).

The open raceway has many drawbacks (Richmond, 1999). Large open raceway ponds can not be operated at a water level much lower than 15 cm otherwise a severe reduction of flow and turbulence would occur. This long light-path results in large areal volumes (150 l m⁻²) and cell concentrations of less than 0.60 g l⁻¹ that facilitate contamination and greatly increase the costs of harvesting. Excessive evaporative losses, particularly in hot dry climates, and lack of temperature control are other major drawbacks of open systems. Although areal productivities of 40 g m⁻² d⁻¹ and higher have been reported many times in experimental algal ponds, typically, well managed raceway ponds may achieve 20–25 g m⁻² d⁻¹ for short periods, while long-term productivity in large commercial raceways rarely exceeds 12–13 g m⁻² d⁻¹. The cost of microalgae biomass production in commercial raceways ranges from €9 to 17 kg⁻¹ of dry wt (Lee, 2001); heterotrophic production in fermenters is estimated to cost less than €6 kg⁻¹ of dry wt (Gladue & Maxey, 1994).

9.3 Photobioreactors

9.3.1 Definition of photobioreactors

Photobioreactors can be defined as culture systems for phototrophs in which a great proportion of the light ($>90\%$) does not impinge directly on the culture surface, but has to pass through the transparent reactor's walls to reach the cultivated cells. Consequently, PBR do not allow, or strongly limit, direct exchange of gases and contaminants (dust, microorganisms, etc.) between the culture and the atmosphere.

9.3.2 Classification of photobioreactors

Photobioreactors can be classified on the basis of both design and mode of operation. In design terms, the main categories of reactors are: (1) flat or tubular; (2) horizontal, inclined, vertical or spiral; and (3) manifold or serpentine. An operational classification of PBR would include (4) air or pump mixed and (5) single-phase reactors (filled with media, with gas exchange taking place in a separate gas exchanger), or two-phase reactors (in which both gas and liquid are present and continuous gas mass transfer takes place in the reactor itself). Construction materials provide additional variation and subcategories; for example (6) glass or plastic and (7) rigid or flexible PBR.

Axenic PBR are reactors operated under sterile conditions. Although a major characteristic of PBR is their ability to limit contamination, it must be made clear that an effective barrier, and thus operation under truly sterile conditions, is not achieved except in the few special designs developed expressly for that purpose.

9.3.3 Tubular photobioreactors

9.3.3.1 Serpentine photobioreactors

Serpentine PBR are systems in which several straight transparent tubes are connected in series by U-bends to form a flat loop (the photostage) that can be arranged either vertically or horizontally. Gas exchange and nutrient addition normally take place in a separate vessel. Circulation between the photostage and the gas exchanger is achieved by the use of a pump or of an airlift. Several reactors of this type have been developed following the original design by Tamiya (Tamiya *et al.*, 1953), but only one serpentine system seems to be in commercial use at present (see Section 9.4.2).

Tamiya and co-workers, in the early 1950s, developed a 40-l serpentine reactor made up of 3-cm diameter glass tubes, which was immersed in a water bath for temperature control. After several months of experimentation with *Chlorella ellipsoidea*, the authors concluded that one of the main limitations was temperature control and that a significant increase of productivity could be attained through cultivation of algae exhibiting higher temperature optima and through vigorous mixing aimed at increasing growth

by providing intermittent illumination. These concepts still guide our research.

In the early 1950s, the Arthur D. Little Company at the Massachusetts Institute of Technology, developed a horizontal tubular unit for the cultivation of *Chlorella*, that may be considered the first pilot plant for microalgae production (Anonymous, 1953). The reactor, of a total surface area of about 56 m², consisted of 4-mm-thick polyethylene tubes that, once filled with algal culture up to a depth of about 8 cm, assumed a more or less elliptic shape of 1.2 m width. Leakage and contamination by *Chlorococcum* and protozoa were the major problems encountered. In a 40-day run the system attained an average areal productivity of 9 g m⁻² d⁻¹.

Following initial studies, little work was carried out with photobioreactors for several decades. Two exceptions were the work of Setlik in Czechoslovakia (Setlik *et al.*, 1967) and Jüttner in Germany (Jüttner, 1982) (see Section 9.3.6). In the early 1980s, Gudin and co-workers (Chaumont *et al.*, 1988; Chaumont, 1993), working at the Centre d'Etudes Nucléaires de Cadarache (France), devised a horizontal serpentine reactor that was immersed in a water pond to obtain temperature control. A 100-m² system of this type was experimented with from 1986 to 1989. It was composed of five identical 20-m² units, each of which consisted of 20 polyethylene tubes, 20 m in length and 6 cm in diameter. The total culture volume was about 7 m³. Temperature control was attained by floating or immersing the reactor in a water bath. Initially, the culture was circulated by means of a pump, but later, airlift systems were adopted to limit damage to shear-sensitive cells and at the same time provide CO₂ supply and O₂ degassing. Productivities from 20 to 25 g m⁻² d⁻¹ were obtained with *Porphyridium cruentum*. The use of rigid rather than flexible tubes permitted operating a self-cleaning system consisting of two plastic balls, one with higher density than the culture medium and one lighter, which were hydraulically pushed through the system. Although flotation and immersion in a water basin can provide efficient thermoregulation, the cost of such a system is prohibitive for most applications.

In the early 1980s, Pirt and co-workers developed a vertical serpentine tubular reactor at the University of London's Queen Elizabeth College (Pirt *et al.*, 1983). The PBR consisted of a photostage formed of 52 Pyrex® glass tubes (each approximately 1-m-long and with a 1-cm ID) horizontally stacked and connected to glass U-bends by silicone-rubber tubing to form a vertical loop. The loop outlet was connected through a vertical riser to a degasser. A second tube connected the degasser to the inlet of the loop. The culture suspension (about 4.6 l) was circulated by either a peristaltic pump, a rotary positive-displacement pump or an airlift; the latter method was preferred because of the adverse effects observed with the two pumps. With a defined consortium consisting of a *Chlorella*-type green alga and three bacteria, cell concentrations in excess of 20 g l⁻¹ and a productivity of 2.2 g m⁻² h⁻¹ were achieved at a light intensity of only 38 W m⁻² (PAR). The work carried out by Pirt and co-workers was remarkable for three reasons: It provided the first detailed analysis of the fundamental engineering parameters of a closed PBR, introduced the concept of high s/v reactors (127 m⁻¹ in this case), and attained extremely high light-conversion efficiencies. Pirt's analysis, however,

did not consider the issue of the accumulation of photosynthetically generated oxygen, which represents a key limiting factor in large-scale tubular reactors (see Section 9.5.3), and ignored the light saturation effect, assuming that the efficiencies observed at low light intensity could be achieved in full sunlight as well. Although Pirt's serpentine reactor did deal with many of the problems encountered in closed systems, the concept of very high *s/v* reactors revealed all its practical drawbacks when the experimental set-up was scaled up to industrial level by Photo Bioreactors Ltd (PBL) (see Section 9.4.1).

Reactors of the type developed by Gudin are now being operated by Molina Grima and co-workers in Spain (Molina Grima, 1999). The systems consist of a tubular photostage, made of Plexiglas® tubes (from 2.6 to 5 cm in diameter), joined to form a 98.8-m long horizontal loop, which is connected to a 3–3.5-m high airlift (Fig. 9.2a). The reactors are immersed in a shallow, white-painted pond for temperature regulation. Dissolved oxygen, pH and temperature probes are connected to an online control unit and to a computer for data acquisition. The Spanish group has carried out extensive

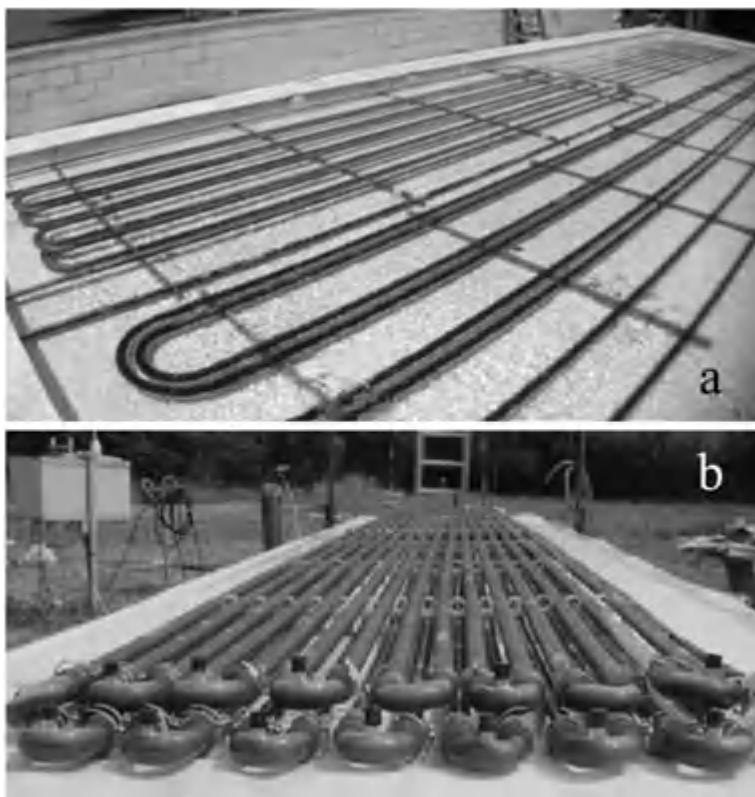


Fig. 9.2. (a) Serpentine reactors at the Department of Chemical Engineering of the University of Almeria (Spain) (photograph courtesy of E. Molina Grima); (b) A two-plane serpentine reactor at the Centro di Studio dei Microrganismi Autotrofi of the CNR (Florence, Italy). Reproduced with permission from Kluwer Academic Publishers.

research activity with serpentine reactors on the influence of the principal parameters that regulate growth of photosynthetic cells, among which average irradiance, gas–liquid mass transfer, temperature control, fluid dynamics and mixing (Acién Fernandez *et al.*, 1997, 1998; Sanchez Miron *et al.*, 1999; Molina Grima, 1999; Molina Grima *et al.*, 1999).

In the mid-1980s at the Centro di Studio dei Microrganismi Autotrofi (Florence, Italy), Florenzano and co-workers started experiments with horizontal serpentine tubular photobioreactors for the cultivation of *Arthrospira* (Torzillo *et al.*, 1986). The productivity was 50% higher than in raceway ponds operated in parallel. More recently, Torzillo *et al.* (1993) developed and experimented with a 145 L two-plane serpentine reactor (Fig. 9.2b). A maximum areal productivity of $27.8 \text{ g m}^{-2} \text{ d}^{-1}$ was achieved with *A. platensis* in July. This study addressed fundamental engineering parameters such as the effect of the rheological behaviour of the algal culture on the mixing characteristics of the system and the energy requirement for turbulent flow.

9.3.3.2 Manifold photobioreactors

In manifold PBR, a series of parallel tubes are connected at the ends by two manifolds, one for distribution and one for collection of the culture suspension. As shown by Pirt *et al.* (1983), about 15% of the energy consumed for recycling the culture in serpentine reactors is spent in moving the culture suspension around the bends; thus in comparison to serpentine reactors in which the culture inverts the direction of motion at each turn, manifold reactors allow a significant saving of energy.

Tredici and co-workers, working at the Department of Agricultural Biotechnology of the University of Florence (Italy), have developed several near-horizontal manifold-type photobioreactors called NHTR (Fig. 9.3a). Units made of rigid or flexible tubes ranging from 6 to 85 m in length and about 5 cm in diameter have been built and used to grow *A. platensis*, *A. siamensis*, *Nannochloropsis* sp. and *P. tricornutum* outdoors (Tredici & Chini Zittelli, 1998; Chini Zittelli *et al.*, 1999). Typically, a NHTR consists of some 12 flexible tubes, 45-m long and 4 cm in diameter, connected by two manifolds; the upper manifold acts as degasser. The tubes are placed side-by-side on white corrugated plastic sheeting, facing south and inclined at a slight angle to the horizontal (4–6°). The corrugated sheeting keeps the tubes well-aligned and at uniform inclination, reflects light onto the culture, and also provides drainage for rain and cooling water. Temperature control during the day is obtained by water spraying. The largest unit experimented with thus far occupies an area of 60 m^2 and contains about 1200 l of culture. Air is injected at rates between 0.02 and 0.11 l min^{-1} by means of a perforated pipe placed in the bottom manifold. Typically one out of four tubes is not gassed and is used as a return flow tube to increase the circulation speed of the culture and obtain better mass transfer. Carbon dioxide addition to the air stream or in the return tubes is regulated through a pH-stat system. Volumetric productivities of up to $1.3 \text{ g l}^{-1} \text{ d}^{-1}$ and areal productivities of more than $28 \text{ g m}^{-2} \text{ d}^{-1}$ have been obtained with *A. platensis* (Tredici & Chini

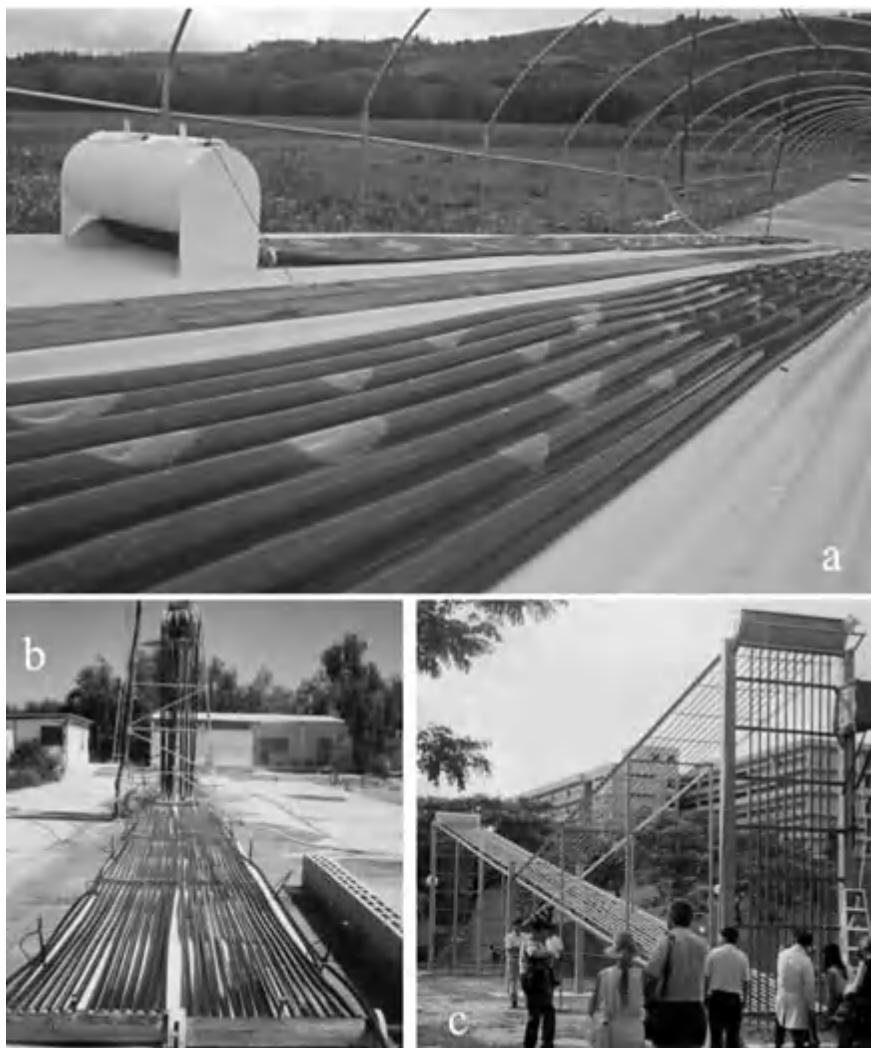


Fig. 9.3. (a) Near-horizontal manifold PBR at the Department of Agricultural Biotechnology of the University of Florence (Italy) (photograph by the author); (b) Parallel flow reactor at the Ben Gurion University of the Negev (Israel) (from Encyclopedia of bioprocess technology: fermentation, biocatalysis and bioseparation/Michael C. Flickinger, Stephen W. Drew; Copyright © 1999 by John Wiley & Sons, Inc. This material is used by permission of John Wiley & Sons, Inc.); (c) α -type PBR at the Biotechnology Department of the National University of Singapore (photograph courtesy of Y.K. Lee).

Zittelli, 1998). Besides simplicity of operation and low maintenance cost, this internal gas exchange reactor has other advantages among which low shear stress and reduced fouling and wall growth due to the scouring effect of the gas bubbles. Length of the tubes is, however, limited to about 40 m due to reduced mass transfer. A cost analysis for a large NHTR system for hydrogen production has been presented (Tredici *et al.*, 1998).

A parallel-flow tubular PBR was devised and experimented with in outdoor cultivation of cyanobacteria at the Microalgal Biotechnology Laboratory of Ben Gurion University (Israel) (Richmond *et al.*, 1993). The system consisted of eight 20-m long, 3-cm ID polycarbonate tubes connected by manifolds and laid parallel on the ground or on a horizontal platform (Fig. 9.3b). Circulation and gas exchange were obtained by means of a 2.2-m high airlift connected to a 20-l gas separator. Small differences in flow rates were measured between the tubes. Temperature control was provided by water spraying. A productivity of $0.55\text{ g l}^{-1}\text{ d}^{-1}$ was obtained with *A. platensis* and *A. siamensis*. The main advantages of the system over serpentine loop reactors are the reduction of head losses and lower oxygen concentrations, two factors that facilitate scale-up to industrial size.

A 300-l elevated manifold system called the ‘ α type tubular PBR’ was devised and experimented with in Singapore (Lee *et al.*, 1995). The reactor, that covered a land area of about 12 m^2 , consisted of two sets of ten parallel transparent PVC tubes (25-m long with a 2.5-cm ID), both placed at an angle of 25° to the horizontal, but inclined in opposite directions (Fig. 9.3c). An areal productivity of $72\text{ g m}^{-2}\text{ d}^{-1}$ was reported on the basis of the occupied ground area.

9.3.3.3 Helical photobioreactors (bio-coil PBR)

Helical PBR consist of small-diameter, generally flexible tubes wound around an upright structure. This design is not new; it was used in the 1950s by Davis to grow *Chlorella* (Davis *et al.*, 1953) and later adopted, in a flattened version made of glass tubes, by Setlik (Setlik *et al.*, 1967), Krüger & Eloff (1981) and Jüttner (1982). A helical PBR called *biocoil* was patented by Biotechna Ltd (Robinson & Morrison, 1987). A *biocoil* consists of a photostage of polyethylene or PVC tubing (between 2.5 and 5-cm diameter) wound helically around a cylindrical support (typically 8 m in height with a core diameter of 2 m). Several parallel bands of tubes may be connected via manifolds to the pumping system. This allows more even flow and shorter tube length thus minimising oxygen build-up.

A 120-l helical bubble reactor was devised and operated by Tredici & Chini Zittelli (1998) to grow *A. siamensis* and *A. platensis* outdoors. The reactor consisted of three 49-m long transparent PVC tubes (3-cm ID, 0.5-cm wall thickness) wound around a 1.65-m high, 1.1-m diameter vertical structure with an inclination of 2° to the horizontal. The higher ends of the tubes were connected to a 20-l degasser. From the degasser, the culture suspension flowed down through a single pipe to the lower ends of the culture tubes. Air was injected at the bottom of each tube and the bubbles flowing up provided mixing and gas exchange and prevented biofouling. Cooling was achieved by water spraying. With *A. platensis*, a mean volumetric productivity of $0.9\text{ g l}^{-1}\text{ d}^{-1}$ and a photosynthetic efficiency of 6.6% Photosynthetic Active Radiation (PAR) were achieved. The system operated with remarkable stability. The cost of a 120-l unit of this type was estimated to be about €150 (Tredici, 1999).

9.3.3.4 Fence arrangement with manifolds

Applied Photosynthetics Limited (APL) (Manchester, UK) developed a photobioreactor known as the *bio-fence* specifically designed for the cultivation of the marine algal species used in aquaculture or for waste water treatment either with microalgae or photosynthetic bacteria (Tredici, 1999). The bio-fence consists of an array of rigid transparent tubes racked together in banks and connected by manifolds in a fence-like structure. The culture suspension is circulated continuously between the photostage and a holding tank by a centrifugal pump or by an airlift. The pH of the culture is controlled automatically by injection of CO₂ in the photostage.

9.3.4 Flat photobioreactors

Flat culture chambers and flat PBR have often been used to grow photo-trophic microorganisms in the laboratory because they greatly facilitate the measurement of irradiance at the culture surface. Despite their apparent simplicity, few such systems have been used for mass cultivation of algae (Tredici, 1999).

9.3.4.1 Early attempts

The first flat culture unit devised for mass production of algae was the *rocking tray* used by Milner (1953) to grow *Chlorella* in a thin turbulent layer.

Anderson & Eakin (1985) used 3-m² inclined flat plates for polysaccharide production from *Porphyridium cruentum*. About 20 g of polysaccharide m⁻² d⁻¹ were produced during the summer, when temperature control was provided.

Samson & LeDuy (1985) cultivated *A. maxima* in a 64-l, 10-cm thick flat reactor vertically arranged. Volumetric productivities of 1.2 g l⁻¹ d⁻¹, corresponding to 60 g m⁻² of illuminated surface area per day, were achieved under artificial illumination (about 360 µmol photon m⁻² s⁻¹) provided from both sides by cool-white fluorescent lamps.

9.3.4.2 Flat alveolar panels

In the late 1980s, two groups working independently in France and in Italy, introduced alveolar panels in algae cultivation. These systems are constructed from commercially available, transparent PVC, polycarbonate or polymethyl methacrylate sheets that are internally partitioned to form narrow channels called *alveoli*. At the Centre d'Etudes Nucléaires de Grenoble (France) Ramos de Ortega & Roux (1986) experimented with 6-m long, 0.25-m wide, 4-cm thick, double layer panels made of transparent PVC for growing *Chlorella*. The plates were laid horizontally on the ground; the upper layer of channels was used for algal growth, the lower for thermoregulation. The culture suspension was circulated through a pump. A productivity of 24 g m⁻² d⁻¹ was achieved in the summer using units of 1.5 m² of surface area.

In 1988, Tredici and co-workers at the University of Florence introduced the idea to make vertical flat reactors from commercially available 16-mm thick Plexiglas® alveolar sheets 95% transparent to PAR. The channels of the panel (and hence the culture flow) run parallel to the ground and a pump was used to circulate the suspension, a design later adopted in Germany by Pulz and co-workers. The same year, the Florence group started to experiment with a different design in which the panel was set up vertically or highly inclined (Fig. 9.4a), with the channels perpendicular to the ground, and mixing and deoxygenation of the culture were achieved by bubbling air at the bottom of the reactor (Tredici *et al.*, 1991). The inner walls forming the alveoli were removed from the top and bottom sections of the panel to ensure free communication of the culture suspension in the entire panel volume. This latter version, referred to as vertical alveolar panel (VAP), has been used extensively by this group for outdoor cultivation of microalgae and cyanobacteria (Tredici *et al.*, 1991; Tredici & Materassi, 1992; Tredici & Chini Zittelli, 1997, 1998). Several VAP reactors were constructed with a panel surface area varying from 0.5 to 2.2 m² and used to study the influence of areal density, turbulence, oxygen tension and



Fig. 9.4. (a) A 2.2 m² alveolar panel at the Department of Agricultural Biotechnology of the University of Florence (Italy) (photograph by the author); (b) PBR system at the IGV Institut für Getreideverarbeitung (Bergholz-Rehbrücke, Germany) (photograph courtesy of O. Pulz); (c) glass plates at the Ben Gurion University of the Negev (Israel) (photograph courtesy of A. Richmond).

inclination of the PBR on productivity and biomass composition (Tredici & Chini Zittelli, 1997). Due to their high s/v (160 m^{-1}), alveolar plates attained high volumetric productivities with *A. platensis* ($>2\text{ g l}^{-1}\text{ d}^{-1}$) and permitted operation at high cell concentrations ($4\text{--}6\text{ g l}^{-1}$). When placed near-horizontally (5°), however, these systems achieved lower areal productivities (about $24\text{ g m}^{-2}\text{ d}^{-1}$) compared to tubular reactors (about $28\text{ g m}^{-2}\text{ d}^{-1}$). The better performance of the latter was attributed to the fact that, tubular reactors, unlike flat surface systems, achieve light dilution, thus reducing the negative effect of light saturation and photoinhibition at noontime (Tredici & Chini Zittelli, 1998).

The flat panel developed in Florence was significantly improved by two groups working independently in Germany and in Israel. At the IGV Institut für Getreideverarbeitung (Bergholz-Rehbrücke, Germany), Pulz and co-workers used 32-mm thick vertical alveolar panels, in which the direction of flow was horizontal, circulation being provided by a pump (Pulz & Scheibenbogen, 1998). The panels were placed 20 cm apart to form a compact structure called PBR which was scaled up to commercial size (Fig. 9.4b). PBR reactors were sold commercially by B. Braun Biotech Int in sizes from 10 to 6000 L. Difficulty in maintaining an adequate turbulent flow in the reactor, build up of oxygen (up to $30\text{--}40\text{ mg l}^{-1}$ at the outlet of the circuit) and fouling were among the main problems encountered. The main merit of the PBR reactors developed in Germany, besides their high degree of technical development, is that parallel rows of plates were closely packed together so that an illuminated surface area of about 500 m^2 could be set up on a ground area of 100 m^2 . This design may seem weak, at first sight, since in this configuration the panels strongly shade each other and this would reduce significantly volumetric productivity. However, despite the packed arrangement and the unfavourable climatic conditions of Germany, daily productivities of 1.3 g l^{-1} and 28 g m^{-2} of illuminated panel surface, corresponding to a record output of 130 g m^{-2} ground area d^{-1} , were reported with *Chlorella* (Pulz & Scheibenbogen, 1998). These areal productivities seem excessive and very likely the calculation suffers from the fact that peripheral effects were not duly considered. It should be noted, however, that the packed arrangement adopted in the PBR series allows a fivefold dilution of solar irradiance at the culture surface, an effect called *lamination*, which greatly enhances the efficiency of conversion of solar irradiance into biomass (Carlozzi, 2000; Richmond & Zhang, 2001).

9.3.4.3 Glass plates

In the mid-1990s, Richmond and co-workers (Hu *et al.*, 1996) developed an inclined modular photobioreactor consisting of a series of flat glass chambers, 0.7-m high and 0.9-m long, connected in cascade and tilted at the proper angle to maximise solar radiation capture (Fig. 9.4c). Reactors with varying thicknesses (1.3, 2.6, 5.2, and 10.4 cm) were tested in the cultivation of *A. platensis*. Air-bubble mixing was adopted by means of two perforated tubes running horizontally along the entire length of the reactor, one at the bottom and the other at mid-height. After having optimised the population

density and adopting vigorous mixing ($2.5 \text{ l air l}^{-1} \text{ culture suspension min}^{-1}$), record productivities of about $50 \text{ g m}^{-2} \text{ d}^{-1}$ of front illuminated reactor area were achieved at the lower reactor thickness (1.3–2.6 cm). When the back and side surfaces of the panels, that received only reflected and diffuse radiation, were covered, productivity diminished of 25%, 30% and about 55% at inclinations of 30° , 60° and 90° , respectively, thus confirming the important role played by diffuse and reflected light in elevated systems.

Using glass panels with a light-path ranging from 1.3 to 17 cm, Zou & Richmond (1999) and Richmond & Zhang (2001) elucidated the influence of the light path on productivity in *Nannochloropsis* cultures (see Chapters 8 and 16). As expected, the shorter the light path, the higher the volumetric productivity. In contrast, areal productivity, as already seen for *A. platensis*, exhibited an optimum curve in relation to the light path, with the highest productivity attained in a 10-cm thick reactor. In summer, at the optimal population density of 2.6 g l^{-1} , the specific growth rate (μ) was about 44% of the maximum and an average productivity on the basis of the illuminated reactor surface of $\sim 12 \text{ g m}^{-2} \text{ d}^{-1}$ ($0.24 \text{ mg l}^{-1} \text{ d}^{-1}$) was achieved. Thus, by varying the thickness of the panel, the influence of the light path on productivity could be investigated and an important result could be achieved: There is a precise light path at which areal productivity is maximal. The optimal light path changes with the algal species cultivated: e.g. it is 10 cm for *Nannochloropsis*.

To develop the methodology for cost-effective mass production of *Nannochloropsis* in quantities required for hatcheries, Richmond and co-workers (Zhang *et al.*, 2001) devised a glass flat reactor that may be readily scaled-up to 1000–2000 l. A 500-l unit of this type, with a 10-cm light path, optimal for mass production of *Nannochloropsis*, was built by gluing together two sub-units made of 200-cm long, 110-cm high, 1-cm thick glass sheets. To prevent the glass from cracking, vertical dividers were glued between the front and back walls of the reactor. Mixing was provided by injecting compressed air through a perforated tube extending all across the bottom of the reactor. Cooling was accomplished by means of sprinklers set-up along the upper part of the reactor. Productivity varied between 10 (in winter) and 14.2 (in summer) g m^{-2} illuminated surface area of the plate per day. For a postulated 2000 l unit of this type, the investment cost was calculated to be about €4 l^{-1} (or €200 per square metre of illuminated reactor surface), one of the lowest available today for medium and large-scale enclosed systems. An analysis of production costs showed that the cost of *Nannochloropsis* biomass produced in a 2000 l reactor of this type would vary between €55 and €120 per kg dry wt, the dominant input being the cost of labour.

9.3.4.4 Critical evaluation of flat plate systems

Researches carried out in Italy, Germany and Israel indicate that vertical flat reactors represent very promising culture devices. Elevated flat panels can be oriented and tilted at optimal angles for maximal exposure at direct beam radiation. As demonstrated by Tredici & Chini Zittelli (1997) and by Richmond and co-workers (Richmond, 1999), at intermediate latitudes, high

angles in winter (ca. 60°) and low angles in summer (ca. 30°) maximise productivity per unit volume and per unit of illuminated surface of the reactor. On the other hand, these systems can be tilted so as to intercept beam radiation with large angles, thus reducing the amount of radiant energy impinging on the culture surface. This increases the efficiency of light conversion and the productivity per unit of ground area. In contrast with horizontal reactors, the entire surface of elevated plates is illuminated. The front surface receives direct radiation and the back surface, as well as the side walls, is illuminated by a rather homogeneous and low-energy photon flux made essentially of diffuse and reflected light very effective for photosynthesis. They also offer the possibility to be closely packed together and thus attain, by a sort of *lamination* of the culture exposed to direct solar radiation, high efficiencies of light conversion and high areal productivities. Which strategy is to be pursued depends on the situation, but undoubtedly elevated flat panels present a high degree of flexibility. Air bubbling can be adopted, obtaining efficient mixing and degassing of the culture. Temperature control is not a problem if evaporative cooling by spraying water can be used. Efficiency of CO₂ utilisation may be low, but small bubbles sparging systems can be used to increase K_{La} .

Some alveolar reactors also have some drawbacks. After some months of use, alveolar panels leak from the glued connections, the numerous internal walls favour wall growth and may cause damage on fragile cells, and last but not least, too many units are needed to scale up the system to commercial size. These drawbacks have very likely prompted the German group to adopt a tubular design, instead of their PBR, for the construction of the ÖPA plant (see Section 9.4.2).

Glass flat plates seem superior to alveolar panels. Glass is highly transparent, easy to clean and resistant to weathering. Using glass sheets, reactors of any desired optical path can be assembled and tailored to meet the specific requirements of any algal species. Glass panels also have some disadvantages, e.g. excessive weight (about 1-cm thick glass sheets are needed to withstand a water column of 100 cm), fragility and, perhaps, cost.

9.3.5 Vertical cylinders and sleeves

Vertical tubular reactors (or column reactors) are simple systems in which mixing is achieved by injecting compressed air. Several vertical tubular PBR have been developed following Cook's first design set-up at Stanford University in California in the late 1940s (Cook, 1950). These first units consisted of glass columns 1.8 m in height and 10 cm in diameter, constricted at the bottom to prevent algal settling. Maximum volumetric productivity of *Chlorella* indoors was 0.48 g l⁻¹ d⁻¹. Outdoors, productivity of *Chlorella* averaged 0.28 g l⁻¹ d⁻¹ with a maximum of 0.35 g l⁻¹ d⁻¹. The lower outdoor productivity was explained by the fact that vertical reactors are always at a large angle to the sun's rays, for which a substantial amount of solar energy is reflected and not available for growth.

A 301 (2-m height, 15-cm ID) tower-type glass reactor was devised by Jüttner (1982). The main characteristic of this system was a wide, hollow

glass finger inserted from above into the column to reduce dark space inside the reactor. Since the finger was open at the upper end, it could also be used to regulate the culture temperature. A thickness of the annular culture chamber of about 3.5 cm was found to be optimal for growth of *Microcystis aeruginosa*.

Miyamoto *et al.* (1988) experimented with vertical tubular reactors (2.35-m height, 5-cm ID) made from the low-cost, mass-produced glass tubes used in the fluorescent lighting industry. Volumetric productivities of about $0.6\text{ g l}^{-1}\text{ d}^{-1}$ were obtained with *Nostoc* and *Anacystis* in outdoor cultivation. Using a similar system consisting of 32 glass tubes 1.5 m in height and with a 2.6-cm ID, Hu & Richmond (1994) obtained a volumetric productivity of $1.6\text{ g l}^{-1}\text{ d}^{-1}$ with *Isochrysis galbana* cultivated outdoors.

The rigid vertical columns, typically 2–2.5 m in height and 30–50 cm in diameter, that are extensively used in hatcheries to produce algal biomass for feeding the larval stages of marine bivalves and fishes must be included in this category. Most commonly, these systems are made of translucent glass fibre sheets formed into cylinders. Air is bubbled at the bottom for mixing. Illumination is provided either by artificial or natural light.

A disposable vertical reactor can be easily built by cutting a suitable length of transparent polyethylene tubing and heat-sealing one end. This *bag* or *sleeve* reactor can be suspended from a framework or supported within a mesh frame. The culture is mixed by bubbling air from the bottom. Such reactors are used indoors with artificial illumination (generally vertically mounted fluorescent lamps) or outdoors under sunlight. Sleeve reactors have a relatively short life because of biofouling, but are easy and inexpensive to replace. Two-metre-long, transparent polyethylene sleeves sealed at the bottom and hung on an iron structure have been used to grow *Porphyridium* and *Dunaliella* outdoors at the Institute for Applied Research (Beer-Sheva, Israel) (Fig. 9.5a) obtaining a significantly higher productivity compared to open ponds (Cohen & Arad (Malis), 1989). The main drawbacks of sleeve reactors are the relatively low s/v and wall growth.

At the Department of Chemical Engineering of the University of Almeria, a 12-l concentric-tube airlift reactor, 9.6 cm in diameter and 2-m high, was developed and used to cultivate *P. tricornutum* outdoors (Garcia Camacho *et al.*, 1999). The system compared favourably with the horizontal serpentine reactors experimented with in the same location (see Section 9.8).

Vertical cylinders illuminated from inside, which may be considered a variation of the annular reactor devised by Jüttner (1982), have been proposed for the production of marine microalgae in hatcheries. Internally lit cylinders typically attain higher volumetric productivities and greater efficiencies of light utilisation compared to completely filled columns, since the photon flux provided is completely trapped by the culture. A vertical annular reactor with internal illumination was recently developed at the University of Florence by Tredici and co-workers (Fig. 9.5b). The reactor consists of two 2-m high Plexiglas® cylinders of different diameter placed one inside the other so as to form a regular annular culture chamber, 3–5-cm thick and 120–150 l in volume. An air–CO₂ mixture is injected at the bottom of the annular

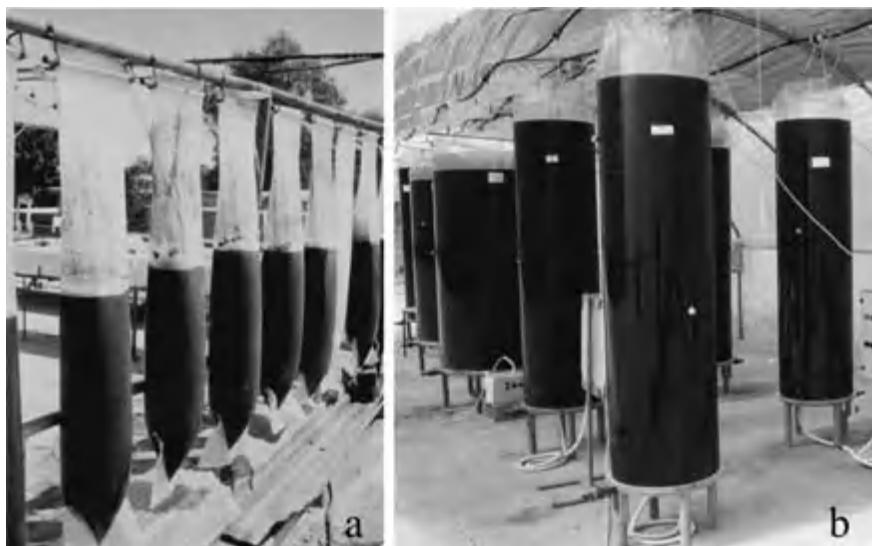


Fig. 9.5. (a) Polyethylene sleeves at the Institute for Applied Research (Beer-Sheva, Israel) (photograph courtesy of S. Arad); (b) Annular columns at the Department of Agricultural Biotechnology of the University of Florence (Italy) (photograph by the author).

chamber for mixing and gas-exchange. To operate the reactor with artificial illumination, lamps or fluorescent tubes are placed inside the inner cylinder. The use of this system for cultivation of *Nannochloropsis* is described in Chapter 16.

9.3.6 Axenic photobioreactors

Setlik (Setlik *et al.*, 1967) and later Krüger & Eloff (1981) and Jüttner (1982) developed glass tubular reactors for the axenic cultivation of microalgae. Jüttner's system, which substantially reproduced Setlik's design, was an all glass, 110-l vertical flat coiled photobioreactor that could be sterilised by steam. The reactor, made of 4-cm ID tubes, had a total length of 79 m. The algal suspension was circulated by a glass centrifugal pump at a flow rate of $20\text{--}60\text{ cm s}^{-1}$. The temperature was regulated through a 2-m water-jacketed section. Gas exchange took place in an external exchanger in which a CO_2 -air mixture was injected at a rate of 300l h^{-1} . The system attained a maximum productivity of about $0.5\text{l}^{-1}\text{ d}^{-1}$ under artificial illumination. Cell damage caused by the centrifugal pump was a problem in the case of fragile cyanobacteria; other problems encountered were foam formation and cell adhesion to the glass walls. Jüttner concluded that cell adhesion was the most discouraging feature and suggested that cultivation ought to be limited to nonsticky algae. A similar 60-l system, composed of twelve 5-cm ID glass tubes each 2 m in length, coupled by U-bends to form a vertical flattened spiral, was operated by Krüger & Eloff (1981), who reported a productivity of about $0.13\text{ g l}^{-1}\text{ d}^{-1}$ with a non-toxic species of *Mycrocystis*.

Buchholz at the Technische Universität Berlin (Germany) has recently developed a sterilisable modular PBR known as Medusa Photobioreactor (Buchholz, 1999). The reactor consists of a photostage made from U-bent borosilicate glass tubes, 2-m long and 5-cm diameter vertically arranged, connected to a stainless steel or glass head space. The head space acts as a degasser and houses electrodes and probes. Illumination is provided by fluorescent lamps regularly interspaced between the culture tubes. Mixing is achieved by gas injection from the lower ends of the tubes. The reactor can be sterilised at 120°C and 1.5 bar.

An 80-l helical glass photobioreactor allowing axenic cultivation of phototrophic bacteria and microalgae has been built at the Wilhelm-Universität Münster (Münster, Germany) (Hal *et al.*, 2000). Standard glass tubes were helically arranged and connected with a degasser.

Although these sterilisable systems appear difficult to scale up and are rather expensive, they are the sole reactors that can guarantee, if properly operated, real axenic conditions for the cultivation of phototrophic micro-organisms.

9.4 Commercial scale photobioreactors

A few commercial-scale PBR have been built and operated, but most were closed after a few months of operation. Only three large commercial systems, the plants built by Ökologische Produkte Altmark GmbH in Germany, and by Micro Gaia Inc and Aquasearch Inc in Hawaii (USA), are apparently in full operation. Unfortunately not much is known about their productivity and general performance. Although not large scale, two other PBR (bio-fence and Advanced Algal Production System [AAPS]) are described in this section, since they are commercialised at present.

9.4.1 Attempts of the past

9.4.1.1 Sagdiana PGT (Tadzhikistan)

A large-scale horizontal manifold reactor was built in 1991 in Javan (Tadzhikistan) by Sagdiana PGT and used for the cultivation of *Chlorella* (Tredici, 1999). The system comprised ten units consisting of two banks of twenty-eight 5.7-cm ID glass tubes, each 73.5-m long, for a total of about 41 000 m of tubing (Fig. 9.6a). The reactor was operated, however, for only three months attaining low productivity ($<0.1\text{ g l}^{-1}\text{ d}^{-1}$). Instability of the production process and contamination by *Scenedesmus* were reported to be the main problems.

9.4.1.2 Photo Bioreactors Ltd (Spain)

Photo Bioreactors Ltd, set up in southern Spain in the late 1980s based on the two different designs patented by Pirt (1983), is one of the biggest disasters in the field of microalgal biotechnology. The quality of the basic work done at Queen Elizabeth College by John Pirt and the high projected productivities

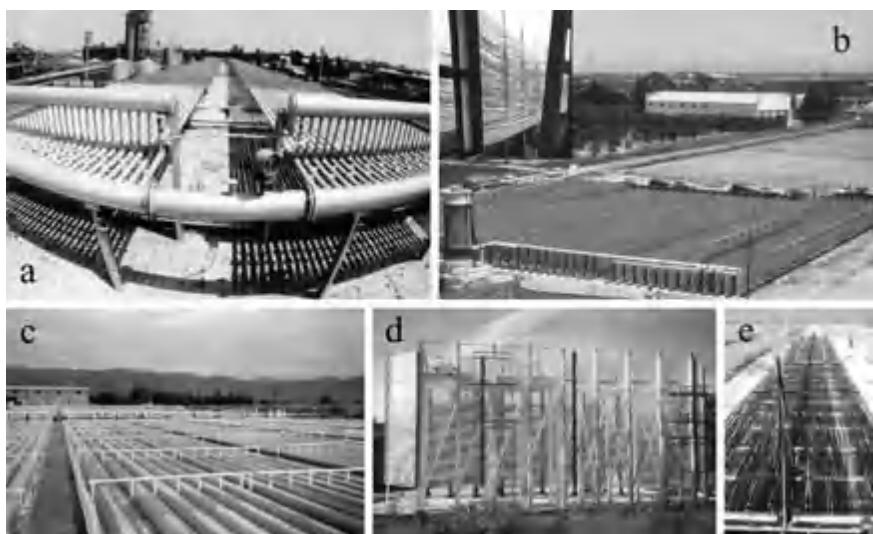


Fig. 9.6. (a) Sagdiana PGT manifold PBR at Javan (Tadzhikistan) (from Encyclopedia of bioprocess technology: fermentation, biocatalysis and bioseparation/Michael C. Flickinger, Stephen W. Drew; Copyright © 1999 by John Wiley & Sons, Inc. This material is used by permission of John Wiley & Sons, Inc.); (b) PBL photobioreactors in Santa Ana (Murcia, Spain) (from Encyclopedia of bioprocess technology: fermentation, biocatalysis and bioseparation/Michael C. Flickinger, Stephen W. Drew; Copyright © 1999 by John Wiley & Sons, Inc. This material is used by permission of John Wiley & Sons, Inc.); (c) The Hydrobiologica SA plant at La Rioja (Argentina) (photograph by G. Chini Zittelli); (d) Coiled PBR built by Inalco S.p.A. (Florence, Italy) (photograph by G. Chini Zittelli); (e) Microalgae S.p.A. PBR near Taranto (Italy) (photograph courtesy of G. Gregorini).

(more than $200 \text{ t ha}^{-1} \text{ year}^{-1}$) attracted investments and led to the creation of Photo Bioreactors Ltd (PBL UK) in 1986. Three years later, PBL Spain (PBL SA) was founded with investments from private industries and public Spanish sources and a commercial plant for the production of *Dunaliella* was established in Santa Ana near Cartagena (Murcia, Spain). Full-scale activity started in May 1990, using a fence-like system made of 1.2-cm diameter, 50-m long polyethylene tubes connected to vertical manifolds for a total tubing length of 125 000 m (Fig. 9.6b). Circulation was provided by an airlift; temperature was controlled by shading the reactors with nets or by water spraying. Using 200 000 m of tubing a full-scale near-horizontal manifold system was built later according to the design patented by Pirt (1983). The culture flowed from a tank through a descending conduit to the higher ends of a set of 50-m long tubes supported by a slightly inclined framework. Each tube communicated at its lowest point with its own riser, which led up to the common reservoir. Several major technical errors are apparent in both systems: The tube diameter was too small for effective mixing, the material degraded quickly under sunlight, degassing was inadequate, wall growth was almost unavoidable, and temperature control was insufficient. The very high s/v in relation to the length of the tubes, together with the insufficient circulation and the improper management of the culture led to poor growth

of the alga, biofouling and heavy contamination. In September 1991, PBL closed without ever entering into production.

9.4.1.3 Hidrobiologica SA (Argentina)

In 1996 Hidrobiologica SA, with an initial investment of about €2 million, built about 15 Km south of La Rioja (northern Argentina) the largest PBR known at that time (Tredici, 1999). The system consisted of 96 polyethylene tubes 120-m long and 25.5 cm in diameter (Fig. 9.6c). The tubes were laid parallel on the ground and arranged like a manifold with feeding, connecting and collecting channels made of concrete. The tubes, only partially filled, were maintained at low positive pressure so that they assumed a roughly elliptical shape (about 35-cm wide and 9-cm high). The surface occupied by the whole plant, including the gaps between modules, was about 5000 m²; the total culture volume was 600 m³. The culture (*A. platensis*) was circulated at a flow speed between 6 and 10 cm s⁻¹ by a single axial flow pump with a capacity of 900 m³ h⁻¹. During the initial period of activity, a productivity of about 0.2 g l⁻¹ d⁻¹ was attained and the biomass produced was of excellent quality (Chini Zittelli, personal communication). In the long run, however, several problems became apparent, among which inadequate mixing and biofouling deriving from low flow speed and unequal distribution of the culture among the tubes. Other problems were the limited capacity to control temperature, a major drawback in summer, and oxygen build up to dangerous levels despite gas outlets that were provided half way along the tubes. Despite complete restructuring (a tubing of lower diameter was adopted and culture circulation was improved), the plant was closed in the summer of 1999.

9.4.1.4 Inalco S.p.A (Italy)

A 3000-l coiled photobioreactor was built in 1996 by Inalco S.p.A near Florence (Italy). The unit, 7-m high and occupying an area of about 40 m², consisted of 5-cm diameter polycarbonate tubes connected in series by Plasson® elbows and secured to an upright iron hexagonal frame with an inclination to the horizontal of about 1.4° (Fig. 9.6d). Despite the fact that the reactor was successfully used for more than six months in the cultivation of *A. platensis*, the company stopped operations in 1998.

9.4.1.5 Microalgae S.p.A. (Italy)

In 1997 Microalgae S.p.A built a 14 m³ fully controlled horizontal manifold PBR in Crispiano near Taranto (Italy). In 1999 the company started its activity with six units made from 80 km of rigid plastic tubes about 3 cm OD and having a total capacity of 85 m³ (Fig. 9.6e). Mixing was attained through an airlift. Inline degassers provided exit for the photosynthetically produced oxygen. For unknown reasons, the company ceased activity in the winter of the same year.

9.4.2 Present enterprises

9.4.2.1 Ökologische Produkte Altmark GmbH (ÖPA) (Germany)

Among commercial PBR currently in operation, special mention goes to the 700 m^3 plant recently built at Klötze near Wolfsburg (Germany) by Ökologische Produkte Altmark GmbH (ÖPA) with an investment of about 16 million DM (Fig. 9.7a). The plant, the largest ever realised, started production in June 2000, after only seven months planning and construction based on the know-how developed by Pulz and collaborators (Pulz, 2001). The aim of ÖPA, which in the same location produces artificial peat by composting pine wood chips, is to recover as algal biomass (*Chlorella* sp., which is sold at €50 per kg dry wt), the CO_2 evolved during the composting process. The plant consists of 20 modules, 35 m^3 each, installed in a $12\,000\text{ m}^2$ greenhouse. The photostage is made from 6-m long, 48-mm diameter borosilicate glass tubes placed horizontally in a fence-like structure to form vertical walls about 3-m high and 0.8-m apart. The glass tubes (500 km total length), produced by Sklárny Kavalier a.s. (Sazava, Czech Republic) and supplied in lengths of 6 m, are joined by a special glue. Each unit is provided with an on-line control system. Mixing is achieved through centrifugal pumps. Harvesting is accomplished by two Westfalia separators, and the biomass is spray-dried. The expected productivity is 150 t year^{-1} , which means achieving a volumetric productivity of about $0.9\text{ g l}^{-1}\text{ d}^{-1}$ during an eight month operation period.

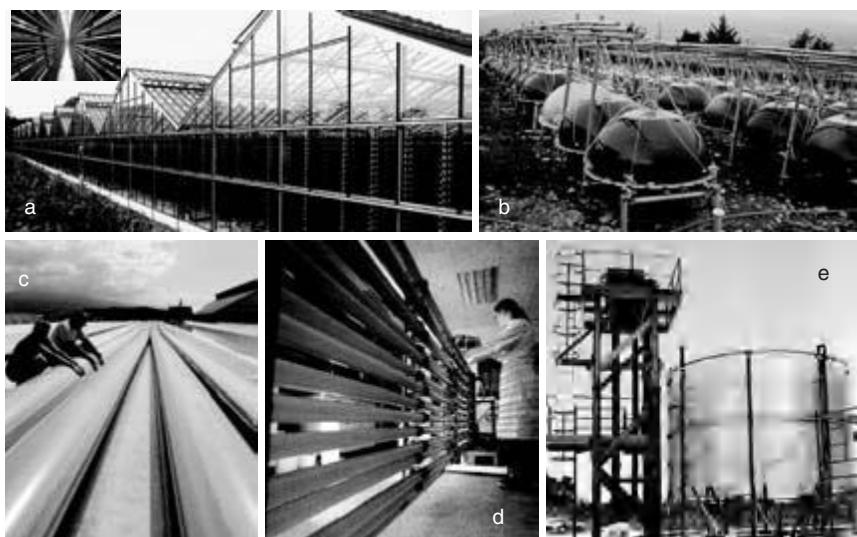


Fig. 9.7. (a) The 700 m^3 plant built at Klötze (Wolfsburg, Germany) by Ökologische Produkte Altmark GmbH (company brochure); (b) Bio-domes installed by Micro Gaia, Inc. at the Maui Research and Technology Park (Hawaii) (photograph courtesy of A. Vonshak); (c) Aquasearch Growth Module (Hawaii, USA) (photograph courtesy of M. Olaizola); (d) A bio-fence PBR (from Encyclopedia of bioprocess technology: fermentation, biocatalysis and bioseparation/Michael C. Flickinger, Stephen W. Drew; Copyright © 1999 by John Wiley & Sons, Inc. This material is used by permission of John Wiley & Sons, Inc.); (e) the AAPS developed by Addavita Ltd (Chesterfield, UK) (company brochure).

This seems a little too optimistic under the unfavourable climatic conditions of northern Germany. Extensive shading among the tubes arranged in a fence-like design mandates a mixotrophic approach. This is one of the best PBR ever designed and, if successful, will put Europe in a prominent position in the microalgal biotechnology field.

9.4.2.2 Micro Gaia, Inc (USA)

In 2000 Micro Gaia, Inc. installed on an area of about 8 ha at the Maui Research and Technology Park on the island of Maui (Hawaii) a plant based on its patented *bio-dome* photobioreactor (Hirabayashi, 1999, 2001). On January 2001, Micro Gaia, Inc. has begun production of astaxanthin for the Japanese market. By June 2001, one thousand 1.2-m diameter bio-domes were installed.

The bio-dome is a rather elaborate system made by coupling at the bottom two hemispheric transparent domes placed one on top of the other, convex surface up, so as to create a hemispheric culture chamber, from 2.5 to 10 cm wide (Fig. 9.7b). The culture is mixed and degassed by air which is bubbled into it by an apparatus that moves, *jumping* along the circular bottom of the bio-dome. The air tube, inserted from a top opening and connected to the moving device, cleans the domes walls by scraping their surface during its circular motion. The cylindrical opening on top of the external dome provides the exit for bubbled air and gases. Cooling is obtained by water spraying from the top of the reactor. An artificial light source can be placed below the bio-dome to integrate solar light. This system has two severe drawbacks: the need to build and connect thousands of units to realise a commercial plant, and difficulty in cleaning.

9.4.2.3 Aquasearch Inc (USA)

Three 25 0001 computer-controlled PBR, called Aquasearch Growth Modules (AGMs), have been recently developed at Aquasearch, Inc. (Kailua-Kona, Hawaii) for astaxanthin production from *Haematococcus pluvialis* (Olaizola, 2000). The AGM is a serpentine reactor made up of low-density polyethylene tubes, 0.18–0.41 m in diameter, laid parallel on the ground (Fig. 9.7c). The velocity of the culture flow is maintained high so as to obtain Reynold's numbers between 2×10^3 and 2×10^5 . Temperature is controlled by immersion in a water pond. During 1999, at standing biomass densities varying from 50 to 90 g m^{-2} , areal productivity rose from $9\text{ g m}^{-2}\text{ d}^{-1}$ in January to $13\text{ g m}^{-2}\text{ d}^{-1}$ in September. Given the high volume of the system, volumetric productivity was rather low (between 0.036 and $0.052\text{ g l}^{-1}\text{ d}^{-1}$). Unfortunately little information is available on important technical aspects such as mixing and reactor's design.

9.4.2.4 Biosynthesis Co (UK)

Biosynthesis Co (UK) commercialises a bioreactor, known as *bio-fence*, which consists of a vertical array of horizontal tubes connected to manifolds

in a fence-like configuration (Fig. 9.7d). The principle at the base of the bio-fence design is that, in order to avoid hydrostatic pressure causing the flow rate to decrease from the upper to the lowermost tube in the vertical tube array and thus maintain a homogeneous flow suitable for algae culture, a plurality of fence-like arrays are stacked one above the other extending between common manifolds. In this way the size of the apparatus may be increased without any increase in pumping capacity. This configuration has been patented both for rigid tubes (Mortimer *et al.*, 1999) and flexible material (Mortimer, 2000).

Bio-fence reactors are sold for waste water treatment and especially for growing microalgae in hatcheries at a cost ranging from about €15 000, for units of 400 l, to about €34 000 for units of 1200 l (i.e. at a cost of about €30 l⁻¹) which is very high (see for comparison the cost of glass plates in Section 9.3.4). The larger units comprise a 20-m long, 2.6-m high photostage, a conical tank, a pump and a control system. Bio-fence reactors suitable for growing fragile microalgae (like *T-iso* and *Chaetoceros* spp.) are offered at a cost about 12% higher.

9.4.2.5 Addavita Ltd (UK)

Addavita Ltd (Chesterfield, UK) commercialises modules of 400, 1000, 2000 and 5000 l of a coiled cultivation system called *Advanced Algal Production System* (AAPS) (Fig. 9.7e). Circulation of the culture is achieved through a pump or by an airlift accordingly to the type of algae grown. The slope of the tubes can be varied to optimise flow (Burbidge & Harper, 2000). The AAPS can be supplied with artificial lighting and control systems for pH and temperature. In 1999, a 2000 l unit of this kind was installed at Harbor Branch (Fort Pierce, USA) and failed because of difficulties in cooling and cleaning the system.

9.5 Design criteria for photobioreactors

Design criteria for PBR should aim at achieving high volumetric productivities and high efficiency of conversion of light energy, providing, at the same time, the necessary reliability and stability to the cultivation process by means of a cost-effective culture system. An efficient PBR can not be properly designed without adequate knowledge of the physiology in mass culture of the organisms to be cultivated. Since phototrophic microorganisms are highly diverse in their morphology, nutritional and light requirements, and resistance to stresses, PBR can not be designed so as to adapt to all organisms and all conditions (Tredici, 1999). The main design criteria for PBR include: surface-to-volume ratio, orientation and inclination, mixing and degassing devices, systems for cleaning and for temperature regulation, transparency and durability of the construction material. Ease of operation and scale-up, and low construction and operating costs also take on particular relevance in relation to commercial PBR.

9.5.1 Surface-to-volume ratio (s/v)

The ratio between the illuminated surface area of a reactor and its volume (s/v) determines the amount of light that enters the system per unit volume. Generally, the higher the s/v, the higher the cell concentration at which the reactor can be operated and the higher the volumetric productivity of the culture. The s/v also influences illuminated surface productivity: As found by Richmond and co-workers there is an optimal s/v (or light path) at which maximum productivity per unit of illuminated surface area is obtained (see Chapter 8). High cell concentrations reduce the cost of harvesting, as well as the cost of medium preparation and of culture handling. For this, high s/v PBR are generally preferred, but it must be emphasised that they may become highly inefficient systems when scaled up to industrial size (Tredici, 1999). In high s/v reactors, volumetric activities such as oxygen evolution, CO₂ absorption, nutrient depletion and metabolite excretion, change at a high rate and may have long-term negative effects on the stability of the culture, as shown by the problems encountered in the 1-cm diameter PBL photobioreactors set-up in Spain (see Section 9.4.1).

9.5.2 Orientation and inclination

Unlike horizontal systems, elevated PBR may be oriented and inclined at various angles to the sun and thus offer the possibility to vary irradiance at the reactor surface. The effect of PBR inclination on productivity has been investigated by Lee & Low (1991), Tredici & Chini Zittelli (1997) and Hu *et al.* (1998). While no great influence of the angle of inclination has been found at low latitudes with reactors placed in an east–west direction (Lee & Low, 1991), it has been shown that the tilt angle exerts a significant effect on productivity at higher latitudes (Tredici & Chini Zittelli, 1997; Hu *et al.*, 1998) (see Chapter 8). Generally, sun-oriented systems (south-facing and tilted so as to intercept maximum solar radiation) achieve higher volumetric productivities, while vertical systems or reactors arranged so as to dilute the impinging radiation attain higher efficiencies of solar energy conversion.

9.5.3 Oxygen accumulation

Accumulation of photosynthetically generated oxygen is one of the main factors that limit the scale-up of serpentine PBR. Oxygen production is directly correlated with volumetric productivity, and dissolved oxygen concentrations equivalent to four or five times saturation with respect to air, which are toxic to many phototrophs, may be easily reached in outdoor cultures, especially in tubes of reduced diameter (high s/v). At maximal rates of photosynthesis, a 1-cm diameter reactor accumulates about 8–10 mg oxygen l⁻¹ min⁻¹ (Weissman *et al.*, 1988). In serpentine reactors, the maintenance of oxygen levels below the toxic concentration requires frequent degassing and thus the adoption of very short loops or high flow rates, making the serpentine design difficult to scale up. Manifold systems

and vertical reactors (columns or flat panels) mixed by air-bubbling offer a significant advantage in this respect. A useful model for predicting dissolved oxygen and carbon dioxide concentration profiles in serpentine reactors has been developed and verified experimentally by Camacho Rubio *et al.* (1999).

9.5.4 Mixing

The type of device used to mix and circulate the culture is essential in PBR design. Mixing is necessary to prevent cells from settling, to avoid thermal stratification, to distribute nutrients and break down diffusion gradients at the cell surface, to remove photosynthetically generated oxygen and to ensure that cells experience alternating periods of light and darkness of adequate length. The fluid dynamics of the culture medium and the type of mixing influence average irradiance and the light regimen to which the cells are exposed, which in turns determine productivity. Typically, in serpentine reactors the culture is circulated at flow rates between 20 and 30 cm s⁻¹. The velocity in the tube controls turbulence and mass transfer capacity, thus greatly influencing the oxygen concentration in the culture and carbon dioxide supply (Molina Grima, 1999). The choice of the mixing device and of the intensity of mixing should be dictated by the characteristics of the organism to be cultivated. Generally, cultures of filamentous cyanobacteria and dinoflagellates cannot be mixed by pumps. Although even air bubbling may cause some stress to the cells at the level of bubble formation and break-up, bubble columns and airlift systems cause lower shear stress and therefore are recommended for fragile organisms. Increasing the aeration rate up to quite high values increases productivity, but damage may occur when the fluid microeddy size approaches cellular dimension (Sanchez Miron *et al.*, 1999). The influence of mixing on cell growth and culture productivity is described in detail in Chapter 8. Mixing devices and power requirement for mixing have been dealt with by Torzillo (1997). Camacho Rubio *et al.* (1999) have well addressed gas–liquid mass transfer and mixing in serpentine airlift bioreactors. A mass transfer model has been developed with which it is possible to predict dissolved oxygen concentrations at the end of the tubular loop, the composition of the outlet gas, the carbon dioxide requirements and the pH of the culture as functions of solar hour. The predictive capacity of the model has been supported in outdoor cultures of *P. tricornutum* (Camacho Rubio *et al.*, 1999).

9.5.5 Temperature control

Maximum productivity can be achieved only at the optimal temperature for growth. While open ponds are limited by low temperatures in the morning, PBR generally require cooling at midday. Shading, immersion in a water bath and water spraying are the most common solutions adopted to avoid overheating of PBR outdoors. Shading, to be effective, requires that a large portion of the reactor (up to 80%) be covered during the hours of maximum insolation, which causes a significant reduction of productivity. Cooling by

immersion in a water bath is efficient, but its cost-effectiveness is rather doubtful. Cooling by water spraying may be reliable and cost effective in dry climates. Economic considerations favour evaporative cooling over the use of heat exchangers.

9.5.6 Supply of carbon dioxide

Carbon nutrition is a major component of the production cost of microalgae. Supplying carbon dioxide in shallow suspensions at near neutral pH is not an easy task, the residence time of the bubbles being insufficient for complete absorption, resulting in great losses of CO₂ to the atmosphere. Gas injection as minute bubbles into a column of downcoming culture in which the culture velocity is adjusted to that of the rising CO₂ bubbles may increase the efficiency of absorption of CO₂. In this way the utilisation efficiency can be increased up to 70% (Molina Grima, 1999).

9.5.7 Materials

One fundamental criterion for construction of a suitable PBR is the material used for the photostage. Materials for PBR must lack toxicity have high transparency, high mechanical strength, of high durability (resistance to weathering), chemical stability and low cost. Ease of cleaning is also another important operational issue. The loss of transparency of several plastic materials exposed outdoors has been investigated (Tredici, 1999). Advantages and drawbacks of the most common tubular materials used for building PBR have been described by Tredici (1999).

9.6 Productivity in photobioreactors

9.6.1 Methods of evaluation and comparison (see also Chapter 8)

There are three parameters commonly used to evaluate productivity in photobioreactors. First is volumetric productivity (VP), i.e. productivity per unit reactor volume (expressed as g l⁻¹ d⁻¹), the second is areal productivity (AP), i.e. productivity per unit of ground area occupied by the reactor (expressed as g m⁻² d⁻¹); the third is illuminated surface productivity (ISP), i.e. productivity per unit of reactor illuminated surface area (expressed as g m⁻² d⁻¹). It must be noted that in vertical systems the illuminated surface area comprises both the front surface receiving beam radiation and the back surface and the side walls receiving reflected and diffuse radiation. The VP is a key parameter that illustrates how efficiently the unit volume of the reactor is used. However, it should be kept in mind that VP is a function of the number of photons that enter the unit reactor volume in the unit time and, as such, it is dependent on the s/v of the reactor. The higher the s/v, the higher the VP. We should be also aware of the fact that high s/v reactors may achieve high VP even if they perform poorly and that a VP of 1 g l⁻¹ d⁻¹ assumes a completely different significance if obtained in a reactor of 1 cm or 5 cm light path. Care should be taken to discern between AP and ISP. In the case of

ponds and horizontal or near-horizontal flat reactors, the ground surface area occupied by the system and its illuminated surface area substantially coincide, and so do AP and ISP. In the case of horizontal tubular reactors, placed with tubes in contact, the illuminated surface area is 1.57 times the occupied surface area, so ISP will be always lower than AP and both parameters can be easily calculated. In the case of horizontal tubular reactors with empty space between contiguous tubes and vertical or highly inclined systems, the situation is more complex. For example, in horizontal serpentine reactors it is difficult to decide whether and how to compute the empty space between tubes and how to account for the fact that horizontal serpentine reactors may intercept a different proportion of the radiation impinging on the horizontal, depending on the elevation of the sun and, hence, on the hour of the day. The performance of elevated systems may be even more difficult to evaluate, unless a fourth parameter for measuring productivity is introduced: the overall areal productivity.

9.6.2 Overall areal productivity

It may happen that, even when all the three parameters defined above (VP, AP and ISP) are provided, a complete evaluation of reactor performance in productivity terms is not attained. This is particularly true in the case of elevated photobioreactors (i.e. reactors set at an angle with the horizontal). To fully appreciate the performance of elevated PBR, a fourth parameter is needed: the productivity obtained from the overall (including empty spaces) ground area occupied by the several reactors that constitute the plant. In this chapter, this output is indicated as *overall areal productivity* or OAP (expressed as $\text{g m}^{-2} \text{ d}^{-1}$). OAP is a useful method to evaluate productivity; it has greater meaning for scale-up and permits comparison between different kinds of reactors, and between reactors and ponds.

Commonly, VP and ISP are calculated from the data obtained from operation of a single or of a few units. Sometimes also AP is given, but AP of elevated reactors is a meaningless parameter and should be always avoided. In fact, a vertical or highly inclined reactor intercepts a much higher amount of radiation than that impinging on the ground surface area it occupies (i.e. the horizontal projection of the system on the ground), and expressing productivity of elevated systems in terms of AP may lead to high unrealistic figures (see earlier sections). There are cases in which the set-up of a single unit is the final goal. For example, a single PBR may satisfy the whole need for microalgae biomass in a hatchery. VP and ISP correctly describe the performance of the reactor in this case, although productivity per reactor and biomass production cost would have a greater significance. When, on the contrary, a single unit is experimented with to obtain data for scaling up the system, to be essentially achieved by setting up a number of units, it has to be realised that the productivities calculated using a single unit may be of limited use, since the outputs will be much influenced by the mode (essentially the distance) of setting up of the several units in the field. In this case, to properly evaluate productivity of vertical or highly inclined systems, an adequate

number of units must be set up and operated, and the OAP must be computed on the basis of the whole ground area occupied by the reactors (including the empty spaces), carefully considering peripheral effects.

If in the final location the reactors are spaced far apart (i.e. a small number of units are placed on the ground allocated to the production process and so that they do not significantly shade each other), their VP and ISP will be high, and not too far from the values observed by experimenting with a single unit. With this type of arrangement, however, the OAP will be low because of the empty non-productive space between the systems. If the reactors are placed at a short distance, they will shade each other, and VP and ISP will be generally reduced in an inverse relationship with the distance; OAP on the contrary will be increased. Which strategy is to be chosen depends on many factors, among which the cost of the land and of the reactor are the most important.

The correct evaluation of OAP in elevated reactors should be one of the principal targets of experiments during piloting. Unfortunately, this is rarely done, and the performance of the final plant is erroneously extrapolated from the data obtained with a single unit. Horizontal reactors differ much in this sense. With ponds and horizontal reactors, OAP may be correctly calculated from the performance (generally from the AP) attained by a single unit, since the increase of the unit size or the close setting of a number of horizontal units on the ground do not influence significantly the light regimen to which the cultures are exposed.

The performance of a PBR can also be evaluated by an economic analysis providing the cost of biomass production in the system under consideration. However, this type of evaluation may be not valid in all situations, since the cost of the biomass may change greatly with different algae and sites. For example, some cost factors that have large impact on the final cost of the biomass (e.g. cost of labour) change considerably from site to site.

9.7 Photobioreactors versus open ponds

Together with the open surface, the major drawback of open ponds is the fact that for practical reasons the water level can not be kept much lower than 15 cm (i.e. 150 l m^{-2}). Optimal standing crops for algae are generally below 100 g m^{-2} , hence the cell concentration of the culture in a pond must be maintained at about 0.65 g l^{-1} or below. These diluted, open algal cultures become readily contaminated by other faster-growing microalgae, bacteria and protozoa. PBR have much higher s/v ratios (typically from 20 to 200 m^{-1}) than open ponds (from 5 to 10 m^{-1}) and can sustain much higher cell concentrations. The higher population density, together with a more protected environment and a better control over growth parameters (pH, pO_2 , pCO_2 and temperature), makes closed reactors relatively safe from invasion by competing microorganisms.

Comparison of performances achieved by open ponds and PBR may not be easy, as the evaluation depends on several factors, among which the algal species cultivated and the method adopted to compute productivity are most important. A couple of examples may help to clarify this point.

A. platensis is a well-known cyanobacterium that requires a specific and selective medium and can be grown outdoors in large-scale open ponds. When *A. platensis* was cultivated in parallel in open ponds and in a serpentine tubular reactor, the culture in the closed reactor achieved a 50–65% higher areal productivity on an annual basis (Torzillo *et al.*, 1986). The reason for this was a better diurnal temperature profile, that permitted higher daily outputs, and an extended cultivation period. In July, when mean areal productivities of $15\text{ g m}^{-2}\text{ d}^{-1}$ were attained in the ponds, mean productivity in the tubular reactor was $25\text{ g m}^{-2}\text{ d}^{-1}$. In the case of *A. platensis*, the higher productivity attainable by the closed system is evident. Why, then, is *A. platensis* not commercially produced in PBR? Despite some attempts carried out even at a commercial level (for example, at Hidrobiologica SA in Argentina), the inevitable conclusion is that the higher cell concentration and higher productivity achieved by *A. platensis* in PBR do not compensate for the higher capital and operating costs. A similar situation applies to *Dunaliella salina*.

In contrast with *A. platensis* and *Dunaliella*, *Nannochloropsis*, a marine eustigmatophyte widely cultivated as feed for rotifers, does not show particular growth requirements. Attempts have been made to cultivate *Nannochloropsis* in 3000 m^2 raceway ponds (Sukenik, 1999), but typically the cultures collapse after a few months from the start due to predation by protozoa and contamination by other algal species. Chlorination and use of antibiotics do not prevent the problem (Sukenik, 1999). Chini Zittelli *et al.* (1999) cultivated *Nannochloropsis* outdoors in near-horizontal tubular reactors (NHTR) from March to September (see Chapter 16). The mean cell concentration was about 5 g l^{-1} , the volumetric productivity averaged $0.6\text{ g l}^{-1}\text{ d}^{-1}$ and the mean areal productivity was $12.5\text{ g m}^{-2}\text{ d}^{-1}$. Contamination by protozoa and other microalgae, a serious matter in open raceways (Sukenik, 1999), was not a problem. With *Nannochloropsis*, the tubular reactor offers no advantage in terms of areal productivity, but largely surpasses the ponds in terms of volumetric productivity (eight times higher) and cell concentration (about 16 times higher). Richmond and co-workers cultivated *Nannochloropsis* in a vertical 500-l glass flat reactor (see Chapter 16). Productivity per illuminated surface area was not better than that attained in the ponds. The system, however, provided year-round sustainable production with no contamination. The closed environment, the higher degree of control over culture parameters, and the higher cell concentration attainable in PBR effectively protect the culture from contamination and make cultivation of this important microalga feasible. The cost of biomass production in closed reactors may be an order of magnitude higher than in the ponds, but, in this case, low enough to be attractive for aquaculture use.

9.8 Scale up of photobioreactors

With two exceptions (Micro Gaia that uses *bio-domes* and Aquasearch that uses serpentine modules made up of very large diameter flexible tubes)

commercial plants adopt manifold PBR. Even the systems that are commercially sold for small scale production of microalgae (bio-fences and biocoils) are based on a manifold arrangement of the tubes. The serpentine design, much experimented with at the research level, is not favoured for scale up. Molina Grima and co-workers at the Department of Chemical Engineering of the University of Almeria (Spain), who for a long time experimented with serpentine reactors, now favour vertical bubble columns and airlift cylinders for scaling up microalgae cultivation (Sanchez Miron *et al.*, 1999). According to these authors, horizontal serpentine PBR reveal limited scalability, because as tube length increases, pH and dissolved oxygen rise to very high values, but above all because of inadequacy of illumination. Oxygen removal capacity is governed by the magnitude of the overall gas–liquid mass transfer $k_{La,L}$, which in bubbled columns is about fourfold the estimated value for a horizontal serpentine. Although vertical bubble columns and airlift cylinders have a low s/v, they have substantially greater gas hold-ups than horizontal reactors and a much more chaotic gas–liquid flow, and consequently can attain substantially increased radial movement of fluid that is necessary for improved light–dark cycling (Sanchez Miron *et al.*, 1999). Thus, cultures in vertical bubble columns suffer less from oxygen inhibition and photoinhibition, and experience a more adequate light–dark cycle. The limitations of bubble columns may be their cost and the fact that, since diameter and height can not be much increased, a large number of units are needed to build a commercial plant.

The vertical plates mixed by air bubbling developed by Tredici and co-workers and improved by Richmond (see Section 9.3.4), seem even better than bubble columns in terms of productivity and ease of operation. It has been shown that vertical flat plates of 1000–2000 l in volume can be built and successfully operated for long periods, so they have potential for scale up. If the findings of Pulz (2001) that closely packed flat reactors achieve very high overall ground-areal productivities through lamination of solar light are to be confirmed, glass panels mixed by air bubbling might become the commercial PBR of the next decade.

A completely different approach to bring light to the cultivated algal cells is adopted in those systems in which light capture is physically separated from cultivation. Light is collected by mirrors, concentrated through lenses and delivered to the culture through optical fibres or transparent rods (Mori, 1985; Ogbonna & Tanaka, 1997). Janssen (2002) has proposed a rectangular airlift PBR, 10-m high, 5-m wide and 5-m thick, containing 80 light-re-distributing plates 3-cm thick and placed 3-cm apart. Solar light is collected by parabolic dishes from a field of about two hectares and conveyed through glass fibres to the light-re-distributing plates so that an average irradiance of $1200 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ is provided at the plate surface. Assuming a 10% efficiency of conversion, the author estimates the productivity of such a conceptual system at $193.5 \text{ t year}^{-1}$. Cultivation of microalgae in optical fibre PBR has been demonstrated only at the laboratory scale, and although an interesting path of research, the very high cost of mirrors, lenses, solar tracking devices and optical fibres, puts a severe limitation to the commercial application of this design.

9.9 Concluding remarks and prospects

Photobioreactors are considered to have several advantages over open ponds: They can prevent or minimise contamination, offer better control over culture conditions, prevent evaporation and reduce CO₂ losses due to outgassing, and attain higher cell concentrations and volumetric productivities. Elevated reactors can be oriented and tilted at different angles and can use diffuse and reflected light, which plays an important role in productivity. PBR can be built with various light paths, a key issue to reach very high productivities and efficiencies of solar energy utilisation, and can be mixed by different types of pumps or by air bubbling. Many different materials can be used for their construction: collapsible or rigid tubes, plastic or glass sheets, and many different designs are available (serpentine, manifold, helical, flat). In conclusion, closed reactors are flexible systems that can be optimised according to the biological and physiological characteristics of the algal species being cultivated. The ultimate and most important advantage of closed systems, however, is that they permit cultivating algal species that cannot be grown in open ponds.

Closed systems, on the other hand, suffer from several drawbacks (overheating, oxygen accumulation, biofouling, cell damage by shear stress, deterioration of the material used for the photostage) that can lead to disasters (see Section 9.4.1) when not duly considered and solved. The main limitation of closed systems seems to be the greater difficulty in scaling up compared to raceway ponds. As a result of these limitations, PBR are expensive to build and operate, and the production cost of algal biomass in PBR may be as high as one order of magnitude higher than in ponds. Thus, despite their advantages, PBR will not have a significant impact in the near future on any product or process that can be attained in large outdoor raceway ponds. PBR and open ponds should not be viewed as competing technologies. The real competing technology is genetic engineering, as shown by the two examples that follow.

At present, heterotrophic cultivation of microalgae in fermenters is of limited application because most microalgae are obligate photoautotrophs, but bringing into photoautotrophs the capacity to use organic molecules as energy and carbon sources is no more a remote target. Zaslavskiaia *et al.* (2001) have recently achieved the trophic conversion of the photoautotrophic diatom *P. tricornutum* by transforming the alga with a single gene encoding a glucose transporter. When this capacity is extended to other microalgae, many high-value products will then preferably be obtained through heterotrophic cultivation of these transgenic microorganisms in fermenters.

A novel anaerobic polyketide synthase-like pathway for the biosynthesis of polyunsaturated fatty acids (PUFAs) has been recently discovered in marine organisms (Napier, 2002). These novel genes can be used to direct the synthesis of PUFAs in heterologous hosts since introducing and regulating this gene cluster is relatively simple compared to the more than five desaturase and elongase genes required for the typical aerobic pathway. This achievement makes the synthesis of PUFAs via transgenic organisms possible.

The ability of photoautotrophic technologies to compete with fermentation technologies and transgenic organisms for the production of high-value compounds will depend on breakthroughs in PBR development able to bring the cost of photoautotrophic biomass production from the €50–100 kg⁻¹ attainable today to about 1/10 of that cost (although in China, Thailand and India reports indicate *Spirulina* sp. dry mass, produced in open raceways, sells for less than €10 kg⁻¹, indicating the cost of production being highly dependent on the cost of labour). This goal can be achieved only by substantial improvement in PBR design and cultivation techniques, development of new low-cost transparent materials and significant increase of productivity.

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10 Downstream Processing of Cell-mass and Products

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10.1 Introduction

Processing is rapidly becoming a major area of R&D in microalgal biotechnology, and could become a greater impediment to commercialization of microalgal products than either cultivation or harvesting. This chapter deals with some of the most usual methods for harvesting the microalgal biomass as well as the most relevant operations used in downstream processing. In each operation, a brief description of the general principles governing the design and operation of the downstream processing equipment is presented. In addition, some relevant works in the field of algal biotechnology concerning each particular operation are summarized. Finally, a case study is analyzed regarding an integrated process of biomass production and product recovery for eicosapentaenoic acid (EPA) production from microalgae, including economic evaluation.

10.2 Harvesting

All downstream processing of microalgal cultures involves one or more solid-liquid separation steps. The biomass may need to be separated from the culture medium, or cell debris removed following cell disruption for release of the metabolites of interest. Biomass is usually harvested by sedimentation, centrifugation or filtration, sometimes requiring an additional flocculation step. Gudin & Therpenier (1986) reported that microalgal cell recovery accounted for at least 20–30% of the total cost of production. The problem is due to a combination of the small size of the microalgae (3–30 µm) and their low concentration in the culture medium (below 500 mg l⁻¹ in some industrial production units).

Algal harvesting is likely to remain an active area of research, and although a *universal* harvesting method does not exist, experience has demonstrated that for all algal species it is possible to develop an appropriate, economical harvesting system. Moreover, each production system will require a harvesting process optimally adapted to both the requirements of the organism and of the processing steps that follow harvesting (Gudin & Chaumont, 1991).

10.2.1 Flocculation

Flocculation is the collection of cells into an aggregate mass by addition of polymers. Aggregated microalgal cells offer advantages by facilitating cell-broth separation. Aggregation as the result of pH adjustment or electrolyte addition is regarded as *coagulation*, whereas aggregation as the result of polymer addition is termed *flocculation* (Mackay, 1996; Boonaert *et al.*, 1999).

Chemicals reduce the cell surface charge and form precipitates that enhance the clustering process and sedimentation. Two major forces are involved: at large distances, electrostatic repulsion dominates (negative cells repel other negatively charged cells). At very short distances, however, intermolecular or Van der Waals attraction occurs. This force is large compared to electrostatic forces, but it acts over a very short range. Fig. 10.1 shows the two forces and their sum, acting on waterborne colloids which a microalgae culture resembles: at larger distances the repulsive forces dominate, whereas at shorter distances the attractive forces dominate. Coagulants can be used to reduce the electrostatic repulsive forces, thereby increasing the distance at which attraction could occur.

The addition of coagulants containing divalent or trivalent cations can both reduce the negative surface charge of the cells and form a precipitate in which to trap additional cells. The reduction in electrostatic repulsion is due to the reduction of the surface charge on negative cells by salts like ferric chloride (FeCl_3), aluminium sulphate or alum ($\text{Al}_2(\text{SO}_4)_3$) and ferric sulphate ($\text{Fe}_2(\text{SO}_4)_3$). The efficiency of electrolytes to induce coagulation of suspended cells is usually expressed by the concentration required to achieve a rapid coagulation, called critical coagulation concentration. This efficiency increases as the counterion valence increases.

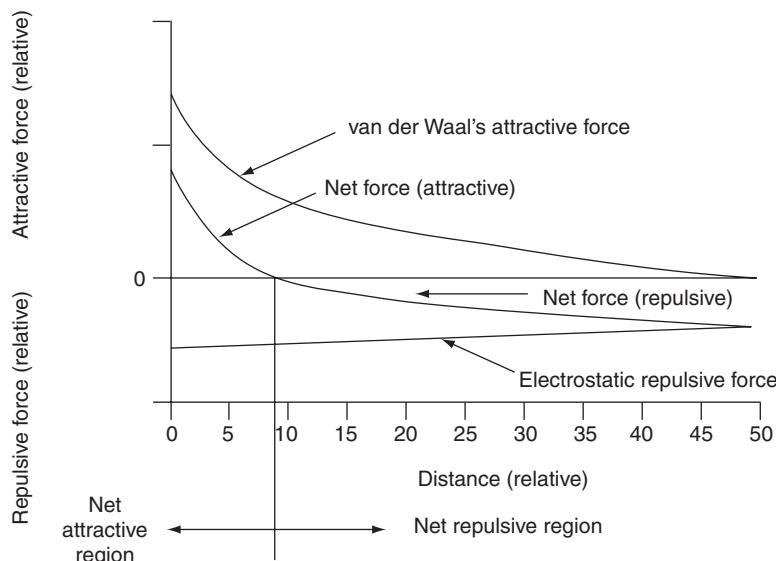


Fig. 10.1. Attractive and repulsive forces acting on waterborne colloids (Ray, 1995). Reproduced with permission.

Harvesting of algal cells by flocculation is more convenient than by conventional methods such as centrifugation or filtration, because it allows large quantities of culture to be treated. Chemical flocculation has become the method of choice in removal of algae from waste treatment ponds and other waste applications. The chemicals and the flocculation process are, however, expensive, being only marginally cheaper than centrifugation. For applications of fine chemicals, flocculation is considered as a pre-treatment previous to other operations (e.g. centrifugation). For aquaculture, flocculation by pH adjustment is currently used. Although flocculation is considered as the most suitable method for microalgal biomass harvesting, this method involves economic or technical drawbacks, such as a high energy cost, flocculant toxicity, or non-feasibility of scaling up (Hee-Mock *et al.*, 2001).

10.2.1.1 Flocculation by pH adjustment

Flocculation techniques based on pH manipulation are being developed, and algal concentrates harvested by this technique are being used to supplement natural microalgae for rearing Pacific oyster *C. gigas* spat, housed in field nursery upseller systems (McCausland *et al.*, 1999). pH values between 11.8 and 12 induce extensive flocculation without adding any electrolyte. In both cases, total solids abatement was more than 95%, producing sludge of excellent settling and good mechanical resistance. Flocculation becomes significant when the pH value is ca. the theoretical value of magnesium hydroxide precipitation, i.e. 11.3, disregarding the suspension ionic strength (Alexeev, 1980), confirming the importance of magnesium hydroxide precipitation in the flocculation mechanism (Elmaleh *et al.*, 1991). The addition of seawater slightly improves the total solids abatement when pH is lower than 12 and seawater concentration under 40%; when pH is higher than 12 no seawater is required. Blanchemain and Grizeau (1999) studied the decantation of *Skeletonema costatum* at different pH values: sedimentation was higher than 80% at pH values higher than 10.2. Although the cells were disrupted and internal metabolites were released, the lipid composition of the biomass was not modified.

10.2.1.2 Flocculation by cationic polymers

Cationic polymers neutralize the negative surface charges of cells, and polymeric flocculants modify electrostatic repulsive interactions, inducing bridging mechanisms. The important properties of a polymeric flocculant are: (1) molecular weight, (2) charge density, and (3) structure configuration. With a cationic polyelectrolyte of high charge density, the coagulant effect and the influence of polymer molecular mass and ionic strength can be explained by the electrostatic patch model. The adsorbed polyelectrolyte represents patches of positive charges surrounded by the original cell surface. The optimum flocculation concentration, therefore, corresponds to a situation where the cell surface is partially covered by patches of cationic polymers. Adsorption of the cationic polymer by the negatively charged cell algae reverses the charge and thus reduces the force of repulsion between cells.

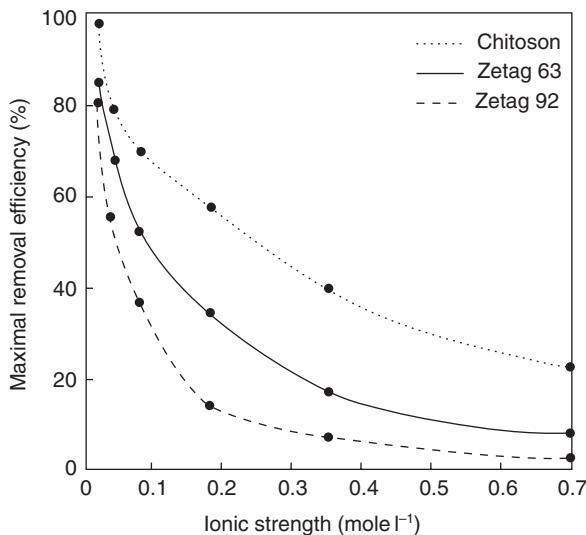


Fig. 10.2. The effect of ionic strength on the maximal removal efficiency of algae by flocculation with commercial polymers. Chitosan (molecular weight = $4 \cdot 10^5$ g mol⁻¹; chemical character = derivative of chitin, cationic), Zetag 63 (molecular weight = $1 \cdot 10^7$ g mol⁻¹; chemical character = cationic polyacrilamides), Zetag 92 (molecular weight = $2 \cdot 10^7$ g mol⁻¹; chemical character = cationic polyacrilamides) (Bilanovic *et al.*, 1988). Reproduced with permission from Elsevier Science.

The adsorbed polymers, because of their size, are able to form a bridge between cells, bind them together, and bring about flocculation.

With cationic polymers of low ionicity, the influence of charge density, concentration and the molecular mass is more complex. The influence of ionic strength is also complex because it affects the screening of segment–segment lateral repulsion and the screening of segment–surface attraction. In practice, increasing ionic strength reduces the concentration at which the flocs initially appear and broadens the flocculation domain (Boonaert *et al.*, 1999). Organic cationic polymers can induce efficient flocculation of freshwater microalgae at low dosages, between 1 and 10 mg l⁻¹, the high salinity of the marine environment inhibiting flocculation with polyelectrolytes (Bilanovic *et al.*, 1988). Effective flocculation was attained at salinity lower than 5 g l⁻¹. At high ionic strength, the polymer shrinks to its smallest dimensions, and fails to bridge between algal cells (Fig. 10.2). Flocculation performance is a function of many factors, including:

- (i) Flocculant molecular weight: the higher the molecular weight the better the bridging.
- (ii) Flocculant charge density: the higher the charge density the more extended the molecular conformation and bridging and/or charge neutralization may be improved.
- (iii) Flocculant dose: overdosing can lead to stabilization of the biomass particles.
- (iv) Biomass concentration: the higher the cell concentration the higher the collision frequency and the higher the rate of flocculation.

- (v) Hydrodynamics: the presence of shear forces at an optimal (e.g. through gentle mixing) can improve the efficiency of biomass flocculation.
- (vi) Cell broth: pH and ionic strength affect the conformation of the flocculant molecules and hence the flocculation process. In practice, the adverse physiological effects on biomass as the result of pH extremes limit the useful range of pH adjustment.

A variety of chemicals have been tested as potential flocculants and the most effective was found to be aluminium sulphate, followed by certain cationic polyelectrolytes (Pushparaj *et al.*, 1993). Thus, chemicals such as lime and ferric-alum, although expensive in large amounts, have been widely used for treating wastewater and effluents (McGarry, 1970; Dodd, 1979; Benemann *et al.*, 1980; Moraine *et al.*, 1980; Koopman & Lincoln, 1983; Lincoln, 1985), although from an aquaculture perspective, these chemicals pose the potential risk of toxic residues if used as a food source. An edible non-toxic alternative is chitosan (polymer of acetylglucosamine), effective on various species of microalgae (Nigam *et al.*, 1980; Lavoie & de la Noüe, 1983; Morales *et al.*, 1985; Lubian, 1989). Lack of reported toxicity, ease of manufacture and low dosage rates for chitosan (Richmond & Becker, 1986) encourage its use, despite reduced efficiency in salt water.

Flocculation by different *polymers* had been extensively used. Tilton *et al.* (1972) studied the flocculation of *Chlorella ellipsoidia* at different algal concentrations ($50\text{--}3000\text{ mg l}^{-1}$), polymer concentrations ($0.01\text{--}1000\text{ mg l}^{-1}$) and pH (4–7). No flocculation at polymer concentrations from 0.01 to 200 mg l^{-1} took place, whereas cationic polyethyleneamine was effective. There was a significant increase in flocculation efficiency as the polymer molecular weight increased from 800 to about 2000 but little improvement above 2000. There was no effect of pH from 4 to 7. Cohen *et al.* (1957) showed that a cationic polymer flocculated the algae *Chlorella* but an anionic polymer did not. Golueke & Oswald (1965) reported that alum and synthetic organic polyvalent polymers were effective flocculants for *Scenedesmus* and *Chlorella*. Jiang & Harvard (1993) studied the effect of polyferric sulphate (PFS) versus traditional flocculants (Fig. 10.3). PFS was superior to other coagulants, believed to be due to the presence of a more highly charged cation species. Different methods for preparation of PFS showed (Odegaard *et al.*, 1990; Jiang & Harvard, 1993) that prepolymerized coagulants have the following advantages over metal salt coagulants: better overall treatment efficiency; better floc separation; wider pH working range; lower sensitivity to low temperatures; lower residual metal-ion concentration. Tilton *et al.* (1972) reported the effects of polymer molecular weight: (1) from 800 to 2000 MW the amount of polymer required to initiate flocculation decreased as the MW increases; (2) above 2000 MW the polymer requirements remained constant as the MW increased; (3) polymers in the 800–2000 MW range either did not produce charge reversal or required very high concentrations to do so; (4) polymers above 2000 MW reverse the charge and (5) high MW polymers disaggregated the flocs.

Optimal dosages of *chitosan* ranged from 40 mg l^{-1} for *Tetraselmis chui*, *Thalassiosira pseudonana* and *Isochrysis* sp. to as high as 150 mg l^{-1} for

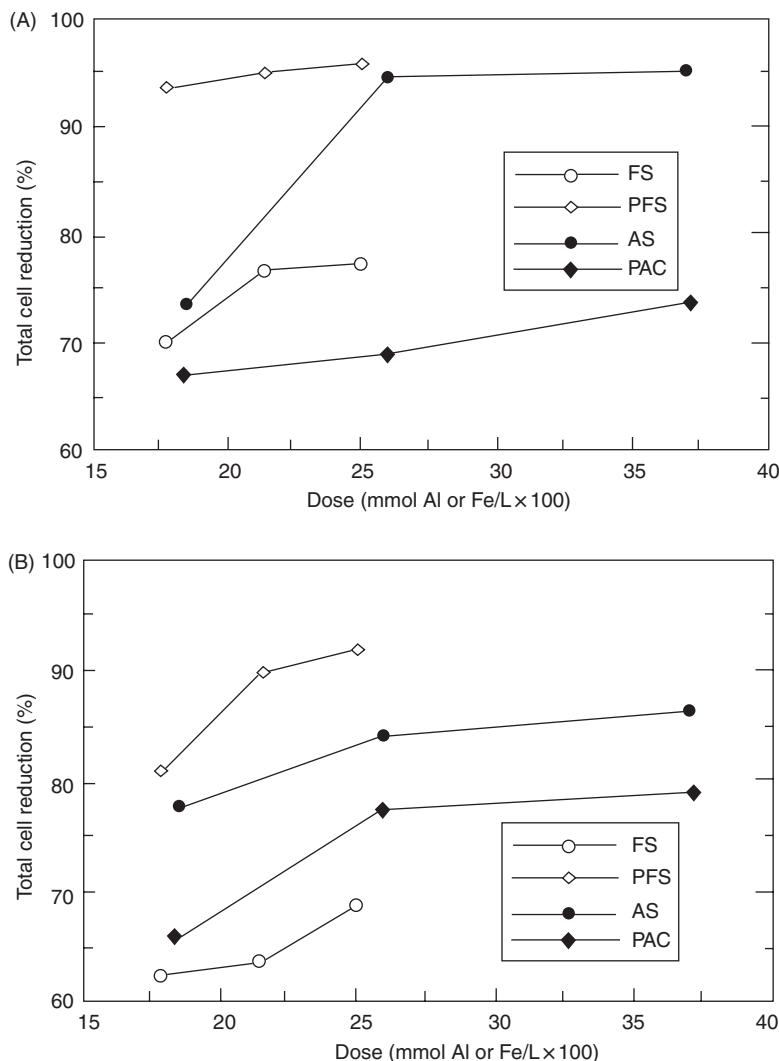


Fig. 10.3. Comparison of four coagulants for cell removal (FS: Ferric sulphate; PFS: Polyferric sulphate; AS: Aluminum sulphate; PAC: Polyacrilamides). (A) *Anabaena*, (B) *Asterionella* (Jiang & Harward, 1993). Reproduced with permission.

Chaetoceros muelleri, with no apparent consistency within algal taxonomic groups and influence of pH being observed (Heasman *et al.*, 2000). Although chitosan is not considered toxic to invertebrate larvae, Heasman *et al.* (2000) referenced a dramatic decrease of survival of oyster larvae when fed with microalgae flocculated with chitosan. Chitosan has been also proposed as a entrainment matrix of microalgae for the tertiary treatment of wastewater (Kaya & Picard, 1996).

Recently, much interest has been shown in the utilization of *bioflocculation* to harvest algae from suspensions of algal cultures (Cannell, 1990;

Suh *et al.*, 1997; Lee *et al.*, 1998). *Bioflocculation* by modifying the culture medium has been essayed for a variety of different microalgae, using both fresh and saltwater systems, the algae induced to flocculate and settle spontaneously. Also a microbial flocculant for harvesting mass cultured microalga has been screened (Hee-Mock *et al.*, 2001). An excellent *bioflocculant* for mass culture of *Chlorella vulgaris* from *Paenibacillus* sp. AM49 was identified (Hee-Mock *et al.*, 2001).

10.2.2 Centrifugation

Almost all types of microalgae can be separated from the culture medium by centrifugation. A centrifuge is basically a sedimentation tank with enhanced gravitational force to increase the rate of sedimentation. Although centrifugation is a unit operation widely treated in text books, the approach of Chisti & Moo-Young (1991) guidelining use of this operation for microbial cultures is strongly recommended. Advances in structural steels have made possible the use of high-speed, corrosion-resistant, centrifuges for large-scale bioprocesses. Easy cleaning and availability of sterilizable machines have made centrifugation the main separation operation of microalgal biomass. The recovery of biomass in a sedimenting centrifuge depends on: (i) the biomass settling rate, (ii) the biomass residence time within the centrifugal field, and (iii) the biomass settling distance. The factors that determine the biomass settling rate will be seen below. The residence time can be increased by reducing the flow rate through the centrifuge and the settling distance can be reduced, thereby enhancing biomass recovery, e.g. in disk stack centrifuges (Mackay, 1996).

Three types of centrifuges can be used: tubular bowl, disc-stack bowl and scroll discharge decanter, depending on particle size ranges (Fig. 10.4). The tubular bowl attains high *g* numbers (see equation (10.2)) that permits good

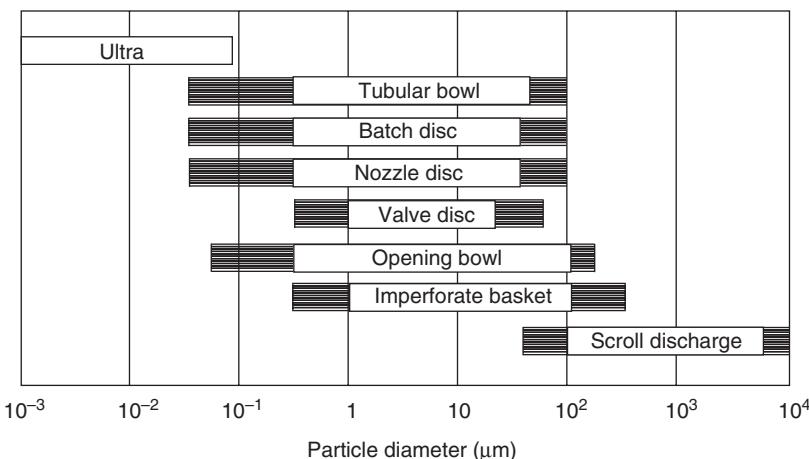


Fig. 10.4. Particle size range for different types of centrifuges.

solids dewatering but the capacity is very limited. This sort of centrifuge can be of interest preferably for diluted small volumes.

Disc-stack centrifuges come in several types depending on whether the solids are retained or discharged and on the mechanism of discharge of solids. The general features are: (i) angle of discs 35–50°; (ii) settling distance 0.5–2 mm; (iii) centrifugal force 4000–15 000 g; (iv) feed biomass content 0.2–20% v/v; (v) the discharge nozzle valve can be opened for brief periods to control the biomass moisture content (Mackay, 1996).

The decanter bowl discharge centrifuge is suitable for slurries with high biomass contents (5–80% v/v). Scroll decanter centrifuges (screw centrifuges) consist of a solid bowl, tapered at one end, and a close-fitting helical screw, which rotates at a slight differential speed to the bowl. The g-forces are smaller (4000–10 000) and the clarity of the fermentation broth is not usually as good as with disc stack machines (Mackay, 1996).

The particle velocity achieved in a particular centrifuge compared with the settling velocity which would occur under the influence of gravity characterizes the effectiveness of centrifugation. The terminal velocity during the centrifugal settling, u_c , of a small spherical particle in dilute suspension is given by Stoke's law:

$$u_c = \frac{(\rho_p - \rho_f)d_p^2\omega^2r}{18\mu_f} \quad (10.1)$$

where ρ_p and ρ_f are the densities of the particle and the culture media, respectively, d_p is the particle diameter, ω is angular velocity of the bowl which is related to N (rpm) by $\omega = \pi N/30$, μ is viscosity of the culture media and r is the radius of the centrifuge drum. The ratio of velocity in the centrifuge to velocity under gravity is called the centrifuge effect or g-number, and is usually denoted as Z . Therefore

$$Z = \frac{\omega^2 r}{g} \quad (10.2)$$

The force developed in a centrifuge is Z times the force of gravity, and is often expressed as so many g-forces. Industrial centrifuges have Z factors of 3000–16 000. From equation (10.1), the settling rate in a given centrifuge can be increased by: (i) increasing the centrifuge speed, ω ; (ii) increasing the particle diameter, d_p ; (iii) increasing the density difference between particle and liquid, $\rho_p - \rho_f$; and (iv) decreasing the viscosity of the suspending fluid, μ . Performance of centrifuges of different sizes can be compared using as parameter the sigma factor, Σ . Physically, Σ represents the cross-sectional area of a gravity settler with the same sedimentation characteristics as the centrifuge. For continuous, Σ is related to the feed rate of material as follows:

$$Q = \Sigma u_g \quad (10.3)$$

where u_g is the terminal velocity of the particles in a gravitational field (given by equation (10.1) by changing the centrifugal acceleration, $\omega^2 r$, by the gravitational acceleration (g), and Q is the volumetric feed rate to the centrifuge required to settle all the particles of gravitational settling velocity u_g . If two centrifuges, 1 and 2, perform with equal effectiveness (Mackay, 1996)

$$\frac{Q_1}{\Sigma_1} = \frac{Q_2}{\Sigma_2} \quad (10.4)$$

Selection of a centrifuge for any application would almost always involve expensive pilot scale evaluations. A few simple laboratory tests of centrifugation and gravitational sedimentation can provide indications about the difficulty of the separation by centrifugation.

Centrifugation is often considered as the preferable microalgae harvesting method (Benemann *et al.*, 1980; Mohn, 1980; Richmond, 1986), especially for developing extended shelf-life concentrates that would collectively meet the requirements of marine hatcheries and nurseries (D'Souza *et al.*, 2000; Heasman *et al.*, 2000). Mohn (1980) compared the efficiency of different centrifugal machines (self-cleaning plate separator, nozzle centrifuge, screw centrifuge and hydrocyclones) to harvesting different microalgal species cultures.

The operational conditions of the centrifugation also determine the efficiency of the process. Heasman *et al.* (2000) studied the influence of centrifugation procedure in the harvest efficiency and cell viability of nine different strains of microalgae. Centrifugation forces ranged from 1300 to 13 000 g. Harvest efficiency was higher than 95% only at the maximum centrifugation force, 13 000 g, decreasing to 60% at 6000 g, and to 40% at 1300 g. Cell viability varied significantly with respect to species, methods of centrifugation and the interaction of the two factors. Several changes in centrifuge technology have impacted the performance of the machines since 1990, providing purer and drier cake, as well as self-cleaning units (Letki, 1998).

10.2.3 Filtration

Rotary vacuum drum filters and the chamber filter press appear to be the commonly employed type of filters in harvesting fairly large microalgae (Mohn, 1980). Rotary filters are available either for suction (or vacuum) operation or for pressure operations. These filters have the advantage of continuous operation and are useful when sterility and containment are not stringent.

A rotary vacuum filter consists of a drum frame covered with filter cloth (canvas, nylon, dacron, metal or glass fibre). The internal volume of the drum is divided into radial chambers to which vacuum may be applied. The drum rotates (0.1–2 rpm) partly submerged in the agitated algal slurry to be filtered. Application of vacuum (250–500 mmHg) to the submerged chambers ($\approx 30\%$ of filter area) of the drum results in the slurry being drawn through the filter cloth. When high filtering capacity and no washing are desired, filter drums with 60–70% submerged filter area may be used.

A precoat filtration scheme employing filter aids was described by Gudin & Therpenier (1986): diatomaceous earth or cellulose is filtered through the filter cloth to form a porous cake of the filter aid. Subsequently the culture is filtered through this cake and as a layer of solids deposits on the cake it is scraped off together with a thin layer of the filter aid thereby exposing fresh filtration surface.

The flux of filtrate, i.e. the volume of filtrate (V) collected per unit time (t) per unit filter area (A), is related to the pressure drop driving force (ΔP_f), the viscosity of the continuous phase (μ) and the cake's resistance (R_c) by the basic expression for filtration

$$\frac{1}{A} \frac{dV}{dt} = \frac{\Delta P_f}{\mu_f R_c} \quad (10.5)$$

assuming the resistance of the filter medium is negligible. R_c for incompressible cakes is described by the equation

$$R_c = \alpha S_0 \left(\frac{V}{A} \right) \quad (10.6)$$

where α represents the specific cake resistance and S_0 is the mass of cake biomass per volume of filtrate.

In summary, cake formation can be altered by carrying either the total cycle time t_{cycle} or the fraction of the total cycle time devoted to cake formation β . In addition, at constant β , the filtration flux is inversely proportional to the square root of the cycle time (Belter *et al.*, 1988).

Harvesting of two different sized algal cultures by using filtration by pressure or vacuum conditions was analyzed by Mohn (1980). The chamber filter press and the belt press were determined as the most adequate devices both operating at pressure or under vacuum conditions (Table 10.1). With a chamber filter press, a concentration factor of 245 for large microalgae *Coelastrum proboscideum* was obtained, allowing cakes with solids content of 27%. This high solids content of the cake strongly reduces the posterior dehydrating process. Filtration, however, is only suitable for harvesting fairly large microalgae (e.g. *C. proboscideum* or *Spirulina platensis*) and fails to separate organisms approaching bacterial size like *Scenedesmus*, *Dunaliella* or *Chlorella* species (Mohn, 1980). Thus, filtration of *Dunaliella* through sand filters, cellulose fibres and other filterable materials has not proved practical (Ben-Amotz & Avron, 1987). The only method to be patented (in Australia) for large-scale filtration of natural growing *Dunaliella* in salt ponds is by passing the diluted culture through diatomaceous earth, the filtered algae then being directly extracted by an organic solvent (Ruane, 1977).

10.2.4 Microfiltration and ultrafiltration

As stated in the previous sections, centrifugation of microalgal suspensions is often quite efficient but expensive, and the centrifugal effect is responsible for cell damage. Flocculation can also induce toxic effects such as chemical-

Table 10.1. Recommendable machines tested for the harvesting of microalgae. Costs do not include labour. Relative harvesting costs are calculated on the basis of operational cost for a self-cleaning plate separator = 1.0.

Machine type	Machine and make	Operational mode	Type of concentration procedure	Algal species	Concentration factor %TSS of the concentrate	Energy consumed per m ³	Relative harvesting cost	Reliability	Recommendable according to present experience
Centrifugal	Self-cleaning Disc-stück Westfalia	Suspension continuous Concentrate discontinuous	One step	Scen. Coel.	120 (12%)	1 kWh	1	Very good	Yes
	Decanter bowl Westfalia	Continuous	For final conc. only in ca 2%	Scen. Coel.	11 (22%)	8 kWh	–	Very good	Yes
Pressure filtration	Chamber filter Rittershaus u. Blech, Netzsich	Discontinuous	One step	Coel.	245 (22-27%)	0.88 kWh	0.4	Very high	Yes
	Filter basket Mann, Seitz Dinglinger	Discontinuous	For preconcentration	Coel.	50 (5%)	0.2 kWh	0.48	Good	Yes
Vacuum filtration	Non precoated vacuum drum filter Dorr Olliver	Continuous	One step	Coel.	180 (18%)	5.9 kWh	3.9	Low	
	Potato starch precoat vacuum drum filter Niroba, Walther	After precoating continuous	Needs preconcentration 2-15	Coel. Scen.	2-18.5 (37%)			Good	Yes
	Belt filter Dinglinger	Continuous	For Preconcentration	Coel.	95 (9.5%)	0.45 kWh	0.88	Good	Yes

contamination of the biomass (Cannell, 1990). For these reasons, low shear microfiltration may be an alternative solution for the concentration of fragile species (Petrusevski *et al.*, 1995). Cross-flow filtration has many advantages over conventional filtration (Meindersma *et al.*, 1997), centrifugation and flocculation–flootation sedimentation processes.

Microfiltration and ultrafiltration can be used both for harvesting biomass (microfiltration) and isolation of product metabolites (ultrafiltration). Both methods rely on porous membrane filter media, the basic difference between the two operations being the particle size range handled.

Note that industrial ultrafiltration and microfiltration systems are physically and operationally similar. The theoretical fundamentals of these two operations are also equivalent. The volume of solvent per area per time (solvent velocity) through a membrane (J_v) is proportional to the applied pressure (transmembrane pressure, ΔP_m). However, in ultrafiltration the actual force must be the applied pressure reduced by the osmotic pressure ($\Delta\pi$), the effect of osmotic pressure being reduced because of solutes which somehow leak through the membrane.

The application of cross-flow membrane filtration for the separation of microorganisms from their culture media has been studied since 1970. Its application to microalgal fields, however, is scarce. Interest in the enhancement of marine microalgae value has increased recently, requiring development of adapted technology such as membrane processes (Borowitzka, 1997). Nowadays, several small aquaculture farms use membrane separation techniques for specific applications. Rossignol *et al.* (1999) studied membrane technology based processes for the continuous separation of microalgae from broth. The ultrafiltration membrane polyacrylonitrile 40 kDa proves to be the most efficient in the peculiar conditions of low pressure and low tangential velocity for a long-term operation. For microfiltration, membranes made of polyvinylidene (PVDF) are usually used, with mean pore diameter from 0.1 to 1.5 µm. For ultrafiltration, membranes made of polyvinylidene (PVDF), polyacrilonitrile (PAN), or polyethersulfone (PES) are used, with molecular weight cut-offs of 40 000, 40 000, and 30 000 Da, respectively.

For centrifugation, the major costs are depreciation and maintenance; for cross-flow filtration the costs of membrane replacement and pumping predominate. According to the rule of thumb established by MacKay and Salisbury (1988), for large scale production ($>20\,000\text{ l}$), centrifugation may be more attractive, whereas at small scale ($<2000\text{ l}$), cross-flow microfiltration may be a better choice.

10.2.5 Criteria for selecting the harvesting method

The main criteria for selecting the harvesting procedure is the *quality* of the product. In photosynthetic treatment of liquid wastes or waste-water treatment, the large volume to process ($\text{m}^3\text{ s}^{-1}$) excludes the use of centrifuges or filter processes, at least at the primary concentration steps. Thus, for sewage-based work sedimentation by natural gravity is recommended, perhaps enhanced by flocculants, in sedimentation tanks or settling ponds

(e.g. Venkataraman, 1978), followed by secondary concentration techniques. Another procedure for true large scale operations is the flocculation-flotation method of pre-concentration (e.g. Shelef, 1978). At this point, the use of alum or ferric salts, as the polycationic flocculant, also eliminates residual phosphates and other components from the effluent, thereby improving the quality and value, although adding appreciably to the overall cost.

If algae of highest quality are to be produced for food or aquaculture applications, continuously operating centrifuges, like the plate separator or the nozzle separator, are recommended. These devices can easily be cleaned and/or sterilized. Bacterial contamination or fouling of the raw product can be avoided effectively. In addition, centrifuges are suitable to concentrate any conceivable type of microorganisms, albeit at considerable cost.

Another basic criterion for selecting the proper harvesting procedure is its potential to adjust the density of the resulting concentrate right to the optimum subsequent process (Mohn, 1978). If the raw product is going to be dehydrated by drum-drying, the overall production cost is reduced if solids content of the concentrate is higher, because the expense involved in thermal dewatering clearly exceed those required for mechanical water removal. Many of the machines available are only suitable if combined with others. This leads, e.g. to two-step harvesting procedures such as pre-concentration by means of a microstrainer and post-concentration through a screw centrifuge.

10.3 Dehydrating

Following harvesting of the algal biomass, which should result in a 50–200-fold concentration, the algal slurry (5–15% dry weight) must be quickly processed, lest it should get spoiled (in only a few hours in a hot climate). Processing represents a major economic limitation to the production of low cost commodities (foods, feeds, fuels) and also to higher value products (β -carotene, polysaccharides). It is difficult to discuss processing, since it is highly specific. Several methods have been employed to dry microalgae such as *Chlorella*, *Scenedesmus* and *Spirulina*. The most common are spray-drying, drum-drying, freeze-drying and sun-drying. The first three have been applied to the β -carotene-rich *Dunaliella* and yielded satisfactory results in terms of uniformity of the powder and stability of the β -carotene (Ben-Amotz & Avron, 1987).

One major problem is the high water content of algal biomass, which makes sun-drying (the method applicable to most crops) difficult and spray-drying too expensive for low cost foods or animal feeds. Spray-drying is the method of choice for higher value products ($>\$1000\text{ t}^{-1}$) where the entire biomass is desired; however, it can cause significant deterioration of some algal components, such as the pigments. The cost of dehydration, when added to the cost of algal growth and harvesting, has formed an economic barrier to the mass production of algal powder produced for low-cost products, such as protein or feed.

10.3.1 Spray-drying

Spray-drying is for rapid, continuous, drying of solutions, emulsions and slurries. Pressure or centrifugal atomizers or gas–liquid jets are used to generate a fine spray of solution droplets, which are brought into continuous contact with hot air in a large (1–10 m diameter) chamber. Large droplet surface area and small droplet size ensure high evaporation rates so that drying times are measured in seconds. The flow of air is usually cyclonic. The dimensions of the drier must be such that the droplets do not reach the walls until sufficiently dry to prevent sticking and burn-on. The dry powder settles to the bottom from where it is removed.

Advantages of spray driers are: (1) continuous operation; (2) powdered product requiring no further size reduction; and (3) rapid drying which leads to good product quality, particularly for heat-labile materials, though relatively low thermal efficiency is a limitation. Aseptic spray-drying equipment is available.

The total drying time of a wet solid particle in a stream of excess dry air can be estimated by the sum of the times of a constant-rate adiabatic drying step and a falling rate step. The constant rate-drying step corresponds to removing the free water and the falling rate-drying step corresponds to removing the bound water. In the constant rate-drying step, moisture is removed from initial water content, w_0 , to critical moisture content, w_c . Using these considerations, the drying time can be estimated by the equation

$$t = \left\{ \frac{C_s d_p}{6k\rho_{\text{air}} C_p(T_{\text{db}} - T_{\text{wb}})} \right\} \left[w_0 - w_c + w' \ln \frac{w_c}{w} \right] \quad (10.7)$$

where C_s is the mass of dry cells per droplet volume, d_p is the particle diameter, ρ_{air} is the density of the dry air, k is a mass transfer coefficient, λ is the specific heat of vaporization, C_p is the specific heat capacity of air, T_{db} is the dry bulb temperature in the bulk air, T_{wb} is the wet bulb temperature, w_0 is the initial mass of water per mass of dry solids, w_c is a critical moisture content, w is the final moisture content and w' is a combined constant (Belter *et al.*, 1988).

Spray-drying is the most extended method for dehydrating microalgae and it is applied to different strains such as *Spirulina*, *Chlorella*, or *Dunaliella* at exploitation scale. Sensitivity of the biomass to oxidative processes imposes, for some applications, the addition of antioxidants.

10.3.2 Lyophilization

Freeze-drying (lyophilization) is the most gentle of the drying algal biomass methods. The algal biomass (usually algal slurry coming from a centrifugation step) to be dehydrated is frozen and the ice crystals sublimed by slight warming without thawing. The ice sublimes directly to vapor by exposing it to a partial pressure of water vapor below 4.6 mmHg, the triple point of water. Below this pressure, adding heat energy can turn ice directly into water vapor. About 2800 kJ of heat are required for each kg of ice removed (Chisti

& Moo-Young, 1991). Because the subliming ice crystals leave cavities, lyophilized dry material contains a myriad of interstices into which water can penetrate to give rapid and complete re-hydration when needed. Because it is frozen, constituents of the algal material are kept immobilized during sublimation drying. Because of the high capital cost of equipment and the high energy costs, freeze-drying is only recommended for fine applications where it is desirable to maintain the biochemical composition of the biomass, and where a breakage of the cells eases the downstream process (Molina Grima *et al.*, 1996).

Lyophilization consists of three phases: (i) freezing to solidify the material; (ii) sublimation drying to reduce moisture to below 20% w/w (primary drying); and (iii) desorption or secondary drying to reduce bound moisture to the required final value (often below 1% w/w). During sublimation, vapor is generated from a distinct interface or freeze-drying front which moves from the outer surface of the cake. Since the subliming vapor must pass through the channels left from the ice crystals formed on freezing, their shape affects the speed of drying. If the crystals are small and discontinuous, the escape path for the vapor is limited; if large crystals are formed, escape is easy and the product can be dried more quickly. The method and rate of freezing are thus critical to the course of sublimation. Quick freezing methods, such as the use of liquid nitrogen, must be viewed with caution because of the formation of very small ice crystals (Snowman, 1996).

The objective of freezing is to convert most of the water from the wet biomass to ice and convert all solutes into solids, either crystalline or glass. The wet biomass to be processed is cooled to a temperature that is always below the solidification temperature of the wet biomass. In practice, wet algal slurries to be frozen display one of two different types of freezing behavior: (1) the liquid phase suddenly solidifies (eutectic formation) at a temperature that depends on the nature of the solids in the sample, or (2) the liquid phase does not solidify (glass formation), but rather becomes increasingly viscous until it finally takes the form of a very stiff, highly viscous liquid. In this latter case, there is no eutectic temperature, only a minimum freezing temperature. At the end of the freezing step, a separation exists between the water to be removed (frozen water in the form of ice crystals) and the solute. In many cases, at the end of the freezing stage about 65–90% of the initial water is in the frozen state, and the remaining 10–35% is in the sorbed (non-frozen) state.

Frozen water should be removed in the primary drying stage, whereas sorbed water should be removed during the secondary drying stage. The total drying time can be estimated by the sum of the times of both steps. After the freezing stage, the chamber pressure is reduced down to a value that allows the sublimation of solvent (water) to take place. The latent heat of sublimation must be provided by a heat source, temperature of heating limited by two constraints: (A) the maximum temperature that the dried product can tolerate without (1) loss of bioactivity, (2) color change, (3) degradative chemical and biochemical reactions, and (B) structural deformation in the dried layer. The maximum temperature that the dried product can tolerate without suffering any of these deleterious is denoted, for a given product, by

the temperature of the scorch point, T_{scorch} . If the material has a eutectic form and if the temperature of the lowest eutectic point is exceeded during the primary drying stage, melting in the frozen layer can occur. Any melting can give rise to gross material faults such as puffing, shrinking, and structural topologies filled with liquid solution. When melting has occurred, water cannot be removed by sublimation, and therefore, there is process failure. The water vapor produced by sublimation of the frozen layer and by desorption of the sorbed water, travels by diffusion and convective flow through the porous structure of the dried layer and enters the drying chamber. The time at which there is no more frozen layer is taken to represent the end of the primary drying stage.

The secondary drying stage involves removal of water that did not freeze (sorbed or bound water). Although the amount of bound water is about 10–35% of the total moisture content, its effect on the drying rate and overall drying time is very significant, and the time that it takes to remove the sorbed water could be as long as or longer than the time required for the removal of the free water. It can be removed by heating the product under vacuum, at a temperature limited only by the maximum temperature allowed for the material, typically between 20 and 40°C. The residual moisture content in the dried material at the end of the secondary drying stage, as well as the temperature at which the dried material is kept in storage, are critical factors in determining product stability during storage life (Bruttini, 1999; Snowman, 1996). Use of lyophilization at commercial levels may be considered only for high market-value products.

10.4 Cell disruption

The disruption of microbial cells is an important unit operation in the preparation of intracellular products from microorganisms. There are a number of ways in which this may be achieved, based on mechanical action, e.g. homogenizers, bead mills and ultrasounds, or non-mechanical action, e.g. freezing, organic solvents and osmotic shock (Middelberg, 1994). The method of cell disruption depends on the microalgae wall and the nature of the product to be obtained.

The influence of different cell disruption processes on encysted cells of *Haematococcus pluvialis* in the recovery of astaxanthin was analyzed by Mendes-Pinto *et al.* (2001). Different methods were studied, including autoclave, acid, base, enzyme reactions, mechanical disruption (cell homogenizers) and spray drying. The best results were obtained from autoclaved and mechanically disrupted biomass, the yield of astaxanthin being three times higher than other methods (Mendes-Pinto *et al.* 2001) (Fig. 10.5). Several methods have been proposed to disrupt algal cells (Ruane, 1977). Bubrick (1991) described a process incorporating cryogenic (-170°C) grinding of dried *Haematococcus* biomass in the presence of butylated hydroxytoluene. Lyophilization breaks up the cells and turns the algal material into a loose, fine powder, making other treatment unnecessary. In this way, lipids were extracted directly from lyophilized biomass of *Isochrysis galbana* by ethanol (96%) or hexane–ethanol (96%) mixtures (Molina Grima *et al.*, 1994).

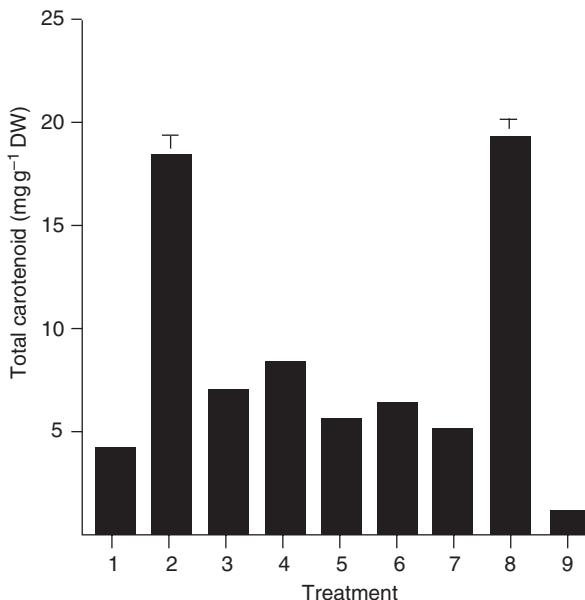


Fig. 10.5. Extraction of total carotenoids into acetone from processed *Haematococcus* biomass. (1) Control; (2) Autoclave; (3) HCl 15 min; (4) HCl 30 min; (5) NaOH 15 min; (6) NaOH 30 min; (7) Enzyme; (8) Mechanical disruption; (9) Spray-drying (Mendes-Pinto *et al.*, 2001). Reproduced with permission from Kluwer Academic Publishers (*J. Appl. Phycol.*).

10.4.1 Cell disruption by chemical methods

Alkaline lysis using hydroxide is an effective and inexpensive method for releasing intracellular products. It is extremely harsh and consequently the product must be resistant to degradation at high pH. This limits the applicability for protein recovery (Middelberg, 1994). For lipid recovery, however, the method may be of interest (Robles Medina *et al.*, 1995; Giménez *et al.*, 1998). Also free fatty acids from *Phaedactylum tricornutum* (Robles Medina *et al.*, 1995) and *Porphyridium cruentum* (Giménez *et al.*, 1998) biomass were extracted by direct saponification of wet biomass with KOH-ethanol (96%).

Organic solvents are also absorbed into the cell wall lipids, swelling and rupturing the wall. The cell's contents are released into the surrounding broth, and separated. Solvents are toluene, alkanes or alcohols. One good way to choose solvents for experiments is to look at their solubility parameters. In practice, solvents with similar solubility parameters should attack cells in a similar manner. Ideally, we should choose solvents whose solubility parameters match those of the wall lipids but are far from those of desired products locked within cells.

10.4.2 Cell disruption by mechanical methods

10.4.2.1 Homogenizers

Mechanical methods are generally seen as most appropriate for large scale disruption, homogenizers being commonplace, generating shear forces suffi-

cient to completely disrupt many types of cell. A common apparatus for homogenization of cells is the Manton-Gaulin homogenizer. This high-pressure pump incorporates an adjustable valve with restricted orifice through which cells are forced at pressures up to 550 atm. The following equation relates disruption of cells to operating conditions in the Manton-Gaulin homogenizer

$$\ln\left(\frac{W_m}{W_m - W}\right) = k_h n_{\text{passes}} P^\gamma \quad (10.8)$$

where W_m is the maximum amount of protein available for release, W is the amount of protein released after n_{passes} passes through the homogenizer, k is a temperature-dependent rate constant, and P is the operating pressure. The exponent γ is a measure of the resistance of the cells to disruption and, for a particular microalgae, depends to some extent on growth conditions. The strong dependence of protein release on pressure suggests that high-pressure operation is beneficial. Cell disruption measured as the release of protein is markedly dependent on temperature; protein release increases at elevated temperatures up to 50°C. Procedures for scale-up of homogenizers are not well developed (Doran, 1995).

A new type of commercial cell disrupter manufactured by Constant Systems Ltd (Warwick, UK) has several advantages over most other disruption systems in that it is sterilizable, is capable of a high-level of containment and may be cleaned in place.

Homogenizers are widely used in aquaculture to enhance the assimilation of carotenoids from *Haematococcus* for fish. Cell cracking increases the bioavailability of the pigment. Thus, if the disruption of the homogenized spores is not complete (60%), their bioavailability and assimilation is reduced in the same percentage.

10.4.2.2 Bead mills

Bead mills consist of either a vertical or a horizontal cylindrical chamber with a motor-driven central shaft supporting a collection of off-centred discs or other agitating elements. Horizontal units are preferred for cell disruption to reduce the fluidizing effect in the vertical units. The grinding action is due to glass or plastic beads typically occupying 80–85% of the working volume of the chamber. The beads are retained in the grinding chamber either by a sieve plate or a similar device. The units require high-capacity cooling systems.

Bead milling has been used for years to disrupt microorganisms and works successfully to disrupt cells such as cyanobacteria, yeast, spores and microalgae – where other techniques have failed. The size of the glass beads has proven to be important. The optimal bead size for microalgae cells is 0.5 mm. The speed of disruption is increased by about 50% using like-sized ceramic beads made of zirconia-silica rather than glass, presumably because of their greater density. The loading of the beads should be at least 50% of the total liquid-biomass volume but can be up to 90% provided that adequate agitation of the bead slurry is still possible. Generally, the higher the volume

ratio of beads to cell suspension, the faster the rate of cell disruption. A bead mill was used successfully by Stuckey & Kune (EPALMAR report, 1998) for disruption of hard cell wall of *Monodus subterraneus* before extracting the EPA enriched lipid fraction. Bead mills have been used to disintegrate the algae *Scenedesmus obliquus* and *Spirulina platensis* (Hedenskog *et al.*, 1969).

10.4.2.3 Ultrasound

Ultrasound has also been used on the laboratory scale for microalgal disruption. The cell disruption mechanism derives from the intensive shear induced by sonicating the suspension at sound frequencies above 20 kHz. A magneto-restrictive or a piezoelectric transducer converts the alternating current of an electric oscillator into mechanical waves that are transmitted to the suspension through a metallic probe (usually made from titanium) vibrating with the same frequency as the oscillator. The sound waves create many micro bubbles at various nucleation sites in the suspension, which collapse impulsively during the rarefaction period of the sound waves. This phenomenon of cavitation (formation, growth, and collapse of the vapor-filled bubbles) produces intense local shock waves, and intense local shear gradients are generated that cause the cells to deform beyond the limit of elasticity and rupture. The rate of microbial cell disintegration is usually low for preparative purposes, and the inclusion of small stainless steel or glass beads will help in the nucleation for cavitation as well as with a grinding action (solid shear) that will increase the efficiency of disintegration (Bermejo *et al.*, 2001).

High working volumes require high acoustic power, which results in violent turbulence and in the formation of large bubbles that cause cavitation unloading. Rate constants were found to decrease linearly with increasing working volumes. The difficulties associated with higher working volumes (100–200 ml) can be alleviated by good design of the disruption vessel. Commonly, a jacketed glass beaker with a continuous flow of cooling fluid is used. In continuous operation, the flow rate determines the residence time of the cells in the vessel and affects the overall yield of the process.

Ultrasound is usually utilized as a cell disruption method in protein extraction from microalgae (Bermejo *et al.*, 2001), because temperature and stress modify the structure of these compounds. Dunstan *et al.* (1992) extracted lipids from green algae with chloroform–methanol–water (1/2/0.8 v/v/v) mixture and between each extraction the samples were sonicated at 20°C.

10.5 Product isolation

After the insolubles are removed, the second step in a bioseparation is usually product isolation. Isolation involves taking a highly dilute aqueous feed and removing most of the water and also removing materials of widely divergent properties compared with the desired product. The resulting concentrate can be purified by a variety of methods which would not be effective in dilute solution. The two key methods for isolation are extraction and adsorption, the latter will be treated in this chapter as expanded bed adsorption form.

10.5.1 Extraction procedures

Liquid–liquid extraction is a method of selective removal of a desired substance from a liquid phase mixture into a second immiscible liquid. The solute, which is usually in aqueous solution, when contacted with an immiscible solvent (extractant), distributes or partitions itself between the two phases. The extent of partitioning is determined by the partition coefficient, k_p , defined as

$$k_p = \frac{C_{\text{solvent}}}{C_{\text{feed}}} \quad (10.9)$$

The chemical properties of the solvent are chosen to facilitate selective uptake of the desired product from the feed phase. Organic phases composed of mixtures of two or more organic solvents have been employed and in these cases k_p can be altered by changing the solvent composition. Addition of inorganic salts, fatty acids, detergents, etc. can be used to manipulate k_p , which is also affected by temperature.

The ratio of the total amount of solute in the two phases is known as the degree of separation (G) and depends on the volumes of the solvent and the feed (Chisti & Moo-Young, 1991):

$$G = \frac{C_{\text{solvent}} V_{\text{solvent}}}{C_{\text{feed}} V_{\text{feed}}} = k_p \frac{V_{\text{solvent}}}{V_{\text{feed}}} \quad (10.10)$$

Lipids and fatty acids have been extracted from the microalgal biomass of *Isochrysis galbana* (Molina Grima *et al.*, 1994; Robles Medina *et al.*, 1995), *Phaeodactylum tricornutum* (Cartens *et al.*, 1996; Molina Grima *et al.*, 1996) and *Porphyridium cruentum* (Giménez *et al.*, 1998) to obtain polyunsaturated fatty acids (PUFAs), such as EPA, docosahexaenoic acid (DHA) and arachidonic acid (AA). Several solvent systems were tested by these authors with the objective of carrying out the extraction quickly, efficiently and gently to reduce the degradation. Moreover the extraction solvents should be inexpensive, volatile, able to form a two-phase system with water (to remove the non-lipids), and be poor extractors of unwanted components. To directly extract free fatty acids from biomass, an alkali was added to the extraction solvent. The best extraction solvent was ethanol (96%), also extracting, however, some cellular contaminants such as sugars, amino acids, salts, hydrophobic proteins and pigments. Therefore, crude alcoholic extract must be treated to remove these hydroalcoholic-soluble contaminants. The crude extract may be treated with apolar solvents such as chloroform, hexane or diethyl ether, in which the non-lipid contaminants are less soluble (Fig. 10.6). These equilibrium data allowed predicting the extraction yield of unsaponifiables and fatty acids that could be obtained. A quantitative extraction of purified fatty acids was possible with a relatively low volume of hexane in several extraction steps. By these procedures, purified extracts of fatty acids were obtained with recovery yields of 80% of EPA and 65% of DHA from *I. galbana* biomass (Robles Medina *et al.*, 1995), 98% of EPA from *P. tricornutum* biomass (Cartens *et al.*, 1996), and 69% of EPA and 68% of AA from *P. cruentum* biomass (Giménez *et al.*, 1998).

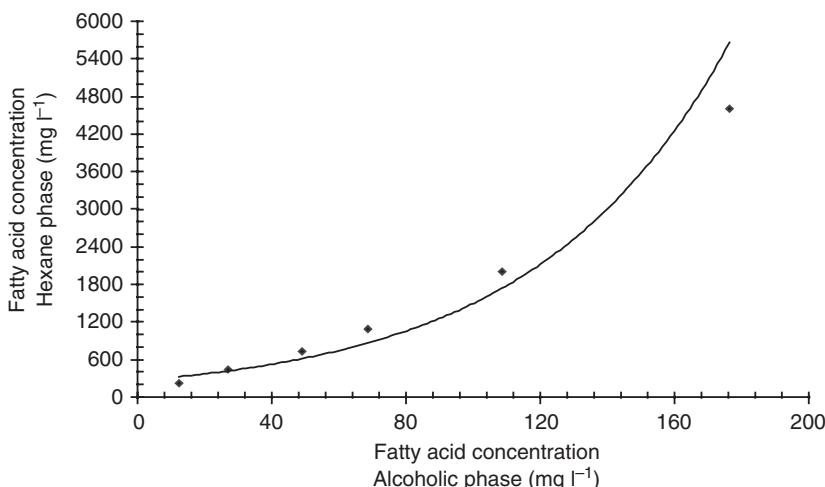


Fig. 10.6. Equilibrium distribution of fatty acids between a hydroalcoholic phase and hexane (Ibáñez González *et al.*, 1998). Reproduced with permission.

10.5.2 Expanded bed adsorption

Expanded bed adsorption is a technique based on fluidization aimed at recovery of solutes directly from un-clarified feedstock without a preceding clarification by filtration or centrifugation. Such solutes include culture broths, cell homogenates, and other solutions containing suspended particulates. When the bed has expanded and is stable, the particulate-containing feedstock is applied, and the solute of interest can bind to the adsorbent particles while the particulates in the feedstock can pass unhindered through the void of the bed.

Expanded bed adsorption was developed for the extraction of proteins from yeast and bacteria, and it has been applied recently to microalgae (Bermejo *et al.*, 2001; Li *et al.*, 2001). Bermejo used expanded bed adsorption with DEAE for the extraction of phycobiliproteins from *Porphyridium cruentum*. Biomass is sonicated and extracted with 50 mM phosphate buffer. The crude extract is circulated directly from the bottom through a column filled with DEAE particles, the rest of the components exhaust by the top. Finally, the phycobiliproteins are eluted by 250 mM phosphate buffer in classical chromatography. Recovery yields of 80% were obtained with purity higher than 95% (Bermejo *et al.*, 2001). Li *et al.* (2001) developed a preparative isolation and purification process of lutein from the microalga *Chlorella vulgaris* by high-speed counter-current chromatography (HSCCC), obtaining a fraction of lutein of 98% purity.

10.6 Product purification

Purification of products can be achieved by a variety of methods (chromatography, ultrafiltration, electrophoresis, etc.). The choice of the most suitable process involves two major considerations. First, the chemistry of the product

and its most troublesome impurities should be considered. Second, the scale at which to operate. Considering both aspects, chromatography is the most useful purification method because of the wide variety of physico-chemical interactions available to carry out the isolation, e.g. gel filtration, ion exchange, affinity, reverse-phase, hydrophobic chromatography, etc.

Beginning from the stage at which a mixture is loaded on a chromatographic column and the flow of eluting solvent is started, a concentration vs time plot of the components emerging from the column can be plotted as in Fig. 10.7. The peaks correspond to the two mixture components, A and B. The extent of separation is measured in terms of the peak resolution, R , defined as the difference between the retention times (t_{rA} and t_{rB}) of the component divided by the average peak width:

$$\phi = \frac{2(t_{rB} - t_{rA})}{\bar{\omega}_A + \bar{\omega}_B} \quad (10.11)$$

the peak width is expressed in time units. When $\phi = 1.0$, the area of overlap of the two peaks is about 2% of the total area of peaks. To reduce the amount of overlap to 0.1%, the resolution must be 1.5 or better. The separation efficiency of a column is measured in terms of the height equivalent of a theoretical plate, H , which is a concept common to all equilibrium stage operations conducted in packed columns.

Polyunsaturated fatty acids (PUFAs) such as EPA, DHA and AA were purified by reverse phase chromatography from PUFA concentrates of the microalgae *Isochrysis galbana* (Robles Medina *et al.*, 1995), *Phaeodactylum tricornutum* (Cartens *et al.*, 1996; Molina Grima *et al.*, 1996; Ibáñez González *et al.*, 1998) and *Porphyridium cruentum* (Giménez *et al.*, 1998). These chromatographic separations were optimized at analytical scale and scaled up to semi preparative (Robles Medina *et al.*, 1995; Cartens *et al.*,

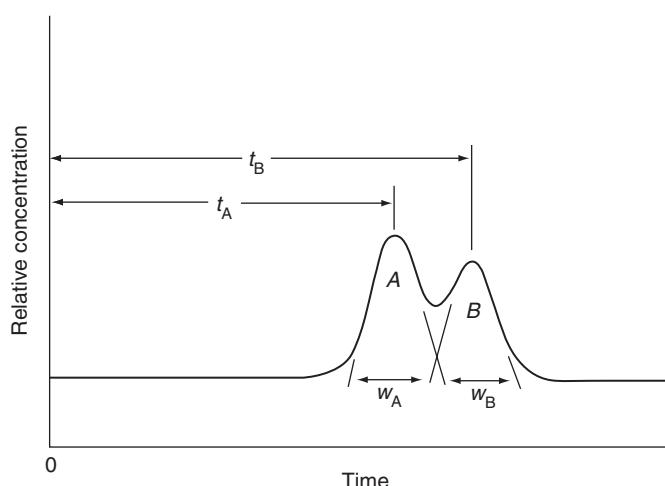


Fig. 10.7. A typical chromatogram showing retention time and peak weights for two components in an elution chromatographic operation.

1996) and preparative gram-scale (Molina Grima *et al.*, 1996; Giménez *et al.*, 1998). The scaling up from analytical to semi-preparative scale was carried out by increasing the column diameter (to increase the sample load), while keeping the superficial velocity, the particle size and the column length to preserve the retention times, peak width and pressure drop. By this procedure EPA and DHA fractions of 94–96% purity were obtained from a PUFA concentrate (39% EPA, 23% DHA) of *I. galbana* biomass. The recovery yields of these PUFAs were always higher than 94% (Robles Medina *et al.*, 1995). By an identical scale-up procedure Cartens *et al.* (1996) obtained highly pure EPA (93%) from the diatom *P. tricornutum*. At preparative scale Molina Grima *et al.* (1996) could obtain about 2.5 g of 96% pure EPA per day (97% yield) from a PUFA concentrate of the microalga *P. tricornutum*. Also, Giménez *et al.* (1998) obtained 94% pure EPA and 81% pure AA from PUFA concentrates of *P. cruentum* (34% AA and 42% EPA).

Bellarbi *et al.* (2000) have purified EPA methyl ester from methyl ester extracts from *P. tricornutum* and *Monodus subterraneus* biomass by argen-tated silica gel column chromatography at atmospheric pressure. The optimal loading of the fatty acid ester mixture on the chromatographic support was about 3–4% (w/w).

10.7 Cost considerations: case-studies analysis

This section will only consider the economics of the production of *P. tricornutum* as well as those corresponding to the harvesting and processing the biomass for obtaining highly pure and concentrated EPA for the pharmaceutical industry.

10.7.1 Recovery of EPA

The process was developed for the wet paste obtained after centrifugation of the biomass of *P. tricornutum* produced in the previous reactors. The process has also been proved with *M. subterraneus* and with fish oil. The effectiveness of EPA recovery from the crude extracts was in the following order: *P. tricornutum* > fish oil > *M. subterraneus*. These differences are associated with differences in the fatty ester profiles of the crude extracts (Bellarbi *et al.*, 2000). The process has three main steps: (i) combined extraction – *trans*-esterification of fatty esters from the algal biomass; (ii) silver ion column chromatography; and (iii) chlorophyll removal. The optimal processing conditions and the scale up of recovery are described in detail in Bellarbi *et al.* (2000).

The sequence of operations in purification of EPA is summarized in Fig. 10.8. The total fatty ester loading with respect to the amount of the stationary phase was about 4% (w/w). The total EPA contained in the various eluent fractions was 92.1% of the EPA that was originally present in the starting total fatty extract (Fig. 10.8). Note that the 500 g moist *P. tricornutum* in Fig. 10.8 was equivalent to 90 g dry biomass. The fatty

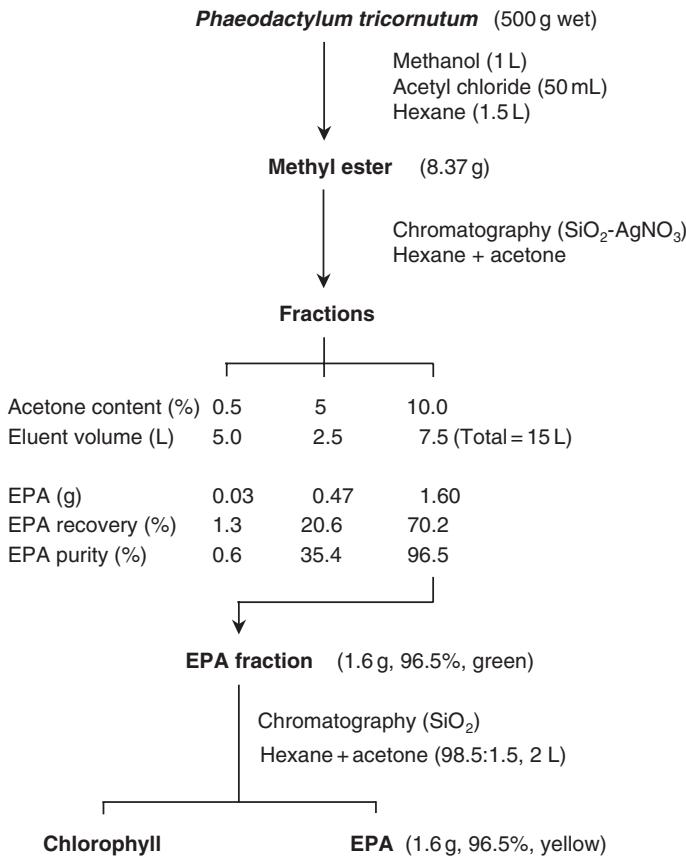


Fig. 10.8. EPA recovery from *P. tricornutum* paste. The per cent EPA recovery numbers for the various solvent fractions are based on the EPA ester content in the crude total fatty ester extract (Belarbi *et al.*, 2000). Reproduced with permission.

ester profile of the various solvent fractions obtained at the silver chromatography stage of Fig. 10.8 are shown in detail in Belarbi *et al.* (2000). Based on the sequence described in Fig. 10.8, an overall schematic process flow sheet for producing microalgal EPA ester is shown in Fig. 10.9. The process operates in batches. Although the flow sheet shows a reactor and a second optional extractor for extended overnight extraction, a single vessel may be designed to serve both functions. The overnight cold storage step, required for quantitative extraction from a relatively static wet biomass slurry, may be eliminated all together by using a normal level of agitation in the reactor. The cooler (Fig. 10.9) would be unnecessary if the reactor is jacketed or provided with a cooling coil. The one-step extraction–trans-esterification eliminated several intermediate processing steps that would be otherwise needed (Rodriguez-Ruiz *et al.*, 1998). The spent biomass of the microalga *P. tricornutum* is potentially useful as a human dietary supplement because of its high amount of mineral elements and its balanced profile of aminoacids. In the subsequent economic analysis, the spent

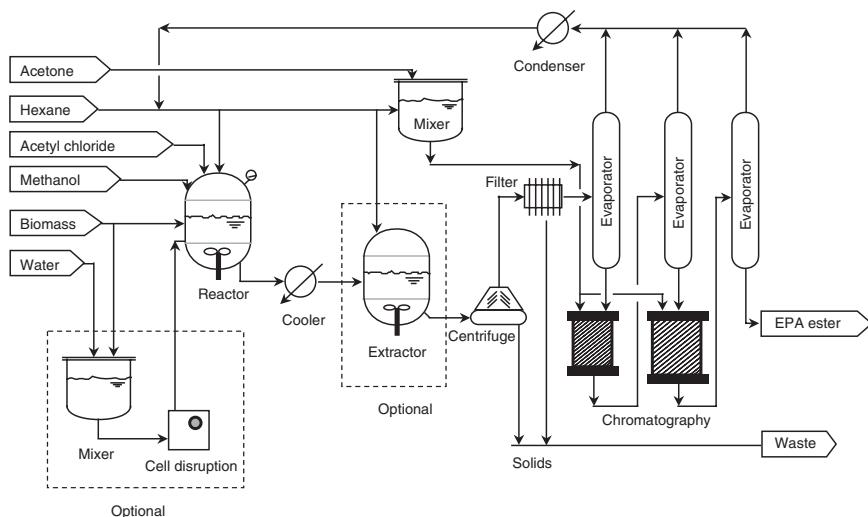


Fig. 10.9. Flowsheet of EPA-from-microalgae process (Belarbi *et al.*, 2000). Reproduced with permission.

biomass is considered as a by-product with a price of $\text{€}20 \text{ t}^{-1}$, against the negative cost of landfill ($\text{€}350 \text{ t}^{-1}$). Also, the low grade EPA products represent an additional income in the economic analysis with an average price of $\text{€}120 \text{ kg}^{-1}$. The flowsheet also shows a cell disruption step that is optional for *P. tricornutum*, but necessary for processing *M. subterraneus* or other algae with thick cell walls. A modification of this procedure using membrane is being tested by Belarbi *et al.* (2002), and an alternative method using supercritical fluid technology has also been developed at Padova (Italy) by Exenia group S.r.L (Petinello *et al.*, 1999).

The presence of any silver contaminant in the product, the peroxide value, and purity were all assessed. The total silver leached from the column during an entire process run was very small, less than 0.01 ppb in the solvent fractions. The minimum peroxide value was rather high at 9.55 mequiv O₂ kg⁻¹ EPA probably as a consequence of the processing conditions. This value does not increase significantly with storage time, provided the sample is kept dissolved in hexane (Belarbi *et al.*, 2000).

10.7.2 Economic assessment of the integrated production system

Below are the cost analyses of producing algal biomass, microalgal oil (crude fatty acid methyl esters) and the enriched EPA from algal biomass when using the silver ion chromatographic process. This study considers an algal plant of 60 m³ (75 photobioreactors, 0.8 m³ volume per reactor, operating with a mean annual biomass productivity of 1.25 g l⁻¹ d⁻¹, thus producing 26.2 t of biomass per year. Harvesting is performed by centrifugation in an disc-stack-bowl centrifuge. The cost of photobioreactors is based on local experience, in Spanish (2001) prices. For the economic evaluation, the components of costs taken into account were:

Fixed capital investment; Property tax + insurance + debt services
+ purchase tax; Production cost; Raw material; Utilities
(steam + process water + cooling water + power);

Other costs:

- Labor
- Supervision (direct plant supervision)
- Payroll charges
- Maintenance
- Operating supplies (lubricant, stationery, cleaning, etc.)
- General plant overheads (administration)
- Marketing
- Contingency
- Taxes.

Cost of algal biomass production is summarized in Table 10.2, whereas cost of producing microalgal oil and pure EPA from this biomass are summarized in Table 10.3. Clearly the cost of the algal biomass is most sensitive to the subsequent oil cost and obviously to the enriched EPA. In short, the economic evaluation presented below is based on the above mentioned assumption that might be different for other algal strains, locations, plant capacity and production systems. An aspect of relevant importance is that the biokinetic parameters (specific growth rate, quantum yield, biomass concentration and EPA content) are those corresponding to that obtained by the author's group all through several years research (Acién *et al.*, 1998). The price of the biomass is mainly influenced by labor cost (Table 10.2a). This effect is repeated in the microalgal oil, the cost of this product being 90% the cost of the raw material, whereas the raw material only represents 67% of the pure EPA (Table 10.3). Total Cost (€) of major equipment for pure EPA production from algal biomass (*Phaeodactylum tricornutum*, Plant size – 430 kg annum, Silver ion chromatography technology) is estimated at slightly above 0.5 million. Two pieces of equipment i.e. chromatography system (SS304) and high volume (1000 ft²) evaporators comprising some 75% of this cost.

The comparative analysis of production costs for EPA give results in contrast to that usually obtained for pharmaceuticals, in which the raw material represents only 20% of the total cost. This fact points out the necessity to reduce the price of the microalgal biomass to make microalgae competitive as an EPA source.

The estimated cost of producing EPA for the plant capacity considered in the baseline, is about twofold the market price of EPA during the year 2000. Thus, the fish oil derived EPA ethyl ester (95% pure) sells for €1200–€1600 kg⁻¹, depending upon manufacturer and specifications. Moreover, the market price includes a profit, but our calculated cost of production does not; i.e. in practice our production cost will be more than twofold the current market price. Clearly, any alternative to existing EPA production technology

Table 10.2a. Cost (€) of algal biomass production (*Phaeodactylum tricornutum*). Plant size 26.2 tonnes/annum. Horizontal tubular photobioreactors.

Fixed capital investment	Total cost	Percentage
1. Major purchased equipment (MEC)	881285	35.39
2. Installation costs (@ 0.4 MEC)	246780	9.91
3. Instrumentation and control (@ 0.15 MEC)	92543	3.72
4. Piping (@ 0.4 MEC)	246780	9.91
5. Electrical (@ 0.1 MEC)	61695	2.48
6. Buildings (@ 0.2 MEC)	123390	4.95
7. Yard improvements (@ 0.1 MEC)	61695	2.48
8. Service facilities (@ 0.2 MEC)	123390	4.95
9. Land (@ 0.06 MEC)	37017	1.49
10. Engineering and supervision (@ 0.3 MEC)	185085	7.43
11. Construction expenses (@ 0.1 Σ items 1–9)	187458	7.53
12. Contractor's fee (@ 0.05 Σ items 1–9)	93729	3.76
13. Contingency (@ 0.06 total fixed capital investment)	149416	6.00
Total fixed capital investment	2490264	2490264
Depreciation (Σ items 1–8 + Σ items 10–12), years	10	230518
Property tax (@ 0.01 depreciation)		2305
Insurance (@ 0.006 depreciation)		1383
Debt service (none, 100% equity capital)		0
Purchase tax (@ 0.16 of items 1–12/10)		37454
Total fixed capital per year		271660
Direct production costs (DPC)		34.16%
Raw materials		
1. Culture medium, kg	0.58825	38530
2. Carbon dioxide, kg	0.4706	45209
3. Medium filters, unit (50 m ³ /unit)	96067	35.05
4. Air filters, unit (50000 m ³ /unit)	210	41.12
5. Other consumables (€100/kg)	105	13.46
Total	117.65	8.97
	13	1.40
	1541	
		109940
		13.83%

Table 10.2a. continued.

	Fixed capital investment	Total cost	Percentage
Utilities			
6. Cooling water included in pump station	15.58	0	0.00
7. Power, kWh	3.81	0.058825	99931
Total	15.58	408115	0
Others			
8. Labor (@ € 16/h), 1 Shift	8400	158122	38.70
9. Supervision (@ 0.2 labor)		31624	7.74
10. Payroll charges (@ 0.25 (labor + supervision))		47436	11.61
11. Maintenance (@ 0.04·MEC)		35251	8.63
12. Operating supplies (@ 0.004 items 1–5 DPC)		440	0.11
13. General plant overheads (@ 0.55 (labor + supervision + maintenance))		123749	30.29
14. Tax (@ 0.16 items 1–7, 11 and 12 DPC)		5756	1.41
15. Contingency (@ 0.05 items 1–7 DPC)		14	0.00
16. Marketing (@ 0.0 items 1–13 DPC)		0	0.00
17. Cost of wastewater treatment	0.59	6164	1.51
Total	10480	408557	51.38%
Total direct production costs (Raw material + utilities+ others)			
Total production costs (Total DPC + Total fix capital per year)		523494	
Unit cost of producing biomass (€/kg)		795153	100%
		30.35	

Table 10.2b. Major equipment list of the algal biomass production unit. Plant size 26.2 tonnes/ annum. Horizontal tubular photobioreactors.

Item	Size	Major equipment list and Costs			
		Delivered cost	No. of units	Total cost	Percentage
1. Photobioreactors, m ³	0.80	3524	75	264334	29.99
2. Centrifuge (24" bowl solids discharge, s.s.), m ³ h ⁻¹	2.99	123949	2	247898	28.13
3. Medium filter unit, m ³ h ⁻¹	5.99	18014	1	18014	2.04
4. Medium feed pumps, m ³ h ⁻¹	0.04	349	75	26196	2.97
5. Medium preparation tank, m ³	19.96	34814	3	104441	11.85
6. Harvest broth storage, m ³	19.96	34814	3	104441	11.85
7. Centrifuge feed pumps, m ³ h ⁻¹	2.99	841	2	1681	0.19
8. Air compressors, m ³ h ⁻¹	240.00	26103	3	78308	8.89
9. Harvest biomass conveyer belts	10.00	7100	2	14199	1.61
10. Sea water pump station, m ³ h ⁻¹	5.99	13661	1	13661	1.55
11. Carbon dioxide supply station, kg h ⁻¹	27.40	3006	1	3006	0.34
12. Weight station, kg d ⁻¹	74.90	2366	1	2366	0.27
13. Biomass silos, m ³	0.07	1370	2	2741	0.31
Total				881285	

would need to be competitive with prices of current fish oil products, clearly therefore microalgae should be produced at about €10 kg⁻¹. Cost sensitive analysis performed based on the above assumptions, demonstrating that the closed photobioreactor technology cannot provide biomass at cost below €25 kg⁻¹. To be competitive with current fish oil products, cost sensitive analysis leads to an algal biomass cost of €10 kg⁻¹ or cell content of EPA higher.

10.8 Concluding remarks

For harvesting biomass, flocculation seems to be the process for treating large quantities of culture. However, centrifugation is considered as the most appropriate microalgae harvesting method, excluding some fragile species. In these latter cases, microfiltration could be an alternative. As a rule of thumb, the selecting criteria for harvesting biomass (Section 10.2.5) might be useful. With respect to biomass dehydration, the sun-dryers are the most inexpensive alternative but for high value algae, both spray-drying and lyophilization may be considered.

The bead mills seem to be a good alternative for breaking algae such as *Haematococcus* and *Monodus*. Finally, for product fractionation and purification, chromatographic techniques are nowadays the first option to be considered.

While a great many factors combine to influence the final outcome, the case study analyzed indicates that the cost of algal production in closed photobioreactors is highly sensitive to subsequent harvesting and recovery processes and that the photoautotrophic production of algal biomass is the key factor to be competitive.

Table 10.3. Cost (€) of highly pure EPA production from algal biomass (*Phaeodactylum tricornutum*). Plant size 430 kg/annum. Silver ion chromatography technology.

Fixed capital investment	Factor	Total cost	Percentage
1. Major purchased equipment (MEC)	0.400	509705	26.11
2. Installation costs (@ 0.4 MEC)	0.150	203882	10.44
3. Instrumentation and control (@ 0.15 MEC)	0.400	76456	3.92
4. Piping (@ 0.4 MEC)	0.100	203882	10.44
5. Electrical (@ 0.1 MEC)	0.450	50971	2.61
6. Buildings (@ 0.45 MEC)	0.120	229367	11.75
7. Yard improvements (@ 0.12 MEC)	0.200	61165	3.13
8. Service facilities (@ 0.2 MEC)	0.050	101941	5.22
9. Land (@ 0.05 MEC)	0.300	25485	1.31
10. Engineering and supervision (@ 0.3 MEC)	0.100	152912	7.83
11. Construction expenses (@ 0.1 Σ items 1–9)	0.050	146285	7.49
12. Contractor's fee (@ 0.05 Σ items 1–9)	0.080	73143	3.75
13. Contingency (@ 0.08 total fixed capital investment)		156187	8.00
Total		1952333	
Fixed capital per year			
Depreciation, years	10.000	179942	84.80
Property tax (@ 0.01 depreciation)	0.010	1799	0.85
Insurance (@ 0.006 depreciation)	0.006	1080	0.51
Debt service (none, 100% equity capital)	0.000	0	0.00
Purchase tax (@ 0.16 of items 1–12/10)	0.160	29363	13.84
Total		212184	17%
Direct production costs			
Raw materials			
1. Algal biomass, kg	60.93	30.3	84.04
2. Ethanol, m ³	0.023	784.7	0.83
3. Acetyl chloride, m ³	0.030	1533.0	2.11
4. Silica gel, kg	2.558	6.6	0.77
5. Silver nitrate, kg	0.233	529.4	5.60
6. Acetone, m ³	0.047	419.4	0.89
7. Hexane, m ³	0.349	364.7	5.78
Total			946215
			74%

	Total	Others	Utilities	Total	Total
8. Steam, kg	1559.12	0.0049	670421	3313	55.22
9. Cooling water, m ³	161.75	0.0294	6.96 E + 04	2.05 E + 03	34.10
10. Power, kWh	25.34	0.0588	10897	641	10.68
				5999	0%
Men/day		Deliv. cost			
	1.5	18.8	4200	79061	31.59
11.1 Labor 1 shifts, 1 workers per shift				15812	6.32
11.2 Supervision (@ 0.2 labor)				23718	9.48
11.3 Payroll charges (@ 0.25 (labor + supervision))					
11.4 Maintenance (@ 0.04 MEC)				20388	8.15
11.5 Operating supplies (@ 0.004 items 1-7)				3785	1.51
11.6 General plant overheads (@ 0.55 (labor + supervision + maintenance))				63394	25.33
11.7 Tax (@ 0.16 items 1-10, 14 and 15)				28997	11.59
11.8 Contingency (@ 0.05 items 1-10)				7853	3.14
11.9 Marketing (@ 0.02 items 1-16)				7264	2.90
				250273	20%
Total direct production costs				1202487	94%
By-product credit (PUFAs concentrate about 50% EPA)				128838	-10%
Total production costs				1282165	100%
Unit cost of producing EPA (€/kg)				2982	

10.9 Nomenclature

A	Area, m^2
C_F	Concentration in the feed, kg m^{-3}
C_{feed}	Concentration in the feed, kg m^{-3}
C_p	Specific heat capacity of air, $\text{J kg}^{-1} \text{K}^{-1}$
C_{Rn}	Concentration in the exhausted reffinate, kg m^{-3}
C_s	Dry cells concentration, kg m^{-3}
C_{solvent}	Concentration in the solvent, kg m^{-3}
D_f	Reciprocal of the fraction or unreleased protein
d_p	Diameter of the particle, m
D_{sm}	Diffusion coefficient of the solute in the mobile phase, $\text{m}^2 \text{s}^{-1}$
g	Acceleration of the gravity, m s^{-2}
G	Degree of separation
H	Heat convection transfer coefficient, $\text{J m}^{-2} \text{s}^{-1}$
H_0	Height of sedimented bed, m
h_{bowl}	Height of the bowl, m
HETP	Height equivalent of theoretical plate, m
HTP _m	Height of theoretical plate minimum, m
J_v	Flow velocity of solvent through the membrane, m s^{-1}
K	Mass transfer coefficient, s^{-1}
k'	constant
k_d	Constant for spray-drying
k_D	Disruption rate constant
k_h	Constant for homogenisation
k_p	Partition coefficient
K_y	Constant
L_{column}	Length of the column, m
L_{conical}	Length of the conical section, m
$L_{\text{cylindrical}}$	Length of the cylindrical section, m
M	Molar concentration of solute, mol m^{-3}
N	Speed of the centrifuge, rpm
N	Number of stirred tank in series
n_{disc}	Number of disks of a disc-stack bowl centrifuge
n_{passes}	Number of passes for homogenisation
NTP	Number of theoretical plates
P	Operating pressure, N m^{-2}
Q	Volumetric feed rate, $\text{m}^3 \text{s}^{-1}$
R	Radius, m
R_c	Resistance of the cake, m^{-1}
$r_{\text{disc.ext}}$	Outer radius of the disc, m
$r_{\text{disc.int}}$	Inner radius of the disc, m
r_{ext}	External radius, m
r_{int}	Internal radius, m
R_{sm}	Solute to mobile phase velocity ratio
S_0	Mass of solids per volume of filtrate, kg m^{-3}
T	Time, s
T	Absolute temperature, K

t_{cf}	Time of cake formation, s
t_{cycle}	Cycling time, s
t_d	Time by spray-drying, s
T_d	Temperature of dry bulb, K
T_{db}	Temperature of dry bulb, K
T_i	Temperature of the wet bulb, K
t_r	Mean residence time, s
t_{rA}	Retention time for component A
t_{rB}	Retention time for component B
T_{scorch}	Temperature of the scorch, K
t_{sd}	Time spent by solute between sorption and desorption
T_{wb}	Temperature of wet bulb, K
u_c	Terminal velocity during the centrifugal settling, m s^{-1}
u_f	Superficial velocity of the fluid, m s^{-1}
u_{fm}	Minimum superficial velocity of the mobile phase, m s^{-1}
u_{fred}	Dimensionless reduced velocity
u_g	Terminal velocity during the gravity settling, m s^{-1}
V	Volume, m^3
V_{cf}	Volume of filtrate collected during the cake formation, m^3
$V_{chamber}$	Total free volume of the grinding chamber, m^3
V_{feed}	Volume of feed, m^3
$V_{solvent}$	Volume of solvent, m^3
W	Final water content
W	Amount of protein release, kg
w'	Constant
w_0	Initial water content
w_c	Critical water content
W_m	Maximum amount of protein available for release, kg
Y_m	Permeability of the membrane, $\text{m}^2 \text{s kg}^{-1}$
Z	g-number
Z	Compressibility of the cake, 0–1
α	Specific cake resistance, m kg^{-1}
α'	Constant
β	Fraction of cycle devoted to cake formation
γ	Resistance of cells to homogenisation
ΔFv	Proportion of particles expanded
ΔH	Increment of height in expanded bed, m
ΔP_f	Pressure drop by filtration, N m^{-2}
ΔP_m	Transmembrane pressure, N m^{-2}
$\Delta\pi$	Osmotic pressure, N nm^{-2}
ε	Voidage of expanded bed
ε_0	Voidage of sedimented bed
θ	Angle of disc, rad
λ	Specific heat of vaporization, J kg^{-1}
μ_{ap}	Apparent viscosity, $\text{kg m}^{-1} \text{s}^{-1}$
μ_f	Viscosity of the fluid, $\text{kg m}^{-1} \text{s}^{-1}$
ρ	Density, kg m^{-3}
ρ_{air}	Density of the air, kg m^{-3}

ρ_f	Density of the fluid, kg m^{-3}
ρ_p	Density of the particle, kg m^{-3}
ρ_s	Mass of dry cells per droplet volume, kg m^{-3}
Σ	Equivalent surface of the centrifuge, m^2
σ	Reflection coefficient, 0–1
σ^2	Variance
τ	Constant
ϕ	Peak resolution
χ	Constant
ψ	Geometric factor
ω	Angular velocity, rad s^{-1}

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Part III

Economic Applications

of Microalgae

11 Industrial Production of Microalgal Cell-mass and Secondary Products – Major Industrial Species

Chlorella

Hiroaki Iwamoto

11.1 Introduction

Chlorella species are encountered in all water habitats exhibiting a cosmopolitan occurrence, having been isolated from widely differing fresh, as well as marine, water habitats (see Chapter 1). A very great breadth of nutritional requirements of *Chlorella* species is indicated.

The species of the genus *Chlorella* have simple life cycles and nutritional requirements. Classification is complex because *Chlorella* species cannot be readily discerned on the basis of morphological features, the taxonomy of Euchlorella, which comprises the most common species is, therefore, incomplete. It has indeed been proposed to use physiological and biochemical rather than morphological criteria, for species identification.

On the basis of their external morphology, *Chlorella* species could nevertheless be placed in four general groups: (1) spherical cells (ratio of the two axes equals one); (2) ellipsoidal cells (ratio of the longest axis to the shortest axis being 1.45 to 1.60); (3) spherical or ellipsoidal cells; (4) globular to subspherical cells (Richmond, 1986). In reproduction, which is exclusively asexual, each mature cell divides usually producing four or eight (and more rarely, 16) autospores, which are freed by rupture or dissolution of the parental walls.

11.2 Industrial background

The first pilot plant for mass cultivation of *Chlorella* was devised and tested in Boston, USA (Little, 1953). This was followed by cultivation devices in Israel (Evenari *et al.*, 1953); Japan (Mitsuya *et al.*, 1953); Germany (Gummert *et al.*, 1953); and Czechoslovakia (Prokes & Zahradník, 1969), successively. Each plant represented a different type of installation for mass cultivation.

Commercial utilization of *Chlorella* was started for the first time in 1961 by Nihon Chlorella Inc., which was established using the mass cultivation installations of the Microalgae Research Institute of Japan (commonly called the Chlorella Institute). This institute was constructed in 1957 on the outskirts of Tokyo, as a pilot plant for research and development of mass cultivation of microalgae, with particular attention to growing *Chlorella*. Total culture area was about 4000 m², composed of three elongated oval ponds based on models designed by Professor Tamiya's group in the Tokugawa Institute for Biological Science (Tamiya, 1957). These ponds were remodelled soon thereafter into eight independent circular ponds, and operated for about six years, beginning from 1959. Part of the culture medium was continuously pumped into tubes, then ultimately returned to the ponds. CO₂ enriched air was introduced into the medium with aspirators which, in the final period of the Chlorella Institute, was an effective method of supplying CO₂ into the medium. Nevertheless, CO₂ was replaced by acetic acid (which is well utilized by *Chlorella* cells and does not induce serious contamination in the culture) both because of the low effectiveness in the utilization rate of CO₂ and because of high CO₂ cost.

This development represented a dramatic change in the mode of *Chlorella* mass culture, that is, from autotrophic to mixotrophic cultivation. Mixotrophy was introduced to cut the cost of production enough to facilitate supply of an inexpensive protein source for food or feed in protein-deficient areas of the world.

No research information existed at the time concerning the mechanism of mixotrophic growth of *Chlorella*. It had been observed, however, that *Chlorella* grows very well with certain organic compounds, with or without light – although better growth was obtained in light. Investigation concerning heterotrophic cultivation started at almost the same time as mixotrophic cultivation was introduced for commercial production.

The Chlorella Institute was directed by Professors H. Iwamoto (Iwamoto, 1958), H. Nakamura and Dr Y. Takechi (Takechi, 1965) in succession, from 1958 to 1964. They struggled with many problems and greatly contributed to the development of practical technology. The Institute supplied ample amounts of dried mass of *Chlorella* cells for nutritional and medical research to the National Institute of Nutrition in Tokyo and to the laboratories of several Japanese universities and hospitals.

Technologies for mass cultivation and production of dried biomass with good digestibility, as well as offering it to the public in easy-to-use tablet form, helped to establish the *Chlorella* industry as a novel health food in Japan in 1964. Also, it has supported many *Chlorella* factories constructed in Japan, Taiwan, Malaysia and Indonesia for the last 35 years. It is worth remembering that commercialization of *Chlorella* as a health food in tablet form was initiated in 1964 by a businessman who believed in the value of *Chlorella* and was encouraged only much later by the nutritional and clinical reports on *Chlorella*.

Chlorella has been mass cultivated commercially for the past 35 years, initially as the material for health food and later as mariculture feed. The total amount produced was about 200 t year⁻¹ by 1975; had exceeded 1000 t

annually in the 1980s; and approached 2000 t year⁻¹ in the 1990s. The majority of production was obtained by mixotrophic cultivation and the remainder was produced by heterotrophic cultivation. The latter mode of production did not grow markedly when first introduced in the market in 1965. The growing demand for *Chlorella* as the basic feed for rotifer and fingerlings in mariculture industry boosted production markedly.

11.3 Nutritional benefits

The most important substance for human health discovered in the *Chlorella* cell was β -1,3-glucan, which is an active immunostimulator and has many other functions, such as a free radical scavenger and a reducer of blood lipids. This kind of active substance was first found in the cell wall of baker's yeast and was named Zymosan in the 1940s in Norway. It was further studied in Italy in the 1960s as β -1,3-glucan (Riggi & DiLuzio, 1961). The same kind of bioactive compound was found in *Chlorella* and studied extensively in Japan around 1970 (Kojima *et al.*, 1971, 1973, 1974; Tanaka *et al.*, 1975). Quite a number of reports on the anti tumor effects of *Chlorella* polysaccharide have appeared in recent years (Nomoto *et al.*, 1983; Tanaka *et al.*, 1984), along with similar polysaccharides found in many kinds of mushrooms. It is now understood that the reputation and flourishing of *Chlorella* as a health food in Japan is based on these fundamental health and clinical effects.

11.4 Mixotrophic production

Mixotrophic mass cultivation, replacing photoautotrophic cultivation, was introduced from the initial stage of commercial production of *Chlorella* in 1964. Almost all commercial *Chlorella* production plants constructed thereafter in Japan and southeast Asia have produced *Chlorella* by mixotrophic cultivation, with only minimal quantities being produced using the heterotrophic mode of cultivation.

One of the major problems in mixotrophic outdoor cultivation is that of bacterial contamination. Acetic acid is preferably used as the carbon source in outdoor cultures, because the types and number of contaminants are rather limited, contamination usually being neither very heavy nor serious. The main contaminant, as a rule, is non-sulfur purple bacteria such as *Rhodopseudomonas* sp., and it sometimes makes the color of supernatant of *Chlorella* culture slightly pink following the first separation of *Chlorella* cells by centrifugation.

The contaminating cells suspended in the medium are readily separated from *Chlorella* cells by centrifugation, an Alfa-Laval type centrifuge usually being used. Further careful washing with water is quite effective in removing most of the bacterial contaminants from the concentrated *Chlorella* cell-mass.

Concentrated and cooled *Chlorella* slurry is rapidly heated and kept at about 100–130°C for a short period of sterilization and deactivation of the enzyme (chlorophylase). Synthetic acetic acid is used as the carbon source,

which is advantageous from the point of view of cost and easy regulation of culture pH in the course of cultivation. Acetic acid solution is added automatically and intermittently to the medium at pH 7 until it declines to 6.5, the pH value of the medium increasing with consumption of acetic acid. Urea is most favorable as the nitrogen source because it affects no change in pH. Two to four grams of acetic acid are required in large scale outdoor mixotrophic cultivation for production of 1 g *Chlorella*. It varies with the outdoor culture conditions, i.e. temperature, amount of sunlight, degree of stirring or aeration as well as extent of contamination. For comparison, the yield of *Chlorella* obtained in heterotrophic cultivation is somewhat less than 50% of the consumed glucose or acetic acid.

Studies on the relationship between organic carbon assimilation and light energy utilization are scarce. One of the few related reports revealed that the growth in mixotrophic cultivation with sufficient supply of O₂, CO₂ and acetate was the sum of heterotrophic growth and the growth induced by incident light (Fig. 11.1). The increased growth of *Chlorella* exposed to light in mixotrophic cultivation coincides with the growth of photoautotrophic cultivation, given the same intensity of light (Endo *et al.*, 1977), hence providing positive indication that growth processes of *C. regularis* consist of autotrophic and heterotrophic processes that proceed noncompetitively. A similar result was also obtained for *C. vulgaris* by Ogawa & Aiba

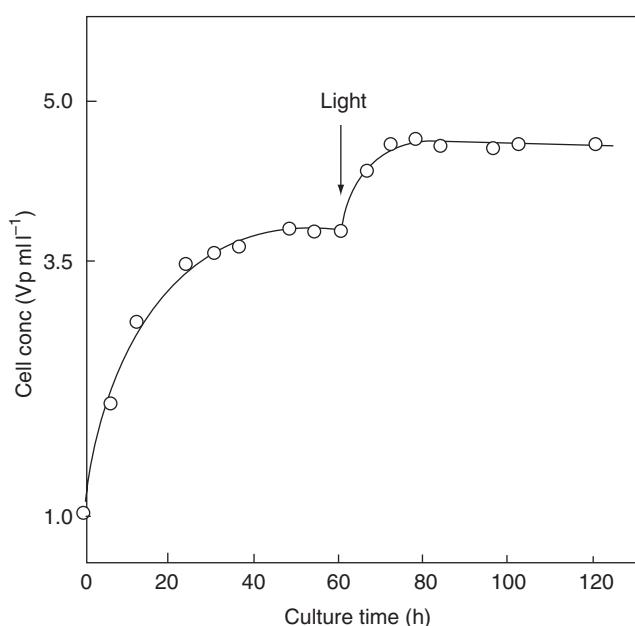


Fig. 11.1. Time course of continuous culture first under heterotrophic, then mixotrophic condition, in a vertically flat culture bottle. Culture medium: 250 ml. Initial concentration of acetic acid: 40 mM, Dilution rate in the continuous culture: 0.1 hr⁻¹, Incident light intensity: 10 klux, Illuminated area of the medium: 95 cm²; Temperature: 36°C; pH: 6.5–6.8; Aeration: 5% CO₂; Air 1.0 vvm throughout the cultivation period. V_p = packed volume. Reprinted with permission from The Japanese Society of Plant Physiologists (*Plant Cell Physiol.*).

(1981), showing that the photosynthetic mechanism and the heterotrophic growth mechanisms seem to function independently.

The practical cultivation conditions in mass production of *Chlorella* do not always coincide with those used in the experiments cited above. That is, oxygen for heterotrophic growth is not supplied by aeration, but is supplied only by stirring the medium by a number of baffle plates placed along the circulating arms for stirring. Oxygen is also supplied through the photosynthetic activity by *Chlorella* during the day. Carbon dioxide is not necessary, since it is supplied through oxidative decomposition of acetic acid by the *Chlorella* cells.

The yield per unit area in mixotrophic cultivation increases by increasing the depth of culture, being greatly different from autotrophic cultivation, in which the yield is determined mainly by the illuminated area and the rate of incidence of photon flux, regardless of culture depth. Deep culture is not advantageous in autotrophic cultivation due to the increased loss of biomass by dark-respiration and other reasons. In mixotrophic cultivation, the yield per unit area increases.

The depth of culture in autotrophic cultivation is ca. 10 cm, 20–30 cm is preferably used in mixotrophic cultivation, and the yield per unit area in mixotrophic cultivation in factories in Taiwan is known to be much larger than obtained in photoautotrophic cultivation. Some data indicate that the yield in summer is $30\text{--}35 \text{ g m}^{-2} \text{ d}^{-1}$ on sunny days, $20\text{--}25 \text{ g m}^{-2} \text{ d}^{-1}$ on cloudy days, and $10\text{--}15 \text{ g m}^{-2} \text{ d}^{-1}$ on rainy days. This is 1.5–2.0 times higher than produced by the photoautotrophic mode of cultivation.

Deactivation of active chlorophylase in fresh *Chlorella* cells is carried out by heating at 110–130°C for a very short time to denature the enzyme. This step is necessary in preparing *Chlorella* powder (or any kind of microalgae for health food) in order to avoid the health hazard caused by phaeophorbide, a derivative of chlorophyll, produced under certain conditions by active chlorophylase. Phaeophorbide induces inflammation of the skin or ear lobe when consumed by humans, or even by animals in large amounts, as soon as the body is exposed to sunlight. Provided chlorophylase in *Chlorella* is denatured, there is essentially no chance of formation of phaeophorbide during processing of *Chlorella* powder.

Fresh *Chlorella* cells, having been harvested and washed, are sometimes broken to improve digestibility for humans. Low digestibility of *Chlorella* has been observed in dried *Chlorella* cake produced by the tray drying method used in the early days of the Chlorella Institute. Digestibility improved through the introduction of the technology of spray-drying, and also quick blanching at over 100°C. Digestibility of *Chlorella* powder in the human body is presently assumed to be ca. 80%. *Chlorella* cells broken by a mill are known to have a slightly higher digestibility than non-broken *Chlorella*, but the difference may not be significant.

11.5 Heterotrophic production (see also Chapters 20 and 31)

Chlorella has long been known to grow not only photoautotrophically, but also heterotrophically. It was also generally accepted that growth and

formation of chloroplasts in *Chlorella* are suppressed under dark-heterotrophic conditions. A special *Chlorella* strain of high chlorophyll content and high growth rate under dark-heterotrophic conditions was found about 30 years ago in Japan (Endo & Shirota, 1972). Successive studies of cell physiology, strain breeding and bioengineering established the biotechnology for growing heterotrophic *Chlorella* cells rich in some phytochemicals such as carotenoids, chlorophyll and tocopherol.

Many *Chlorella* species can be grown heterotrophically, generally having, however, markedly lower growth rates under heterotrophic conditions compared with photoautotrophic conditions (Droop, 1974). A breakthrough in this course was provided by Endo & Shirota (1972), who isolated the strain *C. regularis* S-50 which exhibited a much higher growth rate under heterotrophic conditions than previously reported for any other species or strains. This strain was found to have the highest growth rate and higher content of phytochemicals under heterotrophic conditions compared with other *Chlorella* strains used for mass cultivation (Endo *et al.*, 1974). The maximum specific growth rate ($\mu_{\text{max}} \text{ h}^{-1}$) was 0.30 h^{-1} under autotrophic conditions, whereas 0.24 h^{-1} on glucose and 0.28 h^{-1} on acetate under heterotrophic conditions.

The optimal temperature of this strain is $35\text{--}37^\circ\text{C}$, a very important feature as it is rather high when compared with ordinary *Chlorella* strains. Optimal pH is 6.0–7.0. Thiamin and several amino acids stimulate growth of the strain.

The heterotrophic culture is carried out axenically, using a process which is basically similar to cultivation of aerobic microorganisms in the fermentation industry. The medium is sterilized at 130°C for 30 min. After inoculation, the culture is run axenically under agitation and germ-free aeration at a constant temperature and pH. Starting from the seed culture, culture volume is scaled up with larger fermenters.

The heterotrophic culture stage is controlled in a step-wise manner, i.e. the initial exponential growth stage is followed by a maturation stage, in order to obtain cells rich in chlorophyll and other important phytochemicals. Cell mass increases with consumption of glucose but formation of constituents of chloroplasts is suppressed. After the increase of cell mass ceases due to glucose deficiency, maturation and division of cells proceed rapidly, together with the formation of chloroplasts and their constituents under oxygen sufficiency and glucose deficiency.

The pigment-rich mutant Y21 was induced from *C. regularis* M-1 by means of mutagen treatment (nitrosoguanidine) or UV irradiation, and not by genetic recombination. Pigment-mutants of *Chlorella* can be isolated easily as visible deep-colored colonies on nutrient agar plates. The mutant Y21 produced approximately two times as high content of cellular carotenoids, chlorophyll and other phytochemicals as the parent strain cultivated heterotrophically.

The glucose limited fed-batch culture has been developed as a practical mass production method, under axenic-heterotrophic conditions (Endo & Sansawa, 1994), producing *Chlorella* rich in phytochemicals with a very high final cell density. The principle at the heart of this method involves supplying

glucose in response to its consumption by the growing *Chlorella*. Glucose concentration in culture fluid is analyzed continually and automatically, and glucose concentration is maintained at 1.5%, stopping feeding when cell density reaches the expected value. The culture is maintained for about ten hours in order to enhance cell division and maturation. Maximum cell density is limited by the rate of oxygen supply to the culture vessel. An example of a time course of glucose limited fed-batch culture of *C. regularis* in a 10 m³ culture is shown in Fig. 11.2.

The culture was run for 40 h in total, in the first 30 h of which exponential growth with glucose feeding takes place. The last 10 h are for cell maturation in the absence of glucose feeding. The total cell mass is harvested in the following successive processes: concentration of cells by centrifugation, washing, inactivation of cellular enzyme (chlorophylase) by heating the concentrated slurry (130°C, 3''), and drying by spray-drying to obtain a very fine

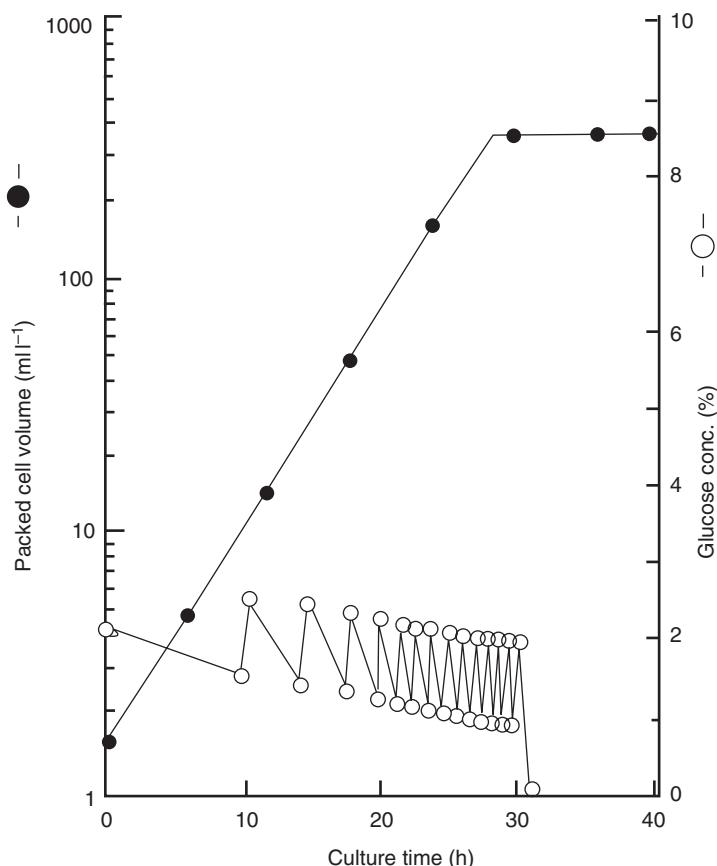


Fig. 11.2. Time course of glucose-limited fed-batch culture of *C. regularis* under heterotrophic conditions. Glucose was fed to culture in response to reduced concentration due to growth. Glucose concentration was controlled at about 1.5% using a glucose analyzer. Culture vessel 10 m³; Temperature 35°C; pH 6.5; Aeration 1 vvm; Agitation 135 rmp; Production terminated after 30 h. Reprinted with permission from The Japanese Society of Plant Physiologists (*Plant Cell Physiol.*).

powder. The final concentration of *Chlorella* cells in the culture was 360 ml l⁻¹ in packed cell volume, or ca. 80 g l⁻¹ in dry weight. The yield of cells as fraction of consumed glucose was 0.45. Each gram of dried cell mass contained 7 mg total carotenoids (3.5 mg lutein, 0.5 mg α -carotene, 0.6 mg β -carotene) and 35 mg chlorophyll. The productivity of dried cells was 48 g l⁻¹ d⁻¹ during a 48 h cultivation period in a 10 m³ culture vessel.

The special significance of heterotrophic mass cultivation of *Chlorella* is that it can be turned into *Chlorella* powder of quite superior quality for health food, rich in valuable phytochemicals and devoid of contamination. Also, it produces live and fresh *Chlorella* biomass with good shelf life as Rotifer feed, essential for growing fry or as direct feed of fry. Most of the demand for this kind of feed in mariculture in Japan is now supplied with live *Chlorella* cell – mass grown heterotrophically. It is thickly concentrated and readily stored at 4°C.

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12 Industrial Production of Microalgal Cell-mass and Secondary Products – Major Industrial Species

Arthrospira (Spirulina) platensis

Qiang Hu

12.1 Introduction

Arthrospira (Spirulina), of which the most popular known species are *Arthrospira (Spirulina) platensis* and *Arthrospira (Spirulina) maxima*, are a group of cyanobacteria characterized by loosely spiral-shaped trichomes arranged in an open helix enclosed in a thin mucilaginous sheath (Castenholz, 1989a). They are ubiquitous, found in a variety of environments, particularly in alkaline, brackish and saline waters, where they frequently become predominant species and form massive blooms (Ciferri, 1983). Of the few cyanobacteria that have the longest history of being utilized as part of the human diet, *Arthrospira platensis* was the first cyanobacterium to be commercially cultivated using modern biotechnology. The annual production of *Arthrospira* biomass worldwide in 2000 was estimated to be ca. 2000 t. The use of *Arthrospira* has expanded from its original application as human food supplements and animal feed to the production of fine chemicals for clinical diagnosis, biological research, and cosmetic applications. More recent studies of the therapeutic and health effects of *Arthrospira* are expected to promote the application of this organism in the pharmaceutical and nutraceutical industries. It is anticipated that *Arthrospira* will continue to act as the single most important species in the cyanobacterial biotechnology industry in the next decade or longer. This section provides an overview of *Arthrospira* and its use in biotechnology. More detailed information about this organism can be found in several review articles and books (Ciferri, 1983; Richmond, 1986; Vonshak, 1997; Vonshak & Tomaselli, 2000).

12.2 Major morphological, cell structure and taxonomic features

The mature trichomes of *Arthrosphaera* are usually a few millimeters long and consist of cylindrical cells of 3–12 µm in diameter (Ciferri, 1983). The helix geometry and overall trichome configuration of *Arthrosphaera* can vary considerably with the species and depend on growth and environmental conditions. The transition of coiled helix to loosened spiral shape or even to a completely straight shape may occur reversibly or irreversibly under different culture conditions (Jeeji Bai, 1985; Vonshak & Tomaselli, 2000).

Cell structure and organization of *Arthrosphaera* is typical of cyanobacteria. The Gram-negative cell wall is composed of four layers, with a major, structural layer of peptidoglycan. The central nucleoplasmic region appears to contain a number of carboxysomes, ribosomes, cylindrical bodies, and lipid droplets. Thylakoid membranes associated with phycobilisomes are distributed peripherally in the cytoplasm. The peripheral region of the cell also contains gas vacuoles and several other subcellular inclusions, such as polyphosphate granules and polyglucan granules.

The life cycle of *Arthrosphaera* is simple. Reproduction is accomplished by fragmentation of a mature trichome into a number of shorter segments through destruction of specialized multiple intercalary cells, called *necridia*. The resulting segments of a few cells then increase in length by binary fission on a single plane along the long axis of the trichome and then assume the helical shape (Ciferri, 1983).

The major classical taxonomic feature of the genus *Arthrosphaera* is the presence of septa, or cross-walls, between the cells in the trichome, which are visible under a light microscope. This distinction separates it from *Spirulina*, another genus with invisible septa, but otherwise sharing the same character of helically coiled filaments. The systematics of *Arthrosphaera* and *Spirulina* has been a subject of long debate over the years. A major difficulty involves the morphological variability of the strains under different environmental conditions. Possible visualization of previously *invisible septa* by using improved light microscopic techniques further complicated the taxonomic situation of the two genera. As a result, the species/strains with regularly stable spiral-shape have been variously placed in the two genera by individual taxonomists. *A. platensis* is, in many cases in the literature, synonymous with *S. platensis*, and *A. maxima* is synonymous with *S. maxima*. In recent years, more evidence based upon 16S rRNA sequence, fatty acid composition, gas-vacuolated cells, and the size of trichomes supports the designation of the large-sized alkalophilic species of commercial interest to be in the genus *Arthrosphaera*, while the designation of *Spirulina* is restricted to small-sized species (Vonshak & Tomaselli, 2000).

12.3 Physiological, biochemical and genetic features

12.3.1 Growth physiology

Arthrosphaera spp. are photoautotrophs endowed with oxygenic photosynthesis. Therefore, any factor affecting photosynthesis will influence the growth

of these organisms. The major factors include light intensity, temperature, salinity, and alkalinity.

Under laboratory conditions, light saturation for growth of *A. platensis* was determined to be 150–200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The optimal growth temperature for *Arthrospira* is in the range of 35–38°C. *Arthrospira* can tolerate low to moderate concentrations (in a range of tens to hundreds of millimole) of NaCl. However, exposure of *Arthrospira* cells to salt concentrations at 0.75 M NaCl and higher may significantly reduce the growth potential (Vonshak & Tomaselli, 2000). *Arthrospira* spp. are regarded as obligatory alkalophiles, with the maximal growth rate being obtained at pH 9.5–9.8. The fact that it thrives in the high pH environment, which makes life for other microorganisms rather difficult, is the key for the success of large-scale monoalgal cultures of *Arthrospira*.

Although *Arthrospira* is generally regarded as a photoautotrophic organism, it may undergo mixotrophic and even heterotrophic growth in the presence of organic carbon sources (such as glucose and acetate). Potential enhancement of cell density and biomass yield by synergistic contribution of photoautotrophic growth utilizing light energy coupled with heterotrophic growth consuming organic carbon substrate/s has promoted the use of mixotrophic culture mode as an alternative strategy for mass production of *Arthrospira* (Chen & Zhang, 1997).

12.3.2 Biochemistry

Proteins and supramolecular complexes involved in photosynthesis of *Arthrospira* have been the subject of extensive studies. Trimeric and monomeric forms of PS I reaction center complexes have been isolated from *A. platensis* (Shubin *et al.*, 1993). Trimeric PS I complexes predominate in the *Arthrospira* membranes, suggesting a potential role for the aggregated form of PS I in protecting the PS I against photodestruction (Kruip *et al.*, 1999). C-phycocyanin and allophycocyanin have been found to be the major light-harvesting pigments present in phycobilisomes of *A. platensis* and *A. maxima* (Boussiba & Richmond, 1979). The crystal structures of *A. platensis* C-phycocyanin and allophycocyanin at 2.2 and 2.5 Å resolution, respectively, have been determined (Moreno *et al.*, 1997; Wang *et al.*, 2001). A study of the distribution of phycobilisomes between PS I and PS II complexes in *A. platensis* has revealed that about 20% of phycobilisomes are bound to PS II, while 60% transfer energy to PS I trimers, and the remaining 20% are associated with PS I monomers (Rakhimberdieva *et al.*, 2001). Several other protein components of the photosynthetic apparatus have been isolated and characterized. These include the cytochrome b_6f complex (involved in the transport of electrons from PS II to PS I and cyclic electron flow around PS I), ferredoxin (a carrier in many electron transport reactions including the photoreduction of NADP⁺), ATP synthase (involved in photophosphorylation), and D-ribulose-1,5-bisphosphate carboxylase/oxygenase (the major enzyme responsible for CO₂ fixation) (Ciferri & Tiboni, 1985; Vonshak, 1997).

12.3.3 Genetics (also see Chapter 16)

Since the 1980s, numerous genes involved in fundamental metabolism and its regulation have been cloned and characterized (Ciferri & Tiboni, 1985; Vonshak, 1997). Moreover, traditional induced mutagenesis methods and selection strategies had been applied to *Arthrospira* for strain improvement. A number of amino acid-analogs-resistant mutants of *A. platensis* were found to overproduce specific free amino acids, such as proline (Riccardi *et al.*, 1981).

However, progress toward expanding of the genetic capabilities of *Arthrospira* and thus its use as a *natural bioreactor* to overproduce novel, recombinant proteins has been limited. A reliable gene transformation system has not yet been established in this organism. This may be partially due to the absence of endogenous plasmids and the lack of suitable constructed vectors. High extra- and intracellular DNase activities represent another major obstacle preventing foreign genes from being stably introduced. Very recently, some small but meaningful progress was made toward this end. Cao *et al.*, (2000) reported that extracellular DNase could be removed by rinsing *Arthrospira* cells several times with Mg²⁺-free Zarrouk medium, while the intracellular DNase activity could also be inhibited by Mg²⁺-free Zarrouk medium containing 1 mM EDTA. Toyomizu *et al.* (2001) demonstrated that electroporation could be an effective transformation tool for *A. platensis*. There is no doubt that development of recombinant DNA methodology for *Arthrospira* would not only advance our current understanding of basic physiological and biochemical phenomena associated with this organism, but also allow genetic manipulation of this organism that would trigger significant changes in *Arthrospira* biotechnology over the long run.

12.4 Commercial production in the world

The first commercial *Arthrospira* production facility was established in the late 1970s. Today, *Arthrospira* spp. are commercially produced all over the world with most of the production facilities located in Asia-Pacific region (Lee, 1997). Mass culture of *Arthrospira* involves basically four major phases: culturing, harvesting, drying and packaging.

12.4.1 Culturing (also see Chapter 9)

Commercial cultivation of *Arthrospira* is performed outdoors in open raceways. A typical open raceway is a long, single looped shallow channel lined by plastic material or concrete and varies in surface area from 1000 to 5000 m². A paddle wheel of various designs is provided to generate mixing of the culture suspension. One exception is a circular-shape pond with a rotating arm to facilitate mixing. The diameter of such a circular pond can be as great as 50 m, covering 2000 m² surface area (Lee, 1997). The key operational parameters affecting the growth and production of algal cultures in these open systems are culture depth, rate of mixing, and algal population density.

12.4.2 Harvesting

Arthrospira biomass is harvested from culture ponds by means of filtration, and the whole process can be automated. Three types of filtration devices are available: inclined gravity screen, horizontal vibratory screen and vacuum table or vacuum belt filter. It should be noted that the filtration process may introduce some risk of filament breakage due to mechanical shearing forces created by filtration and pumping processes, which in turn reduces the harvesting efficiency and may potentially cause bacterial contamination. It has been suggested that operation of all three devices in series may improve the harvesting capacity and efficiency, as well as causing less damage of filamentous trichomes (Vonshak & Richmond, 1988; Belay *et al.*, 1994). The end product of this process is typically a suspension of 8–15% of dry weight.

12.4.3 Drying

Drying is accomplished by spray- or drum-drying, yielding *Arthrospira* powder with moisture content of less than 3–4%. It is essential to have the drying process done in a time frame of seconds or minutes in order to insure preservation of heat sensitive nutrients, such as pigments and certain enzymes (Belay *et al.*, 1994). Spray-drying of *Arthrospira* spp. should be the preferred option when human consumption of the biomass is concerned, though the cost of a spray-drying system is higher than that of a drum-drying facility. Drum-drying is adequate for preparing animal-grade algal biomass.

12.4.4 Packaging

The *Arthrospira* powder coming out of the dryer is immediately sealed under vacuum in an oxygen-barrier bag to minimize oxidation of certain vital pigments like carotenoids. It was suggested that the oxygen-barrier bag is better than polyethylene bag in preventing loss of carotenoids by oxidation (Belay *et al.*, 1994).

12.5 *Arthrospira* biomass and its derived bioproducts

12.5.1 *Arthrospira* for human food and animal feed

Arthrospira has been used in the human diet for at least 700 years, and perhaps much longer, on the continents of America and Africa. About 1300 AD, the Indians in the Valley of Mexico harvested *Arthrospira* from lake Texcoco and made what they called *tecuitlatl*, a type of dry cake made of sun-dried *Arthrospira* biomass (Farrar, 1966). Perhaps around the same time, Africans in the vicinity of Lake Chad were in the habit of eating *Arthrospira* in a way very similar to that of the Mexican Indians, and they called it *dibe* (Abdulqader *et al.*, 2000). Indeed, *Arthrospira* contains high quantities of proteins (up to 65% of dry weight), along with good amounts of essential fatty acids (e.g. γ -linolenic acid [GLA]), polysaccharide, phycobiliproteins, carotenoids,

vitamins (especially B₁₂) and minerals, making it a desired food source. Today, *Arthrospira* products are sold as a dietary/functional food in health food stores worldwide. As animal feed, *Arthrospira* has found its widest use in the aquaculture and poultry industries (Belay *et al.*, 1996; Wikdors & Ohno, 2001).

12.5.2 *Arthrospira* as a source of pharmaceuticals and nutraceuticals

In the past decade, research has been focused on the potential therapeutic effects of *Arthrospira*. Preclinical and clinical studies have suggested a number of health and therapeutic effects of the biomass, ranging from: reduction of cholesterol and nephrotoxicity; inhibition of replication of several pathogenic viruses; enhancement of the immune system and intestinal lactobacilli – to the prevention of cancer/tumor development (Belay *et al.*, 1993; Vonshak, 1997). For example, a sulfated polysaccharide, calcium spirulan, isolated from *A. platensis* was found to inhibit the replication of several enveloped viruses, including Herpes simplex virus type 1, human cytomegalovirus, measles virus, influenza A virus, and HIV-1, and thus could be a potential antiviral agent for human trials (Hayashi *et al.*, 1996).

12.5.3 *Arthrospira* as a source of fine chemicals

Arthrospira is an excellent source of phycobiliproteins, which may consist of up to 17% of *Arthrospira* cell dry weight (Boussiba & Richmond, 1979). Because of a number of unique properties of these proteins, such as high molar absorbance coefficients, fluorescence quantum yields, stokes shift, stable oligomers, and high photostability, phycobiliproteins find their widest use as fluorescence tags for cell surface markers for flow cytometry analyses of cell sorting, high throughput clinical analyses, as well as phycobiliprotein-based assays for reactive oxygen species (Glazer, 1999). The high free-radical scavenging capacity of phycobiliproteins could eventually make them useful as potent antitumor and anticancer drugs. Also, *Arthrospira* spp. are the richest algal source of GLA. GLA has been shown to lower low-density lipoproteins in hypercholesterolemic patients, alleviate the symptoms of premenstrual syndrome, and treat atopic eczema (Vonshak, 1997).

12.6 *Aphanizomenon flos-aquae*

At the end of this *Arthrospira* treatise, another cyanobacterium, *Aphanizomenon flos-aquae*, which has recently drawn much attention as a human food supplement will be briefly described. *A. flos-aquae* is a colony-forming, filamentous, heterocystous cyanobacterium belonging to the family Nostocaceae in the order of Nostocales, that is found in freshwater environments (Castenholz, 1989b). Compared to *Arthrospira*, *A. flos-aquae* has a rather short history of consumption by humans. The exploitation of *A. flos-aquae* did not start until the early 1980s when natural blooms of this organism were first harvested on a large-scale from Klamath Lake in Oregon (USA) (Carmichael *et al.*, 2000). Currently, two main harvesting strategies have been

adopted on Klamath Lake. One involves an off-lake, large series of screens made of nylon mesh to remove the biomass from the water coming out of Klamath Lake and flowing into an aqueduct system. The other employs on-lake barges equipped with either rotating screens or fixed screens coupled with water pumps. The rotating screens can be lowered just under the surface of the lake to collect the biomass, whereas the fixed screens filter the water pumped from below the surface of the lake. It was reported that approximately 2000 t of biomass of *A. flos-aquae* were harvested from Klamath Lake in 1998 (Carmichael *et al.*, 2000).

A. flos-aquae, like *Arthrospira*, is rich in proteins, polysaccharides, carotenoids, phycobiliproteins, vitamins, and minerals. It is sold in the health food market as a nutrient-dense food and dietary supplement. Beyond nutritional value, *A. flos-aquae* has been claimed to have numerous beneficial health effects. These include anti-inflammatory, exhaust relief, assisting digestion, immune-boosting, and general improvement of overall well-being (Jensen *et al.*, 2001). Annual sales of the biomass in 1998 were estimated to be over \$100 million (Carmichael *et al.*, 2000).

Natural harvesting of *A. flos-aquae* requires less capital and maintenance costs associated with cultivation process, which may represent an economic advantage over mass cultures of microalgae in constructed photobioreactors. On the other hand, this process suffers major problems relating to quality and safety issues. The biomass harvested from Klamath Lake is essentially a mixture of multiple species of cyanobacteria and diatoms, though *A. flos-aquae* is the dominant species. The species composition of the bloom, like the biochemical composition of *A. flos-aquae*, varies considerably depending on nutrient availability, weather conditions, and water chemical and hydrological characteristics, which in turn may affect the nutrient profile of the biomass. A more serious concern in the past and even today relates to the toxicological safety of *A. flos-aquae*. Although it becomes less likely that the strains of *A. flos-aquae* in Klamath Lake produce neurotoxins (Li *et al.*, 2000), some other species/strains of this genus have been reported to produce neurotoxins (i.e. neo-saxitoxin and anatoxin-a) and hepatotoxin (i.e. cylindrospermopsin) (Banker *et al.*, 1997; Dias *et al.*, 2002; Rapala *et al.*, 1993). Furthermore, *Microcystis*, *Anabaena* and *Oscillatoria* are regular components of the cyanobacterial communities in Klamath Lake. Potential production of cyanotoxins, such as microcystins and/or anatoxins, by these genera is another source of concern with the safe use of *Aphanizomenon* products. Indeed, the logistics and quality control issues involved in harvesting a safe algal biomass product from natural systems present more obstacles than cultivation of a desired monoalgal population in environmentally and biologically controlled photobioreactors.

The inherent problems and limitations associated with natural harvesting should give rise to the development of large-scale photobioreactors for mass culture of *A. flos-aquae* in the near future. It may be anticipated that quality, yield and safety of *A. flos-aquae* biomass could be greatly improved through screening of desired *A. flos-aquae* strains, better understanding of the growth physiology of this organism, as well as development of highly efficient, cost-effective large-scale photobioreactors.

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13 Industrial Production of Microalgal Cell-mass and Secondary Products – Major Industrial Species

Dunaliella

Ami Ben-Amotz

13.1 Biology and halotolerance

The biflagellated alga *Dunaliella* is classified under Chlorophyceae, Volvocales, which includes a variety of ill-defined marine and fresh water unicellular species (Avron & Ben-Amotz, 1992). *Dunaliella* is characterized by an ovoid cell volume usually in the shape of a pear, wider at the basal side and narrow at the anterior flagella top (Fig. 13.1). The cellular organization of *Dunaliella* is no different than that of other members of the Volvocales presenting one large chloroplast with single-centered starch surrounded by pyrenoid, a few vacuoles, a nucleus and a nucleolus. Cells of the genus *Dunaliella* lack a rigid polysaccharide cell wall, and are enclosed by a thin elastic plasma membrane covered by a mucous surface coat. The lack of a rigid cell wall permits rapid cell volume changes in response to extracellular changes in osmotic pressure with common osmotic treated *Dunaliella* shape from swollen sphere to shrink fiber. Under extreme salt concentrations above saturation, *Dunaliella* loses its flagella and the surrounding mucous and the cell rounds with a buildup of a thick surrounding wall to form a dehydration resistant cyst.

Dunaliella occurs in a wide range of marine habitats such as oceans, brine lakes, salt marshes, salt lagoons and salt water ditches near the sea, predominantly in water bodies containing more than 2 M salt and high-levels of magnesium. The effect of magnesium on the distribution of *Dunaliella* is not clear, but in many *bittern* habitats of marine salt producers, *Dunaliella* usually flourishes. The phenomenon of orange-red algal bloom in such marine environments is usually related to combine sequential growth of *Dunaliella*, brine shrimps and halophilic bacteria, as may be observed in concentrated saline lakes in many places around the globe. *Dunaliella* is recognized as being the most halotolerant eukaryotic photosynthetic

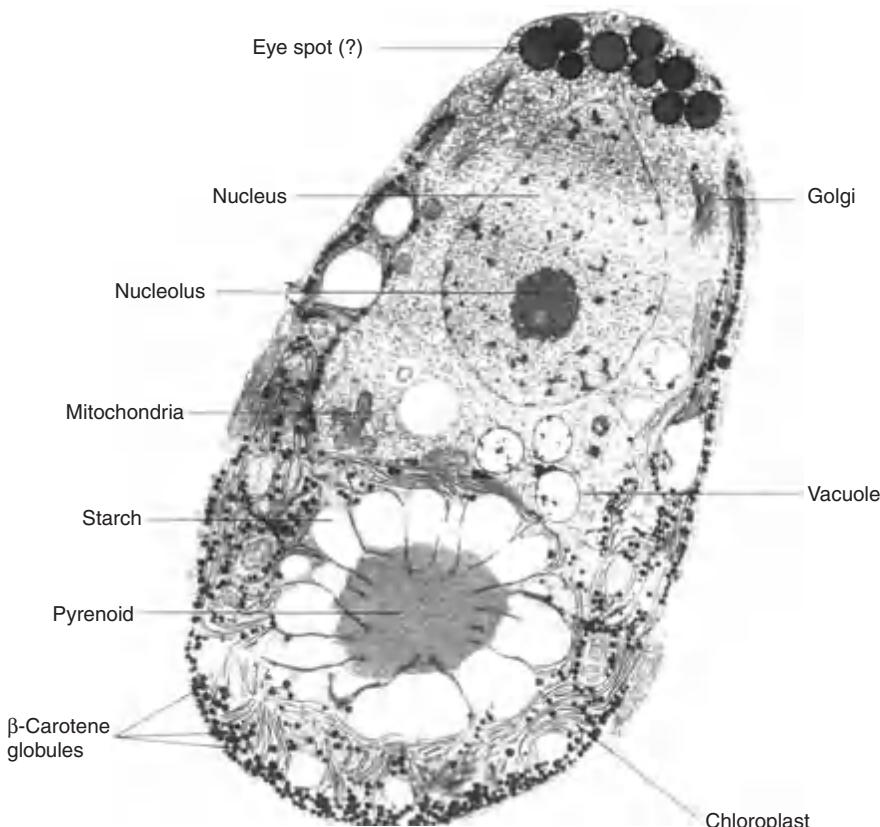


Fig. 13.1. Electron micrograph of *D. bardawil* – a β -Carotene rich alga.

organism known, showing a remarkable degree of adaptation to a variety of salt concentrations from as low as 0.1 M to salt saturation >4 M.

Many manuscripts and monographs were dedicated in the last decade to the osmoregulation of *Dunaliella* (Avron & Ben-Amotz, 1992). The intracellular concentration of glycerol is directly proportional to the extracellular salt concentration and is sufficient to account for all the required cytoplasmic and most of the chloroplastic osmotic pressures. Glycerol biosynthesis and elimination occur under either light or dark conditions by a novel glycerol cycle, which involves several specific enzymes. Accumulation of glycerol, the lack of rigid cell wall, and the enclosing by elastic plasma membrane of special ion transport properties makes *Dunaliella* a model of active osmometer attractive for elucidation research on ion transport, membrane characteristic, and bioenergy.

13.2 β -Carotene biology

Among the genus *Dunaliella*, the few strains that have been shown to change their colors from green to orange produce and accumulate large amounts of cellular β -carotene (Mil'ko, 1963; Semenenko & Abdullaev, 1980; Ben-Amotz

et al., 1982; Loeblich, 1982). In hypersaline lagoons, which are generally low in available nitrogen and exposed to high solar radiation, these β -carotene producing strains of *Dunaliella* predominate over all other organisms to a seasonal orange colored bloom. Under such stressful environmental conditions more than 12% of *Dunaliella* dry weight is β -carotene, usually associated with a sharp decline in cell protein and the chloroplast chlorophyll. The β -carotene in *Dunaliella* accumulates within distinctive oily globules in the interthylakoid spaces of the chloroplast periphery. Analysis of the globules showed that the β -carotene of *Dunaliella* is composed mainly of two stereoisomers: all-*trans* and 9-*cis*, with the rest a few other mono-*cis* and di-*cis* stereoisomers of the β -carotene and no xanthophylls. Both the amount of the accumulated β -carotene and the 9-*cis* to all-*trans* ratio depend on light intensity and on the algal division time, which is determined by the growth conditions. Thus, any growth stress, which will slow down the rate of cell division under light, will in turn increase β -carotene production in *Dunaliella*. In fact, high light and many environmental stress conditions such as elevated salt, extremes of pH, low temperature, nutrient deficiencies, and others affect the content of β -carotene in *Dunaliella*.

Dunaliella follows the same biosynthetic pathway of carotenoids with the same substrates and same intermediates as found in other eukaryotic organisms and plants (Zechmeister, 1962; Goodwin, 1988), however, the biological isomerization reaction, which eventually produces 9-*cis* β -carotene in *Dunaliella*, is not identified as yet (Shaish *et al.*, 1990, 1991; Ebenezer & Pattenden, 1993).

A few speculative hypotheses have been postulated for the function of the β -carotene globules in *Dunaliella* (Ben-Amotz & Avron, 1990). The most accepted hypothesis suggests that the β -carotene globules protect the cell against injury by high intensity radiation under limiting growth conditions by acting as a screen to absorb excess radiation. Strains of *Dunaliella* and other algae unable to accumulate β -carotene bleach and die when exposed to high-levels of radiation while the β -carotene-rich *Dunaliella* flourishes. Moreover, protection against photoinhibition by the massively accumulated β -carotene is observed only when the photoinhibitory light is composed of wavelengths absorbed by β -carotene, i.e. in the blue region.

13.3 Biotechnology of β -carotene production by *Dunaliella*

Dunaliella is the most suitable organism for mass cultivation outdoors in open ponds. The ability to thrive in media with high sodium, magnesium, calcium and the respective anions, chloride and sulfate, in desert high solar irradiated land with access to brackish water or sea water at extreme temperatures from around -5° to above 40°C , all make *Dunaliella* most attractive for biotechnologists and venture capitalists. In fact, since 1980 several firms, government authorities, and industries have invested capital in the application of *Dunaliella* for the production of natural β -carotene. Large-scale *Dunaliella* production is based on autotrophic growth in media containing inorganic nutrients with carbon dioxide as exclusive carbon sources. Attempts to commercially develop heterotrophic strains or mutants of *Dunaliella* for growth on glucose or acetate, e.g. as *Chlorella* or

Chlamydomonas, respectively, were not successful. Due to the demand for high light intensity for maximal β -carotene production beyond that required for normal growth, production facilities are located in areas where solar output is maximal and cloudiness is minimal. Most of the present *Dunaliella* production plants are located close to available sources of salt water, e.g. sea/lake-salt industries, usually in warm and sunny areas where the rate of water evaporation is high and non-agricultural land is abundant.

Four modes of cultivation have been used in large-scale production of *Dunaliella*:

- The first, termed *extensive cultivation*, uses no mixing and minimal control of the environment. To decrease fungal contamination (Tonka & Toncheva-Panova, 1997) and attacks by zooplanktonic predators, such as certain types of ciliates, amoebae, or brine shrimp (Brock, 1975; Post, 1977; Post *et al.*, 1983), the growers employ very high salt concentrations. *Dunaliella* grows slowly in shallow lagoons in nearly saturated brine and predators are largely eliminated. The naturally selected strain of *Dunaliella* is well adapted to nearly salt saturation conditions, partially loses its flagella and produces a thick cell wall on the transformation to a cyst form. Extensive cultivation productivity is low, as it is based on less than $0.1\text{ g } \beta\text{-carotene m}^{-3}$ and the area needed for commercial production is very large; however, the low operating costs of such facilities have led to the development of two commercial plants in Australia.
- The second, termed *intensive cultivation*, uses high biotechnology to control all factors affecting cell growth and chemistry. The ponds are usually oblong, lined, constructed raceways varying in size up to a production surface area of approximately 3000 m^2 . The use of long arm, slow revolution paddle wheels is presently common in the large-scale facilities in Israel, USA, China, and Chile. One production-sized shallow water pond of 20 cm on an area of 3000 m^2 (600 m^3) containing $5\text{--}15\text{ g } \beta\text{-carotene m}^{-3}$ yields $3\text{--}9\text{ kg } \beta\text{-carotene}$ on total harvest. The current large-scale production of β -carotene under intensive cultivation is around $200\text{ mg } \beta\text{-carotene m}^{-2}\text{ d}^{-1}$ on a yearly average; thus, a modern intensive plant of $50\,000\text{ m}^2$ produces $3650\text{ kg } \beta\text{-carotene}$ per year.
- Between the extensive and intensive modes there are examples in Australia and China of the third type, *semi-intensive mode*, where the ponds are enlarged ten times, to about $50\,000\text{ m}^2$ each, with partial control and no mixing.
- The fourth is *highly intensive* cultivation in closed photo-bioreactors. Many trials have been initiated in the last decade to grow *Dunaliella* in different models of closed photobioreactors with attempts to design the best sunlight-harvesting unit for β -carotene optimization. The different designs include: narrow, very long, plastic tubes, plastic bags, trays and more. However, as of today, none of these trials have taken production beyond the laboratory or small pilot plant volume, mainly due to economic limitations and non-feasible large-scale development. The few industrial ventures of high intensity-closed photobioreactors became insolvent and no longer exist.

Generally, large-scale optimization of β -carotene production is achieved in all modes of cultivation by high salt stress and by nitrogen deficiency. For pragmatic reasons, the first is applied in the extensive mode, while nitrogen starvation controls the intensive mode. Most species of *Dunaliella* grow optimally in a medium containing 1–2 M NaCl in accordance with the medium temperature, exhibiting closely similar growth rates at high temperatures of $>30^{\circ}\text{C}$ in >3 M NaCl at moderate temperatures of 25°C in 2 M NaCl, and at low temperatures of 15°C in 1 M NaCl. The algal composition changes respectively by selective accumulation of glycerol and starch. This unique environmental adaptation of *Dunaliella* allows successful intensive outdoor growth in cold seasons and in cold areas. Most commercial *Dunaliella* ponds employ evaporated concentrated seawater, lake salt water, or seawater augmented with dry salt to reach the desired concentration in the medium. Favored sites for *Dunaliella* cultivation are along the seashore or close to salt lagoons and salt producing industries for the use of a mixture of seawater and concentrated salt water in order to obtain the desired salt concentration by season and temperature. The use of recycled high salt medium is common in a few plants after harvesting and separation of the algae by oxidative treatment of the algal free medium to reduce the organic load. Use of recycled medium enriches the medium with higher concentrations of magnesium, calcium and sulfate. *Dunaliella* was found to grow well in seawater based media containing around 1.5 M NaCl, more than 0.4 M MgSO₄ and 0.1 M CaCl₂ under pH control.

13.4 Biotechnology of phytoene production

Dunaliella bardawil, the halotolerant β -carotene species, was recently mutated and treated by the bleaching herbicide norflurazon to select sub-species rich with a mixture of 9-cis and all-trans phytoene and phytofluene. The selected phytoene/phytofluene-rich *Dunaliella* was transferred to NBT Ltd, Eilat (Fig. 13.2) south of Israel, and inoculated and cultivated in small ponds outdoors and the most adapted phytoene species was scaled up gradually into large body open raceways of 3000 m² (Wermau *et al.*, 2002). The success in large-scale production of new carotenoids other than β -carotene by *Dunaliella* opens a new path in applied phycology.

13.5 *Dunaliella* market products

Dunaliella natural β -carotene is widely distributed today in many different markets under three different categories: β -carotene extracts, *Dunaliella* powder for human use and dried *Dunaliella* for feed use. Extracted purified β -carotene is sold mostly in vegetable oil in bulk concentrations from 1% to 20% to color various food products and for personal use in soft gels, usually 5 mg β -carotene each gel. The purified natural β -carotene is generally accompanied by the other carotenoids of *Dunaliella*, predominantly: lutein, neoxanthin, zeaxanthin, violaxanthin, cryptoxanthin, α -carotene comprising approximately 15% of the carotene concentration and is marketed under the title carotenoids mix. A variety of such formulations of natural β -carotene



Fig. 13.2. Commercial cultivation of *Dunaliella*. Up, extensive open ponds, Betatene Ltd, Adelaide, Australia; Down, intensive open raceways, NBT Ltd, Eilat, Israel.

are presently found and distributed in health food stores mainly in the western world under the market sections of vitamins, *health food* or *food supplement*. The second category covers a line of dried low salt *Dunaliella* powders, algae harvested, processed, washed and dried as described above for marketing in the form of tablets or hard capsules containing between 3 and 20 mg mix of all-*trans* and 9-*cis* β-carotene per unit. The tablets are coated with sugar and the capsules are packed separately in aluminum/polyethylene blisters to avoid oxidation and extend the shelf life of the product at room temperature. Dry *Dunaliella* is distributed popularly in the Far East where the consumers are more familiar with edible algae and

accustomed to macroalgae, such as *Chlorella* and *Spirulina*. The third category covers a line of dried *Dunaliella*, harvested and dried with no salt wash to algal powder of about 2% β-carotene and varying concentration of salt. The powder is shipped under vacuum in aluminum/polyethylene bags for feed coloration and pro-vitamin A use in cattle, poultry, fishes, shrimps and more. Pricing of natural β-carotene varies from \$300 to \$3000 kg⁻¹ according to specifications and demand.

13.6 Commercial producers

At present, the following companies are actively engaged in cultivating *Dunaliella* for commercial purposes. These are (listed with mode of cultivation):

1. Betatene Ltd, Cheltenham, Vic. 3192, Australia, a division of Cognis Ltd, Australia. *Extensive mode* (Fig. 13.2).
2. Cyanotech Corp., Kailua-Kona, HI 96740, USA. *Intensive mode*.
3. Inner Mongolia Biological Eng. Co., Inner Mongolia, 750333, P. R. China. *Intensive mode*.
4. Nature Beta Technologies (NBT) Ltd, Eilat 88106, Israel, a subsidiary of Nikken Sohonsha Co. Gifu, Japan. *Intensive mode*.
5. Tianjin Lantai Biotechnology, Inc. Nankai, Tianjin, in collaboration with the Salt Scientific Research Institute of Light Industry Ministry, P. R. China. *Intensive mode*.
6. Western Biotechnology Ltd. Bayswater, WA 6053, Australia, a subsidiary of Cognis Ltd, Australia. *Semi-intensive mode*.
7. Parry agro industries Ltd, Murugappa group, India. *Intensive mode*.
8. AquaCarotene Ltd, Subiaco, WA 6008, Australia. *Extensive mode*.
9. Small plants are also located in Chile, Mexico, Cuba, Iran, India, Taiwan, and Japan.

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14 Industrial Production of Microalgal Cell-mass and Secondary Products – Species of High Potential

Haematococcus

G.R. Cysewski and R. Todd Lorenz

14.1 Introduction

Natural astaxanthin produced by *Haematococcus* has become a commercial reality through new and advanced technology. Cultivation methods have been developed to produce *Haematococcus* containing 1.5–3% astaxanthin by dry weight, and it has applications as a pigment source in feeds and in the worldwide nutraceutical market.

Astaxanthin is ubiquitous in nature, especially in the marine environment and is probably best known for eliciting the *pinkish-red* hue to the flesh of salmonids, as well as shrimp, lobsters and crayfish. Because these animals are unable to synthesize astaxanthin *de novo*, it must be supplied in their diet (Steven, 1948; Goodwin, 1984). In the marine environment, astaxanthin is biosynthesized in the food chain within microalgae or phytoplankton, as the primary production level.

The major market for astaxanthin is as a pigmentation source in aquaculture, primarily salmon, trout and seabream. Astaxanthin sells for approximately US\$2500 kg⁻¹ with the annual worldwide aquaculture market estimated at US\$200 million. Although over 95% of this market consumes synthetically derived astaxanthin, consumer demand for natural products makes the synthetic pigments much less desirable and provides opportunity for the production of natural astaxanthin by *Haematococcus*.

14.2 Chemistry of astaxanthin

The astaxanthin molecule has two asymmetric carbons located at the 3 and 3' positions of the benzenoid rings on either end of the molecule. Different enantiomers of the molecule result from the way that the hydroxyl groups are attached to the carbon atoms at these centers of asymmetry (Fig. 14.1). When the hydroxyl group is attached so that it projects above the plane of the molecule it is said to be in the R configuration and when the hydroxyl group is attached to project below the plane of the molecule it is said to be in the S configuration. Thus, the three possible enantiomers are designated R,R', S,S' and R,S' (meso). *Haematococcus* primarily contains monoesters of astaxanthin linked to 16:0, 18:1 and 18:2 fatty acids. All of the free astaxanthin and its mono- and diesters in *Haematococcus* have optically pure (3S,3'S)-chirality (Renstrom *et al.*, 1981; Grung *et al.*, 1992). Fatty acids are esterified onto the 3' hydroxyl group(s) of astaxanthin after biosynthesis of the carotenoid, increasing its solubility and stability in the cellular lipid environment.

The composition of astaxanthin esters in *Haematococcus* is similar to that of crustaceans, the natural dietary source of salmonids. That is, the astaxanthin pool of encysted *Haematococcus* is approximately 70% monoesters, 25% diesters and 5% free. *Haematococcus* algae included in aquaculture feeds provide the same 3S, 3'S configuration of astaxanthin as their natural counterparts. Whereas *Phaffia* yeast contains pure 3R, 3'R astaxanthin, and synthetic astaxanthin is a mixture of all three isomers (Turujman *et al.*, 1997).

14.3 Industrial applications

Haematococcus algae meal has been approved in Japan as a natural red food color and as a pigment for fish feed. In addition, the Canadian Food Inspection Agency and the US Food and Drug Administration (FDA) have recently granted approval for *Haematococcus* algae to be used as a pigment in salmonid feed. *Haematococcus* algae have also been cleared for marketing by the US FDA as a dietary supplement ingredient and is approved in a number of European countries for human consumption.

14.3.1 Feed applications

The use of carotenoids as pigments in aquaculture species is well documented, and it appears their broader functions include a role as an antioxidant.

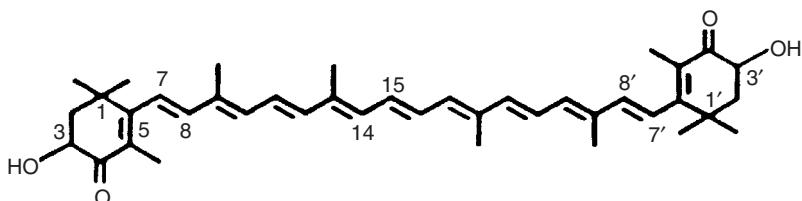


Fig. 14.1. Structure of astaxanthin with numbering scheme. Reprinted with permission from Elsevier Science Ltd (*Trends Biotechnol.*).

and provitamin A activity as well as enhancing immune response, reproduction, growth, maturation and photoprotection.

Perhaps the largest potential use of natural astaxanthin produced by *Haematococcus* is for salmonid aquaculture feeds. The continued growth of salmonid farming has created an enormous demand for pigments. The flesh color of salmonids is the result of the absorption and deposition of dietary astaxanthin. Salmonids are unable to synthesize astaxanthin *de novo*, therefore carotenoid pigments must be supplied in their artificial aquaculture diet (Steven, 1948; Goodwin, 1984).

As a natural pigment, the efficacy and value of natural astaxanthin from *Haematococcus* has been proven in numerous feed applications. In aquaculture these feed applications include: salmon (Arai *et al.*, 1987; Mori *et al.*, 1989; Sommer *et al.*, 1991; Choubert & Heinrich, 1993), Sea Bream (Nokazoe *et al.*, 1984; Ito *et al.*, 1986), shrimp culture (Yamada *et al.*, 1990; Chien, 1996), and ornamental fish (Ako & Tamaru, 1999). In poultry feed *Haematococcus* has been shown to be useful in coloration of both egg yolks (Elwinger *et al.*, 1997) and muscle tissue (Inbbor & Lignell, 1997; Inbbor, 1998). As with the studies in salmonid feed, *Haematococcus* was found not only to provide pigmentation but also to improve health and fertility. Further, all of the studies indicate that *Haematococcus* spores must be cracked to provide adequate bioavailability of astaxanthin in the microalgae.

14.3.2 Metabolic and health effects

There is an increasing amount of evidence that astaxanthin surpasses the antioxidant benefits of β -carotene, zeaxanthin, canthaxanthin, vitamin C and vitamin E. Animal studies have also shown that astaxanthin can protect skin from the damaging effects of UV radiation, ameliorate age-related macular degeneration, protect against chemically induced cancers, increase high density lipoproteins and enhance the immune system. Epidemiological studies have demonstrated a correlation between increased carotenoid intake and the reduced incidence of coronary heart disease and certain cancers, macular degeneration, and increased resistance to viral, bacterial, fungal and parasitic infections. Studies indicate that the mechanism for this protective attribute is partly due to the direct enhancement of the immune response by carotenoids. Anticarcinogenic effects of carotenoids are likely attributable to its antioxidant effect, insofar as oxygen radicals are related to the process of cancer initiation and propagation.

A number of studies have demonstrated the potent radical scavenging and singlet oxygen quenching properties of astaxanthin. Due to its particular molecular structure, astaxanthin has both a very potent neutralizing or *quenching* effect against singlet oxygen, as well as a powerful scavenging ability for free radicals and serves as an extremely potent antioxidant against these reactive species. The antioxidant activities of astaxanthin have been shown to be approximately ten times greater than other carotenoids such as zeaxanthin, lutein, canthaxanthin and β -carotene, over 500 times greater than α -tocopherol, and it has been proposed as the *super vitamin E* (Ranby & Rabek, 1978; Miki, 1991; Shimidzu *et al.*, 1996).

Astaxanthin has a significant enhancing action on the production of immunoglobulins A, M and G, and on T-helper cell antibody production even when suboptimal amounts of antigen is present (Jyonouchi *et al.*, 1995a,b). Consequently, at the initial stage of a pathogen invasion, doses of a particular antigen may be suboptimal to elicit an effective immune reaction and astaxanthin appears to enhance this response.

14.4 Production technology

In principle, the production of *Haematococcus* algae meal as a source of natural astaxanthin is relatively straightforward as cultures grow readily in a very simple nutrient media. However, because *Haematococcus* grows in a neutral culture medium, contamination by other strains of microalgae, amoeba and protozoa can present problems. These challenges are magnified as processes are scaled up, and therefore require advanced technology to control.

New techniques have been developed for the production of natural astaxanthin from *Haematococcus*. In Sweden, completely closed photobioreactors with artificial light are being used for astaxanthin production. In Hawaii, outdoor closed photobioreactors as well as a combination of closed photobioreactors and open culture ponds are being successfully used to commercially produce *Haematococcus*.

In the large-scale outdoor system, the production of astaxanthin-rich *Haematococcus* is a two-step process (Fig. 14.2). First, vegetative cells must be produced under near optimal growth conditions with careful control of pH, temperature and nutrient levels. This can be achieved in various sizes and designs of closed bioreactors transparent to the sunlight. Regulation of pH can be controlled automatically with metered solenoids, and carbon dioxide fed on demand. Temperature control can also be maintained automatically with metered solenoids and thermocouples. In Hawaii, deep ocean water at 10°C is utilized to cool culture systems by means of heat exchangers.

After a sufficient volume of vegetative cells is produced, the culture is subjected to environmental and nutrient stress. Commercial systems induce astaxanthin production by deprivation of nitrate and phosphate, increasing temperature and light, or by the addition of sodium chloride to the culture medium (Sarada *et al.*, 2002). Within two to three days after the culture is stressed, haematocysts are formed and begin accumulating astaxanthin (Fig. 14.3). Within three to five days following formation, haematocysts contain approximately 1.5–3% astaxanthin and are ready for harvest. The change in a *Haematococcus* culture is striking when haematocysts accumulate astaxanthin (Fig. 14.4).

Since haematocysts are denser than water, harvesting of the haematocysts is accomplished by settling and subsequent centrifugation. The haematocysts are then dried and cracked to ensure maximum bioavailability of the astaxanthin. Milling can then be used to crack haematocysts, although the exact details of the techniques are proprietary to companies producing *Haematococcus* algae.

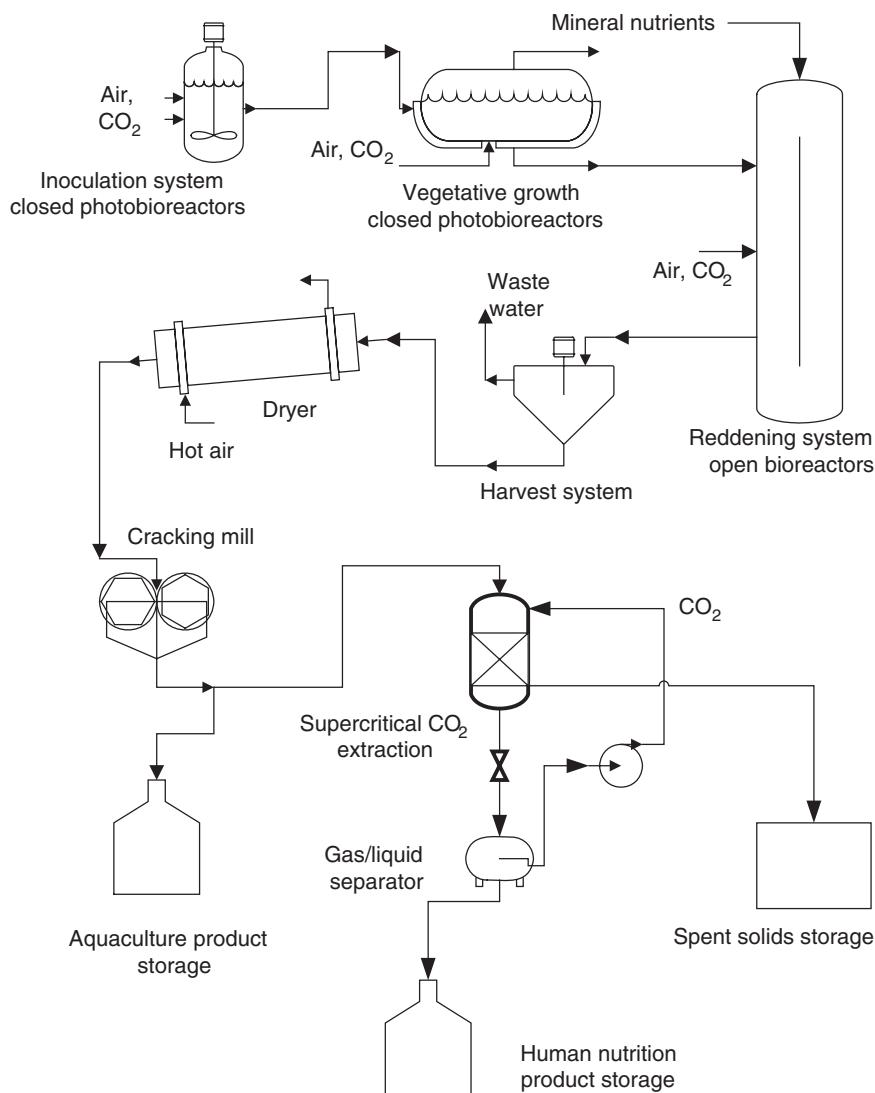


Fig. 14.2. Process flowsheet for the production of natural astaxanthin by *Haematococcus* showing aquaculture product and extraction with supercritical CO₂ for human nutrition product. Reprinted with permission from Elsevier Science Ltd (*Trends Biotechnol.*).

Since astaxanthin is a potent antioxidant, it is readily oxidized when exposed to air and the final astaxanthin-rich *Haematococcus* algal meal must be stabilized to yield a commercial product. This can be done by adding antioxidants to the final product, dispersing *Haematococcus* algal meal in vegetable oil, or extracting the lipid fraction of the algal meal to produce an oleoresin rich in astaxanthin. Further, the astaxanthin-rich oleoresin may be microencapsulated with a combination of gelatin and starch to produce a

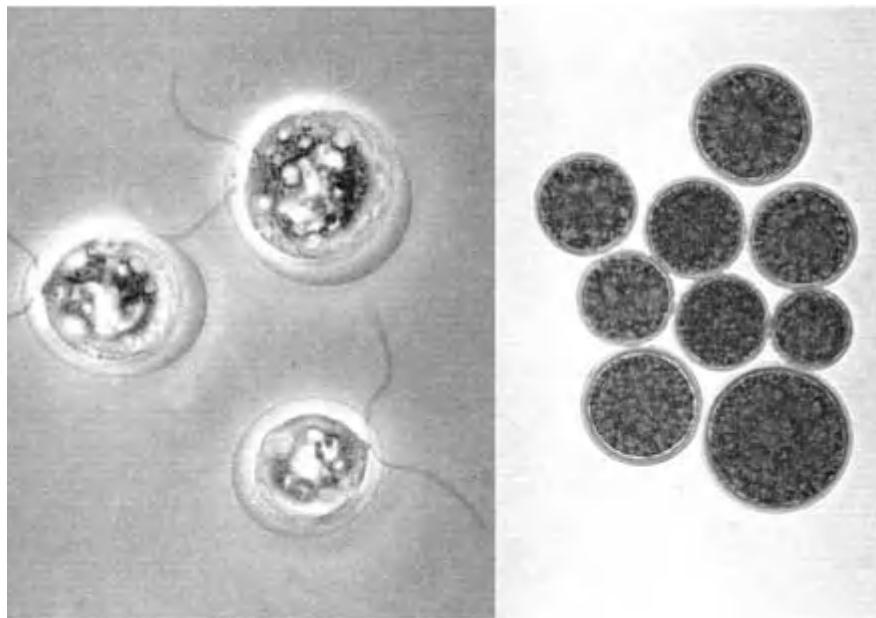


Fig. 14.3. Left, vegetative actively growing *Haematococcus* cells. Right, *Haematococcus* haematoxysts, which have accumulated astaxanthin. 400 times magnification.



Fig. 14.4. 500 000 Liter Culture Ponds at Cyanotech Corporation with fully developed haematoxysts ready harvest.

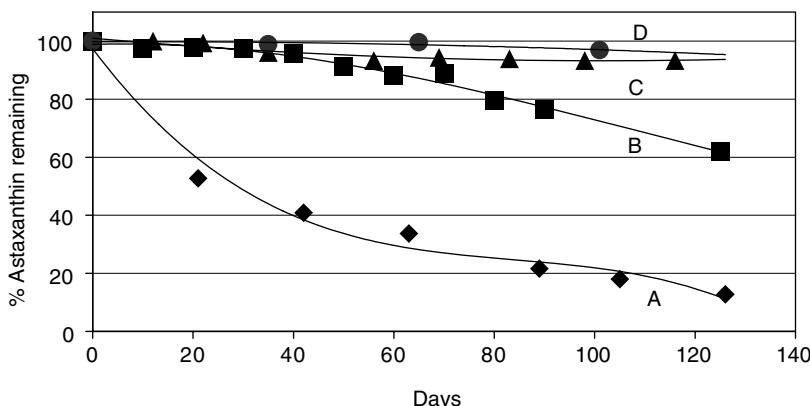


Fig. 14.5. Stability of different astaxanthin products derived from *Haematococcus*. Products left in open petri dishes at ambient temperature in the dark. **A** *Haematococcus* algae meal with 2% rosemary oil; **B** *Haematococcus* algae meal with 2% ethoxyquin; **C** oleoresin extracted from *Haematococcus* algae meal; **D** Oleoresin microencapsulated with gelatin and starch.

stable powder. A comparison of the stability of various astaxanthin products derived from *Haematococcus* is shown in Fig. 14.5.

14.5 Conclusion

The production of natural astaxanthin by *Haematococcus* requires advanced technology but has already become a commercial reality. As a source of natural astaxanthin, *Haematococcus* algae meal has a large potential market in the salmonids, seabream, and poultry feed industries. A potentially large market could also develop for *Haematococcus* algae meal as a nutraceutical in the dietary supplement industry.

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15 Industrial Production of Microalgal Cell-mass and Secondary Products – Species of High Potential

Porphyridium sp.

Shoshana Arad and Amos Richmond

15.1 Polysaccharides of red microalgae: general

The cells of the red microalgae are encapsulated within a sulfated polysaccharide in the form of a gel. During growth in a liquid medium, the viscosity of the medium increases due to the dissolution of polysaccharide from the cell surface into the medium (soluble polysaccharide). The polysaccharide capsules are thinnest during the logarithmic phase of growth and thickest during the stationary phase (Ramus, 1972, 1986).

Knowledge of the structure of polysaccharides of the red microalgae is very limited, due to their complexity and the lack of specific enzymes that degrade them. It is, however, known that the polysaccharides of the different species are all heteropolymers, having different sugar compositions and varying amounts of sulfate (Evans *et al.*, 1974; Medcalf *et al.*, 1975; Heaney-Kieras *et al.*, 1977; Geresh & Arad, 1991; Dubinsky *et al.*, 1992; Simon *et al.*, 1992). In all the species studied, the main sugars of the polymers are xylose, glucose and galactose in various ratios. Additional minor sugars, i.e. rhamnose, ribose, arabinose, mannose, 4-O-methyl galactose, and 3-O-methyl pentose, have been detected. The polysaccharides are negatively charged due to the presence of glucuronic acid and half-sulfate ester groups. The polysaccharides also contain protein (Heaney-Kieras *et al.*, 1976, 1977), for example, the species-specific tightly (non-covalently) bound glycoprotein of 66 k Da found in *Porphyridium* sp. (Shrestha *et al.*, 1995). The polysaccharides of *Porphyridium* sp., *P. cruentum*, *P. aerugineum* and *Rhodella reticulata* contain a basic disaccharide building block, which is an aldobioronic acid, 3-O-(α -D-gluc-

pyranosyluronic acid)-L-galactopyranose (Geresh *et al.*, 1990). In these species, the sulfate group is attached to glucose and galactose in the six or three positions (Lupescu *et al.*, 1991). The molecular mass of the polysaccharides of various species of red microalgae has been estimated to be $2\text{--}7 \times 10^6$ Da (Heaney-Kieras & Chapman, 1976; Heaney-Kieras *et al.*, 1976, 1977; Arad, 1988; Geresh & Arad, 1991; Simon *et al.*, 1992; Arad *et al.*, 1993a).

In concentrated solutions ($1\text{--}2\text{ g l}^{-1}$), the viscosity of *Porphyridium* sp. polysaccharide was found to be stable over a wide range of pH values (2–9), temperatures (30–120°C) and salinities. From the dependence of the intrinsic viscosity on ionic strength, it was estimated that the stiffness of the *Porphyridium* sp. polysaccharide chains is in the same range as that of xanthan gum and DNA (Eteshola *et al.*, 1996). On the basis of rheological studies, it was hypothesized that the biopolymer chain molecules adopt an ordered conformation in solution and that the polysaccharide has the form of double or triple helix.

There are no known carbohydrolases capable of cleaving red microalgal polysaccharides. However, two crude activities capable of degrading the polysaccharide have been found: the first in the dinoflagellate *Cryptocodium cohnii*, which preys specifically on *Porphyridium* sp. and degrades its polysaccharide (Ucko *et al.*, 1989), and the second in a mixture of soil bacteria (Arad *et al.*, 1993b). Although the molecular weights of the degradation products were similar to that of the native polysaccharide, the viscosities of these products were significantly lower than the viscosity of the native polysaccharide (Simon *et al.*, 1992, 1993; Arad *et al.*, 1993b).

15.2 Environmental effects on polysaccharide production

Ambient environmental conditions influence polysaccharide production, for example, nitrate and sulfate starvation of *Porphyridium* sp. not only enhance production and solubilization of the polysaccharide (Kost *et al.*, 1984; Wanner & Kost, 1984; Thepenier *et al.*, 1985; Adda *et al.*, 1986; Arad *et al.*, 1988, 1992), but also influence its chemical composition (Ucko *et al.*, 1994). In *Porphyridium* sp., carbon dioxide concentration in the medium (bubbling in air, or 0.03 or 3% CO₂) influenced growth, cell-wall polysaccharide production, and the ratio of soluble to bound polysaccharide. The latter ratio was highest when carbon dioxide was withheld from the culture. Carbon dioxide concentration also influenced the chemical composition of the cell-wall polysaccharide, the ratio of galactose to xylose being doubled when carbon dioxide was supplied at a lower concentration. It was thus suggested that carbon dioxide concentration affects polysaccharide composition by changing the partitioning of fixed carbon (Li *et al.*, 2000). Kroen & Ramus (1990) suggested that polysaccharide production is controlled at the carbon fixation level. Studies in the laboratory of S. Arad on the effect of environmental conditions on the polysaccharide indicate, however, that exacellular (soluble) polysaccharide production is controlled at the level of carbon partitioning rather than by total photosynthetic loading (Friedman *et al.*, 1991; Arad *et al.*, 1992; Li *et al.*, 2000).

15.3 Partitioning of fixed carbon

Partitioning of fixed carbon in *Porphyridium* sp. has been studied extensively by the group of S. Arad. A pulse-chase technique was used to follow ^{14}C flow into the major carbon fixation compounds under various environmental conditions. ^{14}C -NMR and GC analysis showed that the main low molecular weight product is floridoside, which is a disaccharide composed of galactose and glycerol (Li *et al.*, 2001). The large amounts of carbon incorporated into floridoside are subsequently used for the synthesis of macromolecular components, e.g. cell-wall polysaccharides (Fig. 15.1). Although red algae are known to be obligatory photoautotrophs, *Porphyridium* sp. was shown to assimilate and metabolize both exogenous floridoside and floridoside present in a crude extract of a cell-free system, as was shown by incorporation of the label from [^{14}C]floridoside into the extracellular sulfated polysaccharide. The ability of *Porphyridium* sp. cells to utilize exogenous floridoside for the synthesis of sulfated polysaccharide provides confirmation for the premise that this compound plays a role as a carbon precursor in the production of the sulfated cell-wall polysaccharide. It was thus suggested that the carbon metabolic pathway in *Porphyridium* sp. passes through the low molecular weight photoassimilatory product floridoside, which, in turn, channels the fixed carbon toward the synthesis of the sulfated cell-wall polysaccharide according to environmental conditions, e.g. nitrogen starvation.

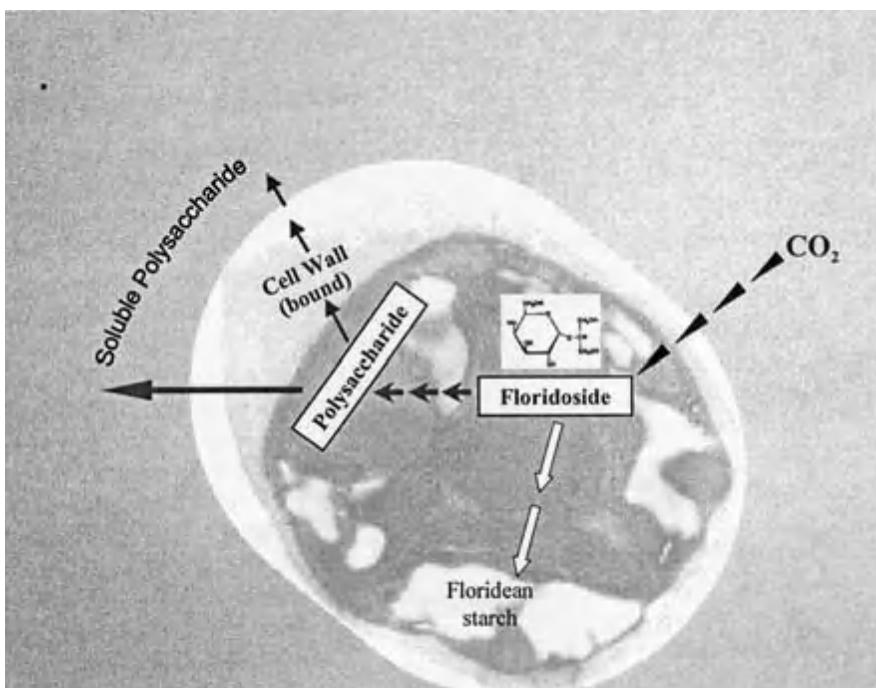


Fig. 15.1. Proposed partitioning towards polysaccharide production in *Porphyridium*.

15.4 Functions of the cell-wall polysaccharide

Various functions have been proposed for the sulfated cell-wall polysaccharide of red algae. It is thought that they serve as ion reservoirs (Kloareg & Quatrano, 1988) and/or as a buffer layer around the cells to protect them against drought or enable them to survive under extreme conditions (Arad, 1988). Ucko *et al.* (1999) have recently shown that at least one of the functions of the 66 kDa glycoprotein in the cell wall of *Porphyridium* sp. is biorecognition. When *Porphyridium* cells were treated with antibodies to the 66 kDa glycoprotein (which blocked the glycoprotein), the cells were not recognized by its predator, the dinoflagelate *C. cohnii*.

In order to properly understand the functions of the sulfated polysaccharide of the red algae, we need to examine the native habitats of the different species. *Porphyridium* sp., for example, was isolated from sea sand. The environmental conditions in this habitat are subject to large fluctuations, which impose harsh conditions on the microalgae, specially exposure to high light and drought during ebb tides. At these times, the gel layer of the sulfated polysaccharide may provide the moisture required by the cells. Such a water-rich gel layer is indeed characteristic of algae whose natural habitat is soil. Although the role of the sulfated polysaccharides in protecting the red algae cells against drought should not be overlooked, it seems that their major function is in the protection of the cells against high solar irradiation. In an attempt to elucidate the manner in which the polysaccharide exerts its protective effect, the group of Arad conducted an experiment in which light damage to the cells was prevented by supplementation of the growth medium of *Porphyridium* sp. with additional polysaccharide. They also showed that degradation of pigments in *Porphyridium* sp. cells fixed to glass could be prevented by covering the cells with polysaccharide before exposure to light. In parallel, they showed that the polysaccharide has antioxidant activities, as measured by the FOX (Wolf, 1994) and TBA (Wasowicz *et al.*, 1993) methods. The group of Arad (Li *et al.*, 2001) also showed that when *Porphyridium* sp. cells were exposed to NaHCO₃, a labeled polysaccharide appeared in the medium within 30 min. Thus, they put forward the hypothesis that under stress conditions the polysaccharide acts as a free radical scavenger that takes the free radicals outside the cells. This hypothesis is currently under further study.

15.5 Basic physiological responses of outdoor cultures of *Porphyridium* sp.

Fuentes Rebollosa *et al.* (1999) conducted a detailed investigation on outdoor continuous cultures of *P. cruentum*, providing a quantitative analysis of the daily cyclic variations of basic culture parameters, i.e. oxygen generation rates, carbon consumption rates, photosynthetic activities, growth rates and biochemical composition of the biomass.

A linear relationship between the external irradiance and the average irradiance inside the culture was clearly observed (see Chapter 8). Most important, the oxygen generation and carbon consumption rates were found to be a function of the average irradiance, indicating the average irradiance

(which is proportional to the quantity light cell^{-1}) to be the most important parameter in controlling culture performance. A reduction in photosynthetic activity of the cells at noon and recovery in the afternoon of bright days was also observed, indicating the cells to have a short-term response, as reflected in oxygen generation rate as well as carbon consumption rate, to both external and average irradiance. The higher the photosynthesis rates, the lower were the carbon losses during the night. The growth rate showed a linear relationship with the daily mean average irradiance and likewise, a linear relationship between the oxygen generation rate and the growth rate was observed. With respect to the biochemical composition of cell mass, a long-term response of metabolic routes to mean daily culture conditions was measured. During the illuminated period, energy was stored as carbohydrates and synthesis of proteins was low, stored carbohydrates being consumed during the night. The fatty acid dry weight decreased during daylight, whereas the fatty acid profile was a function of growth rate. A short-term variation of exopolysaccharides synthesis with solar irradiance was observed, i.e. the higher the irradiance, the higher the excretion of exopolysaccharides, judged to provide protection against adverse conditions.

15.6 Environmental effects on cell composition and polyunsaturated fatty acids

P. cruentum contains the relatively rare polyunsaturated fatty acids (PUFA) arachidonic acid (20:4, n-6) and eicosapentaenoic acid (20:5, n-3), which are essential in human, as well as animal nutrition. Cohen *et al.* (1988) have shown the fatty acid composition to be strongly influenced by environmental conditions. Under optimal growth conditions supporting exponential growth, the dominant PUFA was the 20:5 acid. In contrast, growth rate limiting conditions, e.g. high cell density or low light intensity, below optimal temperature or pH as well as high salinity, resulted in the 20:4 and 18:2 acids becoming dominant. Likewise, high temperature (30°C) resulted in a rise of the 16:0 and 18:0 saturated acids and a decline in the 20:5 acid. A good correlation was indeed found between the specific growth rate μ as affected by cell concentration, salinity and pH, and the 20:4/20:5 ratio (R) (Table 15.1).

Table 15.1. Effect of light intensity, cell concentration and temperature on fatty acid composition in *P. cruentum* (after Cohen *et al.*, 1988, *J. Phycol.* 24: 238–43).

Temp. (°C)	Cell conc. (Mg chl l^{-1})	Growth rate (day^{-1})	Fatty acid composition (%)						
			16:0	18:1	18:2	20:4	20:5	R*	
20	2.0	0.65		35.0	3.1	8.3	19.3	30.0	0.6
	28.0	S**		32.7	1.0	8.3	28.8	24.1	1.2
30	1.5	0.98		38.9	1.5	11.0	18.6	23.7	0.8
	17.0	S**		31.0	7.7	18.5	32.7	2.9	11.0

* Ratio of % 20:4 to % 20:5.

** Stationary phase.

Fuentes Rebolloso *et al.* (2000) provided a rather detailed profile of cell nutrients. On an average, the biomass contains 32.1% (w/w) available carbohydrates and 34.1% crude protein. The mineral contents in 100 g dry biomass were: Ca (4960 mg), K (1190 mg), Na (1130 mg), Mg (629 mg) and Zn (373 mg). Contents of toxic heavy metals were negligible. The fatty acid contents were (in per cent dry wt): 1.6% of 16:0; 0.4% of 18:2 ω 6; 1.3% of 20:4 ω 6 and 1.3% of 20:5 ω 3. Nutrient composition of biomass was, however, highly influenced by the dilution rate and irradiance, biomass collected from short dilution rates was higher in protein and eicosapentaenoic acid.

15.7 Mass production of *P. cruentum* outdoors

Gudin and colleagues (Chaumont & Thepemier) have pioneered as early as the late 1970s, the biotechnology involved in mass cultivation of *Porphyridium* sp. outdoors. Yields of continuous culture of *Porphyridium* in 3 m² tubular reactors correlated well with irradiance, lowest ranging from 597 Kcal m⁻² d⁻¹ to 1812 Kcal m⁻² d⁻¹. Chaumont *et al.* (1988) developed and operated a singular 100 m² tubular photobioreactor and proposed a production cost of *Porphyridium* in tubular reactors to be rather comparable with the cost in open raceways, i.e. 26 FF kg⁻¹ in 1988, being highly dependent on the productivity (Chaumont *et al.*, 1988).

Cohen *et al.* (1991) also identified the main problems in the large-scale cultivation of microalgae outdoors in open ponds as low productivity and contamination. They developed a closed system consisting of polyethylene sleeves hung on a frame and a stream of compressed air, introduced separately into the bottom of each sleeve, was used for stirring and CO₂ introduction. In a study conducted outdoors in the Negev area of Israel, the closed system was found to be superior, in a number of species, to open ponds with respect to growth and production. In both closed and open systems, growth and production under continuous operation were higher than in batch cultivation. In continuous cultures the respective daily yields of dry matter and polysaccharides from the red microalga *Porphyridium* sp. were 17.7 and 7.4 g m⁻² in the sleeves compared with 7.6 and 2.4 g m⁻² in the ponds. Attempting to optimize growth and production, the effect of sleeve diameters (10, 20 and 32 cm) on these parameters was investigated. Growth and polysaccharide production were superior in the narrow sleeves than in the wider sleeves. To scale up the system a unit of connected sleeves each 200 cm long and 20 cm in diameter was developed, and the performance of various microalgae in the unit was investigated.

More recently Singh *et al.* (2000) addressed large-scale cultivation of *Porphyridium* sp. outdoors. The impact of the optical path or light-path length (LP) on cell growth and production of soluble sulfated polysaccharides was studied in flat plate glass reactors. The LP of the plate reactors ranged from 1.3 to 30.0 cm, corresponding to culture volumes of 3–72 l. The side-walls of all reactors were covered, ensuring similar illuminated surfaces for all reactors. Maximal day temperature was maintained at 26 ± 1°C. Growth condition of pH (7.5), stirring (with compressed air) and mineral nutrients, were optimal. Maximal volumetric concentration of the soluble sulfated

polysaccharide (2.32 g l^{-1}) was obtained in winter with the smallest LP reactor (1.3 cm) at a cell density of $1.37 \times 10^{11} \text{ cells l}^{-1}$. Under these conditions, the viscosity of the culture medium was also highest, being inversely proportional to the culture's LP. Highest areal concentration of soluble polysaccharides (60 g m^{-2}) and areal cell density ($3.01 \times 10^{12} \text{ m}^{-2}$) were recorded in the 20 cm LP reactor, progressively lower values being obtained as the LP became shorter. A similar pattern was obtained for the areal productivity of polysaccharides, the highest being $4.15 \text{ g m}^{-2} \text{ d}^{-1}$ (considering the total illuminated reactor surface), produced in the 20 cm LP reactor. The main sugar composition (i.e. xylose, galactose and glucose) of the sulfated polysaccharides was similar in all reactors. As viscosity increased with time during culture growth, there was a substantial decline in bacterial population. Cultivation throughout the year provided good evidence that a LP length of 20 cm in flat plate reactors under these conditions is optimal for maximal areal soluble polysaccharide production of *Porphyridium* sp. The optimal harvesting regime in semi-continuous cultures was replacing 75% of culture volume every 12 days.

Gudin & Chaumont (1991) calculated the theoretical production maxima in their 100 m^{-2} tubular reactor and suggested the maximal productivity would be $60 \text{ g m}^{-2} \text{ d}^{-1}$. The highest output they recorded experimentally, however, was $30 \text{ g m}^{-2} \text{ d}^{-1}$, obtained at a cell concentration of 6 g l^{-1} and a residence time of 12 days, i.e. a very low dilution rate. Gudin & Chaumont (1991) suggested the discrepancy between actual and theoretical yields stems from reduced growth rate related to hydrodynamic stress. Their conclusions prompted the development of bubble columns, i.e. sleeves, tanks and flat plates, in which mixing with compressed air associated with the low shearing forces is used. The future will perhaps tell whether or not the predicted yield of $60 \text{ g m}^{-2} \text{ d}^{-1}$ is feasible.

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16 Industrial Production of Microalgal Cell-mass and Secondary Products – Species of High Potential

Mass Cultivation of *Nannochloropsis* in Closed Systems

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16.1 Introduction

Nannochloropsis is a marine microalga commonly cultivated in fish hatcheries as feed for rotifers and to create a *green water effect* in larvae tanks (Fulks & Main, 1991; Lubzens *et al.*, 1995). Because of its high content of eicosapentaenoic acid (EPA), *Nannochloropsis* has also been proposed as a source of this important dietary polyunsaturated fatty acid (Chini Zittelli *et al.*, 1999; Sukenik, 1999).

Mass cultivation of *Nannochloropsis* is at present carried out in different kinds of large outdoor ponds and tanks (Okauchi, 1991; Sukenik, 1999), and in 50–500 l polyethylene bags or glass fibre cylinders usually kept indoors with artificial light (Fulks & Main, 1991). Several problems have been evidenced by the use of these systems, the most severe of which are low cell concentrations and productivity, and susceptibility to contamination (Borowitzka, 1997). Recently, research efforts have been directed toward the development of efficient closed photobioreactors, which have been successfully used for *Nannochloropsis* cultivation (Chini Zittelli *et al.*, 1999, 2000; Richmond & Zhang, 2001). This review presents the major results obtained in the cultivation of *Nannochloropsis* in closed systems, under either natural or artificial illumination or a combination of both (see Chapter 20 for *Nannochloropsis* in open raceways).

16.2 Indoor culture systems

Indoor cultivation of *Nannochloropsis* for aquaculture use is generally carried out in polyethylene bags suspended from a framework (Fig. 16.1a), or supported within a mesh frame, and illuminated by banks of fluorescent lamps. Reactors of this type, from 50 to 500 l in volume, are easily built using polyethylene tubing, 0.2 mm thick and 30–50 cm in diameter, which is cut into pieces of variable length and sealed at the bottom (Lim, 1991). Mixing is obtained by air bubbling. The cultures are generally maintained at low cell densities ($50\text{--}100 \text{ mg l}^{-1}$) to reduce light limitation, and typically achieve volumetric productivities in the range of $25\text{--}125 \text{ mg l}^{-1} 24 \text{ h}^{-1}$ (Fulks & Main, 1991). Although polyethylene bags have a relatively short life because of biofouling, they are easy and inexpensive to replace.

A more permanent solution is the use of glass fibre cylinders. Transparency of this material is not excellent, but the vessels so constructed are durable. James & Al-Khars (1990) cultivated *Nannochloropsis* in an airlift photobioreactor made of five 200-l, 30 cm diameter glass fibre cylinders connected in series and operated continuously (Fig. 16.1b). A mean volumetric productivity of $50 \text{ mg l}^{-1} 24 \text{ h}^{-1}$ was attained at a light intensity of $330 \mu \text{mol photon m}^{-2} \text{ s}^{-1}$.

Chini Zittelli *et al.* (2000) have developed and experimented with a modular flat panel photobioreactor (MFPP) consisting of six removable alveolar panels, 1.6 cm thick, placed vertically back to front to form a closely packed unit contained in a thermoregulated cabinet (Fig. 16.1c). The total working volume of the system was 123 l and the total illuminated surface area was 20.4 m². Illumination was provided by fluorescent tubes placed between the panels. Cell concentrations at harvesting of about 7 g l^{-1} and a mean productivity of $1.5 \text{ g l}^{-1} 24 \text{ h}^{-1}$ were attained when both sides of the panel were illuminated with $230 \mu \text{mol photon m}^{-2} \text{ s}^{-1}$.

Using 2-l glass plates, 1.4-cm-thick, and vigorous stirring, Zou *et al.* (2000) obtained in batch cultures, record population densities of 60 g l^{-1} and productivities ranging from 0.8 to $2.3 \text{ g l}^{-1} 24 \text{ h}^{-1}$ at high irradiance levels. When the plate was illuminated with $300 \mu \text{mol photon m}^{-2} \text{ s}^{-1}$ from one side and $1700 \mu \text{mol photon m}^{-2} \text{ s}^{-1}$ from the other side, and continuous culture was adopted, productivity increased to $2.9 \text{ g l}^{-1} 24 \text{ h}^{-1}$. The achievement of extremely high cell concentrations in batch culture depended on frequent (every 48 h) replacement of the growth medium to remove auto-inhibitory substances.

16.3 Outdoor culture photobioreactors

The outdoor cultivation of *Nannochloropsis* in closed systems has been recently accomplished using two reactor designs: the near-horizontal tubular reactor (NHTR) (Tredici, 1999) and the vertical glass plate (Zou & Richmond, 1999).

Typically, the NHTR consists of eight flexible plastic tubes laid parallel on a white corrugated sheet facing south and inclined at very small angles to the horizontal ($5\text{--}7^\circ$). The tubes are connected to a degasser and to a bottom manifold. An air/CO₂ mixture is injected into the bottom manifold to provide

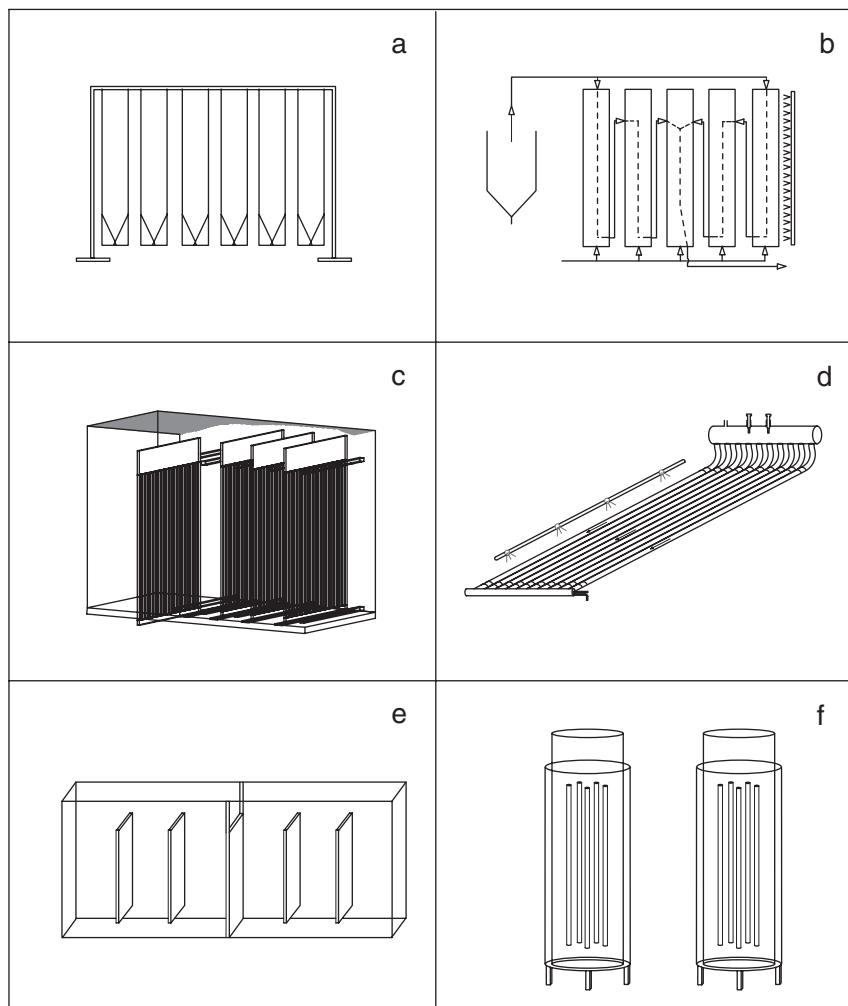


Fig. 16.1. Schematic drawing of closed systems used in the cultivation of *Nannochloropsis*. (a) polyethylene bags; (b) glass fibre cylinders; (c) modular flat panel photobioreactor (MFPP); (d) nearly horizontal tubular reactor (NHTR); (e) glass plate reactor; (f) annular reactors.

mixing and gas exchange. Cooling is obtained by water spraying. The largest NHTR tested in the cultivation of *Nannochloropsis* occupied an area of 30 m^2 and contained about 600 l of culture (Fig. 16.1d). *Nannochloropsis* has been cultivated in NHTR of different sizes from March to September. During the spring, the mean productivity increased from 0.5 to $0.8\text{ g l}^{-1}\text{ d}^{-1}$, reaching the maximum in May. During the summer, when higher productivities were expected, the cultures performed poorly because of prolonged exposure to supraoptimal diurnal temperatures. Although profound modifications of the fatty acid content and profile were observed, the biomass EPA content was barely affected. The average EPA productivity essentially reflected culture productivity and ranged between 16 and $32\text{ mg l}^{-1}\text{ d}^{-1}$ (Chini Zittelli *et al.*, 1999).

Outdoor mass cultivation of *Nannochloropsis* in glass plates of 0.5 m² illuminated surface area was carried out by Richmond and co-workers. The volumetric productivity increased from 0.12 to 0.85 g l⁻¹ d⁻¹, with the decrease of the light path from 17 to 1.3 cm (Richmond & Zhang, 2001). A maximal productivity of 12.1 g m⁻² illuminated surface area per day was attained with a 10 cm thick plate. To test the feasibility of prolonged large-scale outdoor production of *Nannochloropsis*, a 500-l glass plate with the optimal light path of 10 cm and about 8 m² of illuminated surface area was developed (Richmond & Zhang, 2001; Zhang *et al.*, 2001). The reactor consists of two units, 200 cm long and 110 cm high, glued together and equipped with one system for mixing by means of air bubbling and one system for cooling by water spray (Fig. 16.1e). At the optimal harvesting regimen of 10%, productivities of 0.21 and 0.27 g l⁻¹ d⁻¹ (corresponding to 10 and 14.2 g m⁻² illuminated surface area per day) were attained in the winter and in the summer, respectively. EPA content was not affected by season in agreement with Chini Zittelli *et al.* (1999).

To be able to produce *Nannochloropsis* biomass and supply hatcheries in the winter, a vertical annular reactor, which can be operated with either artificial or natural illumination or a combination of both, was developed at the University of Florence (Rodolfi, 2000). The reactor consists of two 2 m high Plexiglas® cylinders of different diameter placed one inside the other so as to form a regular annular culture chamber, 3–5 cm thick and 120–150 l in volume (Fig. 16.1f). The total illuminated surface area of the system varies between 5.3 and 9.3 m². An air/CO₂ mixture is injected at the bottom of the annular chamber for mixing and gas exchange. To operate the reactor with artificial illumination, metal halide lamps or fluorescent tubes are placed inside the inner cylinder. When the reactor is used under natural illumination, cooling water can be circulated inside the internal cylinder for thermoregulation. Under artificial illumination, provided by a 400-W metal halide lamp, a productivity of 0.25 g l⁻¹ 24 h⁻¹ was attained. With natural illumination under a polyethylene roofed greenhouse, productivity increased from 0.12 g l⁻¹ d⁻¹ in the winter to 0.25 g l⁻¹ d⁻¹ in the spring. A maximum productivity of 0.36 g l⁻¹ d⁻¹ was achieved in May, under combined illumination (Rodolfi, 2000).

16.4 Concluding remarks

Polyethylene bags and glass fibre cylinders, currently used in hatcheries with artificial light for *Nannochloropsis* production, are easy and inexpensive to build and operate. These systems are, however, characterised by low volumetric productivities and low cell concentration cultures that are susceptible to contamination, with the consequence that a low quality product is generally obtained at very high costs (Benemann, 1992). A significant breakthrough in *Nannochloropsis* cultivation under artificial light has been achieved with the use of narrow light path flat systems (the MFPP and glass plates). Due to a very high s/v ratio, volumetric productivities 30 times higher than in bags or vertical cylinders were attained in the MFPP, and cell

concentrations as high as 60 g l^{-1} could be achieved in glass plates when autoinhibitory substances were removed. A single 123-L MFPP unit can produce about 180 g of high quality biomass per day on a ground area of only 1.7 m^2 and could be profitably used for *Nannochloropsis* production in hatcheries, with substantial savings in terms of culture operation and space.

Indoor cultivation under artificial illumination ensures a much more reliable production process and offers the possibility to modulate the chemical composition of the biomass. Artificial light adds, however, a significant burden to the production cost. Chini Zittelli *et al.* (2000), assuming an electricity cost of $\text{€}0.1\text{ kWh}^{-1}$, estimated the cost of artificial illumination at $\text{€}35\text{--}50\text{ kg}^{-1}$ of dry *Nannochloropsis* biomass. An interesting compromise is offered by annular reactors that can be operated under a combination of natural and artificial light. Using artificial light, annular reactors allow *Nannochloropsis* production during the winter, when this microalga is required by hatcheries. On the other hand, cost of production is lowered, especially in the spring and in the summer, by the contribution to productivity by solar radiation. Compared to bags or completely filled cylinders, the internally lit annular reactors achieve a much higher efficiency of artificial light conversion since light is completely trapped by the culture (Tredici, 1999). Several reactors of this type are successfully being operated in southern Italy to produce *Nannochloropsis* biomass for sea bream fingerlings rearing.

Outdoor cultivation in open ponds or tanks might improve significantly the economy of *Nannochloropsis* biomass production, because larger scale systems can be adopted and the cost of light has not to be paid for. However, productivity and biomass quality are penalised by the low s/v ratio of these systems and by variability of climatic conditions. The main problem in outdoor open ponds is, however, contamination of the culture, which cannot be prevented by the use of antibiotics, herbicides or chlorination (Gonen-Zurgil *et al.*, 1996; Suenik, 1999) and leads to frequent culture crashes. These problems can be solved by the use of closed photobioreactors, which offer a protected environment and ensure a sustainable cultivation process. Among the closed reactors experimented with for outdoor *Nannochloropsis* cultivation, the NHTR attains higher volumetric productivities, but the glass plate seems superior in terms of ease of operation and cleaning. The cost of *Nannochloropsis* biomass production in outdoor flat plates was calculated to vary from $\text{€}57$ to $\text{€}125\text{ kg}^{-1}$ (Zhang *et al.*, 2001), which is rather low compared to the production cost of *Nannochloropsis* in the hatcheries (Benemann, 1992).

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17 Industrial Production of Microalgal Cell-mass and Secondary Products – Species of High Potential

Nostoc

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17.1 Morphology

The natural colony of *Nostoc* varies with different species. Whether this reflects genetic diversity or different phenotypes responding to various physical conditions is not clear. A colony of *N. flagelliforme* appears hair like with voluble thallus, and the filament about 50 cm long and 0.2–1 mm in diameter. *N. commune* may excrete quantities of gelatin in which the colony looks like a gluey sphere at the beginning of its growth. Also, the formation of spherical colonies is not a passive process, different from other cyanobacteria. In persistent rainfall, it will flatten, as the colony develops. Study of the factors regulating the morphological transformation during different phases of growth is useful for mass cultivation of *Nostoc* spp. (De *et al.*, 2000).

The vegetative cells of *Nostoc* are approximately spherical, forming unbranched and large twisted filaments. The protoplast is differentiated into a peripheral pigmented region and a central colorless one. Heterocysts, with a thick cell wall, often occur in the middle or at the end of the filaments, which appear paler and larger than the vegetative cells. The heterocysts are associated with nitrogen fixation and formation of hormogonia. In addition, when growing in unfavorable conditions, the vegetative cell becomes larger and full of inclusion. Dormant cells, due to unfavorable conditions, are known as akinetes, which can germinate to form new filaments (Jiang, 1990).

The sheath of *Nostoc* plays an important role in protecting cells from environmental stresses. The thicker the sheath is, the higher the probability for survival (Cheng & Cai, 1988). In acid media ($\text{pH} < 6$), sheath formation was inhibited (Liu & Pan, 1997). Studies of sheath structure suggest that it may

serve to concentrate metals and protect cells from predation by other organisms. The sheath material was found to retard gas exchange and water loss.

17.2 Ecology

Having great tolerance to extreme environments, *Nostoc* is distributed widely on the earth including the polar region, hot springs and deserts. For example, *N. flagelliforme* grows in arid and semiarid areas, often located 1000–2800 m above sea level, where annual evaporation is 10–20 times over rainfall. The annual mean temperature is about 2.2–8.6°C, ranging from a low of –17°C to a high of 35°C. Surface temperature on the arid bare lands may range 65.7°C to –29.1°C, i.e. *N. flagelliforme* experiences an annual temperature differential as large as 95°C (Qian *et al.*, 1989). The temperature difference is necessary for the growth of *N. flagelliforme*. *Nostoc* spp. are often available in seasonally inundated environments, e.g. *N. sphaericum*, endemic in flooded rice paddies in some areas of China.

Nostoc spp. share habitats with many species of plants, bacteria and actinomycetes. In hydrophytic environments, *Nostoc* is agglomerated and associated with some benthos. Evidence for the plant origin of the factors inducing heteromorphic changes in *N. muscorum* has been obtained. Filtrations of the culture and intracellular contents of *N. commune* may reduce the number of local lesions caused by *tobacco mosaic virus* (TMV) on tobacco plants by at least 50%. It seems however that *N. commune* did not activate any systemic defense mechanism on tobacco plants (Di Piero *et al.*, 2000). *N. flagelliforme* is often associated with *Salsola passerina* + *Cleistogenes* + *Allium polyrhizm* population in desert steppes (Cui, 1985). In such communities, the vegetation cover reached 30%, and *N. flagelliforme* accounted for 1% in 18–37 mats m⁻². Symbiotic cyanobacteria have the capacity to form motile hormogonia thought to be important for establishment of an association with plants (Tandeau de Marsac, 1994).

The soil in which *Nostoc* usually grows is strongly alkaline with plenty of calcic deposits. Due to its strong coherence and retention of water, which increases the concentration of nitrogen and phosphorus and lowers H⁺, *N. sphaericum* is usually cultivated in rice paddies for soil amelioration.

17.3 Physiology

17.3.1 Temperature

The optimum temperature for growth is from 15 to 25°C (Cui, 1983). *Nostoc* spp. show great adaptability to a large range of temperatures. A strain of *N. commune* has been collected from the Antarctic at –1°C and another was found in hot springs of 65°C. *N. flagelliforme* can survive high temperatures, especially in a dry environment.

High temperature has little effect on photosynthesis and respiration. Experiments with *N. flagelliforme* showed dark respiration was not affected by 24 h pretreatment at temperatures from –5 to 85°C. Photosynthesis was not significantly affected as long as the temperature was not above 45°C for more than

one day. Pretreatment at various temperatures showed different results between wet and dry samples. Pretreatment at high temperature may result in electrolyte leakage in wet conditions. However, cell membrane acquired heat resistance to dryness, which is the reason that rapid water loss helps to dissipate the heat of solar radiation and avoid heat damage after rain in summer (Gao, 1998).

17.3.2 Cell-water content

Desiccation tolerance which is associated with a newly discovered protein is the key for *Nostoc* survival in various terrestrial environments (Potts, 1997). When *N. commune* suffers water stress, the protein will be secreted from the cells. There are indications that the role of water stress protein may be related to the synthesis or modification of carbohydrate-containing components (Hill *et al.*, 1994a,b). It appears that the capacity to maintain proteins in a functional-stable state is a central feature of desiccation tolerance. It seems that production of extracellular polysaccharide (EPS) represents a mechanism related to desiccation tolerance. The capacity of EPS to prevent membrane leakage upon exposure to desiccation constitutes a protective mechanism (Hill *et al.*, 1997).

17.3.3 Nutrition

Nostoc can make use of various nitrogen sources, including inorganic nitrogen (NO_3^- , NO_2^- , NH_4^+), amino acids and N_2 . Nitrogen fixation is beneficial to *Nostoc* in environments poor in nitrogen. The activity of nitrogenase, however, can be affected by many factors. The main source of ATP and reductant for nitrogen fixation is production of intermediates of photosynthesis. Under certain conditions, the nitrogenase activity of some species is sensitive to high temperatures. Long-term exposure of hydrated *N. flagelliforme* filaments to 45°C causes a rapid decline in nitrogenase activity. Under dry conditions, however, *N. flagelliforme* is highly resistant to heat. Dry filaments exposed to 55°C for 5 h daily for 21 days show no marked change in nitrogenase activity. Repeated desiccation–rehydration cycles induced a gradual increase in nitrogenase activity, enhancing resistance of nitrogenase to desiccation (Zhong *et al.*, 1992). These data imply that nitrogenase activity is regulated by cell-water content. When the water content reaches six times the weight of the filaments, nitrogenase activity attains its maximum. *N. flagelliforme* is endowed with strong retention of water to meet with the minimum quantity for nitrogen fixation. The optimal temperature for the nitrogenase activity is 21–28°C, optimal water content being 10–15 times the weight of the dry alga (Zhong *et al.*, 1992). High NaCl concentration (0.17–0.43 M) decreases nitrogenase activity quickly, being slightly enhanced by 1% glucose sprayed on the algal mats (Wang *et al.*, 1981).

17.4 Reproduction and development

Reproduction of *Nostoc* takes place in four different ways: (1) a single cell fragmented from filaments forming new colonies; (2) via akinetes;

(3) hormogonia dispersing and forming new colonies; (4) large colonies budding off small colonies (Dodds *et al.*, 1995). Formation of hormogonia is the main method of reproduction. Provided with enough light, hormogonia (the filament between two heterocysts) can be dispersed from the filaments. The process can be triggered by water absorption (Hua *et al.*, 1994). In darkness, when organic carbon is sufficient, filaments will break into single cells and a hormogonia with a few cells. Once exposed to light, the hormogonia from different filaments can amalgamate to form a new filament.

The life cycle of *Nostoc* is differentiated into colony stage and motile hormogonia stage. The life history of *N. commune* was studied using hormogonia and filaments from hormogonia colonies as the starting development point. Within 24 h of transferring the hormogonia into fresh media, heterocysts were clearly observed. The extracellular polysaccharide of single filaments becomes prominent, except around heterocysts. The single filament tends to contort, and the degree of contortion increases continuously. The heterocysts do not divide or grow but remain as connection point of aseriate spheres (Potts, 2000). Whether it was cultured by aerating or not, all the hormogonia would develop in the following pattern: initially the gas vesicle disappears, then followed by formation of sheath-enclosed filament; after the heterocysts differentiated, the cells start to divide on a plane perpendicular to polar cells' axis and also form aseriate colonies, eventually reaching the filament colony stage.

The relationships between hormogonia differentiation and nutrients, as well as light conditions and signal transduction are intricate. When colonies of *N. commune* in exponential phase of growth were transferred into fresh BG-11₀ medium, the hormogonia differentiated no matter what kind of preculture was used. This provided evidence that differentiation was not controlled by the phosphorous or potassium status, nor the osmotic pressure in the media. In contrast, all the cultures in the stationary phase of growth had no hormogonia differentiation after being transferred into a fresh medium.

Under white, red or green light, the differentiation rates of hormogonia were 100%, 80% and 0–10% respectively, reaching maximum at light intensity ranging from 5 to 10 µmole photon m⁻² s⁻¹. No hormogonia differentiation occurs when colonies are cultured in the dark. In presence of sucrose or glucose, however, differentiation rate could reach 100% in the dark. Once sucrose or glucose was substituted with NaCl of the same osmotic potential as the organic counterpart, no differentiation started. The result suggested that hormogonia differentiation was a process that requires energy and not induced by osmotic potential changes (Li, 2000).

17.5 Chemical composition

The history of *Nostoc* in human diet started 2000 years ago, when the Chinese used *N. commune* to survive during famine. It was also recorded that the pharmaceutical value of *N. flagelliforme* was recognized by the Chinese 400 years ago, using it to treat diarrhea, hypertension and hepatitis. To date, it is realized that *Nostoc* has economic value, meeting

the concept of healthy food for its high protein and natural pigment, as well as low fat content. The typical species with economic value of *Nostoc* are *N. flagelliforme* and *N. commune*.

N. flagelliforme contains abundant natural pigments, such as echinenone, myxoxanthophyll, different carotenoids, as well as allophycocyanin, phyco-cyanin and chlorophyll (Lu *et al.*, 1990). It also contains 19 amino acids, eight of which are essential for human health amounting to 35.8–38.6%. In addition to its basic composition, vitamins C, B₁ and B₂, also contribute to its medical value. The main nutrient content is shown in Table 17.1.

The long history of Chinese use of *N. flagelliforme* indicates that the alga is seemingly safe to eat. Nevertheless, exhaustive studies would be valuable to establish beyond doubt that no adverse effects occur with material obtained from any growth- or storage-conditions. A hot-water extract of *N. flagelliforme* showed significant macrophage activity, indicating potential for pharmaceutical use (Takenaka *et al.*, 1997). The cells of *Nostoc* spp. are used to trap deleterious minerals, such as sulfur and copper, in polluted atmosphere or water. *Nostoc* was found to be effective in treating effluents from the polyfibre industry (Schaefer & Boyum, 1998). A sulfur group of the exopolysaccharides of *Nostoc* spp. was considered as a promising anti-HIV natural product to be used in pharmacy. It was found that *Nostoc* spp. are effective against *Cryptococcus* sp. a causal agent of secondary fungal infections in patients with AIDS.

The EPS of *Nostoc* is a most promising component for industrial and pharmaceutical purposes. One principal function of the EPS is that it provides a repository for water. The content of polysaccharide is very affluent in some species, accumulating to more than 60% of the dry weight of *N. commune* colonies. The second function of exopolysaccharide is to specif-

Table 17.1. Chemical composition of *N. flagelliforme*.
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Chemical composition	Mean
Protein %	21.3
Carbohydrate %	56.0
Lipid %	5.64
Nitrogen %	3.16
Phosphorus %	0.12
Calcium %	1.83
Magnesium %	0.27
Iodine %	0.25
Iron %	0.03
Zinc (ppm)	12.8
Copper (ppm)	4.95
Boron (ppm)	14.0
Cobalt (ppm)	2.78
Nickel (ppm)	10.6
VB ₁ (ppm)	0.81
VB ₂ (ppm)	6.87
VC (ppm)	23.32

ically inhibit the fusion of phosphatidylcholine membrane vesicles when they are dried *in vitro*. Freeze-fracture electron microscopy resolved complex changes in the structure of the EPS and the outer membrane in response to rehydration of desiccated cells. The capacity of the EPS to prevent membrane fusion, and the changes in rheological properties of the EPS in response to water availability constitute seemingly important mechanisms for desiccation tolerance in *Nostoc* (Hill *et al.*, 1997).

There are two distinct UVR-absorbing pigments in the EPS of *N. commune*. One has the spectral properties of scytonemin, 544 molecular weight dimeric molecule of indolic and phenolic subunits. The other is a complex mixture consisting of mycosporine amino acids with chromophores, linked to galactose, glucose, xylose and glucosamine, which have absorption maxima 335 and 312 nm (Böhm *et al.*, 1995). The scytonemin is not lost upon water extraction in contrast to mycosporine, which is released upon rehydration to constitute a significant loss of cellular carbon and nitrogen (Potts, 1994). Scytonemin may likely have a much more important role than the aqueous UVR-absorbing pigments in protecting cells upon dispersal of colony fragments.

17.6 Cultivation

Large-scale cultivation of *Nostoc* is presently being experimented with and depends on progress in fundamental studies. The conditions for growth show periodical changes in nature, indicating the importance of understanding conditions of growth in the laboratory and outdoors. Indeed, development of *Nostoc* takes a complex course, controlled by many factors.

One method of cultivation has *N. sphaericum* harvested in liquid medium by aeration. Hormogonia or filaments dispersed from a colony were transferred into the BG-11 medium. Humid and sterile air was aerated into the liquid at a velocity of 200 ml min^{-1} and 28°C temperature. It shaped into a typical spherical colony in ten days, small spheres budding off from the colonies. A massive amount of EPS were produced in liquid media and identified as carbohydrate-modifying enzyme (Hill *et al.*, 1994a). Some metabolic products will prevent the colony from growing continuously, so it is necessary to transfer the alga into fresh medium.

N. flagelliforme is an edible blue-green alga, being used as a popular food in Asia. *N. flagelliforme* has been directly collected from the field. The natural resource is becoming less significant as the market demand increases. Growth of *N. flagelliforme* has been reported to be very slow and difficulties in cultivation were encountered. Cells divided three to four times in ten days when *N. flagelliforme* was cultured on solid medium (Cheng & Cai, 1988). The average elongation of *N. flagelliforme*, when cultured in various media in the laboratory, was 10% in half a year (Dai, 1992). The growth of *N. flagelliforme* was enhanced, as high as 43% in 12 days, by watering with a dilute solution obtained from its habitat (Cui, 1983). Soils from the Chinese chive and wheat fields, however, gave rise to better growth than the soils from the alga's habitat. The elongation rate on the wheat field medium was about $105 \pm 24\%$ over 30 days.

N. flagelliforme, on the other hand, prefers day–night temperature fluctuations. Growth was better at a temperature regime of 21°C by day and 3–9°C at night than of 29°C by day and 10–13°C at night. Under laboratory conditions, *N. flagelliforme* grew better in scattered dim light (800 lux) than radiated bright (3000 lux) light. The maximum rate was about 14% in 14 days. It has been shown in various culture experiments that *N. flagelliforme* grew faster at the beginning, later slowing down. Moisture is needed for the growth of *N. flagelliforme*, but it also leads to its disintegration due to enhanced bacterial growth. Periodic desiccation seems important to prevent *N. flagelliforme* from being disintegrated by bacteria (Gao, 1998).

The authors are trying to mass cultivate *N. flagelliforme* in the liquid medium. It stopped developing into the natural hair-like colony, however, once the formation of hormogonia took place. Without the gluey sheath, it showed poor resistance to the environment and became flimsy. Further studies are needed to comprehend the growth patterns of *N. flagelliforme* so as to solve practical problems in mass cultivation.

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18 Microalgae in Human and Animal Nutrition

Wolfgang Becker

18.1 Introduction

Since the early fifties, intense efforts have been made to explore new, alternate and unconventional protein sources and/or food supplements, primarily in anticipation of an increasing world population and a repeatedly predicted insufficient protein supply. For these new sources, mainly yeast, fungi, bacteria, and algae, the name single cell protein (SCP) was coined to describe the protein production from biomass, originating from different microbial sources.

Some aspects listed here (also see Becker, 1986, 1994) give a general impression about the necessary prerequisites for the utilization of algal biomass for humans and animals.

- Proximate chemical composition (protein, carbohydrates, fat, crude fiber, vitamins, minerals, etc.);
- biogenic toxic substances (phycotoxins, nucleic acids, other toxicants);
- non-biogenic toxic compounds (heavy metals, pesticides, residues from harvesting and processing);
- protein quality studies (protein efficiency ratio, PER; net protein utilization, NPU; biological value, BV; digestibility coefficient, DC);
- biochemical nutritional studies;
- supplementary value of algae to conventional food sources;
- sanitary analyses (microbial analyses for contamination);
- safety evaluations (short- and long-term feeding trials with experimental animals);
- clinical studies (test for safety and suitability of the product for human consumption);
- acceptability studies (these evaluations are optional since finally the consumer will decide if a product is acceptable or not).

The potential for microalgae to enhance nutritional content of conventional food preparations and to act as probiotic agents that positively affect the health of humans and animals has a broad spectrum.

Today, microalgae, marketed as health food or food supplement, are commonly sold in the form of tablets, capsules, and liquids. Algae are also incorporated in pastas, snack foods, candy bars or gums, in drink mixes, and beverages, etc., either as nutritious supplement, or as source of natural food colorant.

18.2 Chemical composition of algae

As with any other higher plant, the chemical composition of algae is not an intrinsic constant factor, but varies from strain to strain and from batch to batch, mainly depending on environmental parameters such as temperature, illumination, pH value, and mineral content of the medium, CO₂ supply, mixing velocity, etc. To obtain an algal biomass with a desired composition, the proportion of the different algal constituents can be modified to a certain extent by varying culture conditions, or changing of physical parameters such as radiation intensity, population density, light, or dark growth, etc.

Many analyses of gross chemical composition have been published in the literature. In order to give a general overview on these estimations, selected data have been compiled in Table 18.1 and compared with data of some conventional food stuffs.

18.2.1 Protein

The high protein content of various microalgal species was one of the main reasons for considering these organisms as an unconventional source of protein. Most of the figures published in the literature on concentrations of algal proteins are based on estimations of so-called crude protein, commonly used in evaluating food and feed. This figure is obtained by hydrolysis of the algal biomass, estimation of the total nitrogen and subsequent multiplication of the value by the factor 6.25. It has to be kept in mind, however, that this calculation involves certain errors. Since in addition to protein there are other nitrogenous constituents, e.g. nucleic acids, amines, glucosamides, and cell wall materials consisting of nitrogen, the content of non-protein nitrogen amounts to 11.5% in *Spirulina* and 6% in *Dunaliella*. As a rule of thumb about 10% of the nitrogen found in microalgae consists of non-protein nitrogen. Although since long, the protein content of microalgae alone is not regarded anymore as the major argument to propagate their utilization as

Table 18.1. General composition of human food sources and different algae (% of dry matter) (Becker, 1994).

Commodity	Protein	Carbohydrates	Lipids
Baker's yeast	39	38	1
Meat	43	1	34
Milk	26	38	28
Rice	8	77	2
Soybean	37	30	20
<i>Anabaena cylindrica</i>	43–56	25–30	4–7
<i>Chlamydomonas rheinhardtii</i>	48	17	21
<i>Chlorella vulgaris</i>	51–58	12–17	14–22
<i>Dunaliella salina</i>	57	32	6
<i>Porphyridium cruentum</i>	28–39	40–57	9–14
<i>Scenedesmus obliquus</i>	50–56	10–17	12–14
<i>Spirulina maxima</i>	60–71	13–16	6–7
<i>Synechococcus</i> sp.	63	15	11

food or food supplement, some basic information shall be given below on the nutritive value of algal protein and the methods applied to quantify the same. Most of these nutritional tests have been performed several years ago and nothing really new has been added since then, indicating that all important parameters have been worked out thoroughly and comprehensively, forming the foundation on which all other modern applications of algal biomass rest. Today, these former nutritional investigations may be seen from a more historical point of view, and also should demonstrate that the algae have been tested under all possible aspects much more carefully than most of any conventional food commodities. Protein quality is estimated commonly by animal feeding tests in which intake and losses are measured under standardized conditions.

In the following, a short description of the common methods used to determine the quality of a given protein is given.

The simplest method to evaluate proteins by animal feeding tests is the determination of the PER, which is based on short-term (3–4 weeks) feeding trials with weanling rats. The response to the diets fed is expressed in terms of weight gain per unit of protein consumed by the animal, i.e. $\text{PER} = \text{weight gain (g)} / \text{protein intake (g)}$.

However, the test needs strict adherence to certain conditions: the calorie intake must be adequate, and the protein must be fed at a sufficient but not excessive amount (commonly at 10% of total diet) because at high levels of dietary protein, the weight gain does not increase proportionally with protein intake. The PER value obtained is normally compared with a reference protein such as casein. Because of the differences of response to the same standard protein, even in the same animal house, the PER values for casein are customarily adjusted to a figure of 2.50, which requires a corresponding correction of the experimental data.

To overcome the shortcomings of the PER estimation, the following more specific nitrogen balance methods can be applied to evaluate the nutritive quality of a protein and to distinguish between the digestibility of the proteinaceous matter and the quantity of nitrogen retained for storage and/or anabolism.

One of these principles is the estimation of the BV, which is a measure of nitrogen retained for growth or maintenance and is expressed as quotient between nitrogen retained and nitrogen absorbed. The absorbed nitrogen is defined as the difference between the ingested and excreted fecal and urinary nitrogen: $BV = [I - (F - F_0) - (U - U_0)]/[I - (F - F_0)]$, where I = nitrogen intake, U = urinary nitrogen, F = fecal nitrogen, F_0 and U_0 fecal and urinary nitrogen excreted when the animals are maintained on a diet, free or low in nitrogen. This calculation accounts for metabolic (or endogenous) nitrogen losses. If the correction factors F_0 and U_0 are not considered, the BV obtained is designated *apparent biological value*.

Another parameter, which reflects the quality of a protein, is the DC, sometimes also called *true digestibility*. It expresses the proportion of food nitrogen that is absorbed by the animal, calculated by the following equation, using the parameters already used for the calculation of the BV: $DC = I - (F - F_0)/I$.

The NPU can be calculated by the simple expression: nitrogen retained divided by nitrogen intake. It is equivalent to the calculation $BV \times DC$ and is a measure of both the digestibility of the protein and the BV of the amino acids absorbed from the food. NPU can be estimated by using the following equation: $NPU = BV \times DC = (B - B_k)/I$, where B = body nitrogen, measured at the end of the test period on animals fed with the test diet, and B_k = body nitrogen, measured on another group of animals fed with a protein free/low diet.

18.2.2 Amino acids

Proteins are composed of amino acids and hence the nutritional quality of a protein is determined by the content, proportion and availability of its amino acids, the so-called amino acid profile. Whereas plants are capable of synthesizing all amino acids, humans and animals are limited to the biosynthesis of certain amino acids only (non-essential amino acids); the remaining (essential) ones have to be provided through food.

Selected data on the amino acid profile of various algae are compiled in Table 18.2 and compared with some basic conventional food items. The amino acid pattern of almost all algae compares favorably with that of the reference and the other food proteins, with minor deficiencies among the sulfur-containing amino acids methionine and cysteine, a fact that is characteristic for many plant proteins.

In this context it has to be stressed that the amino acid profiles, which are analysed from protein hydrolysates, allow certain conclusions to be drawn about their nutritional value, but fail to differentiate between the total amount and the degree of availability of a certain amino acid, which is of special importance in the case of methionine and lysine. During prolonged storage or excessive heat treatment lysine tends to form compounds with reducing carbohydrates, resulting in the non-availability of lysine. This reaction, the so-called Maillard reaction, has to be considered in connection with the various drying steps applied during the processing of algal biomass. In the case of *Spirulina*, a considerable reduction of the digestibility was found due to the interaction of heat treatment, pH and lysine (Adrian, 1975).

Two methods are common for estimating the quality of a given protein by its amino acid composition, i.e. the *chemical score* (CS) and the *essential amino acid index* (EAA). The estimation of the CS involves the comparison of the amino acid composition of the test protein with that of a high quality protein such as egg, milk, or a reference pattern according to the equation:

$$CS = \frac{\text{mg of the amino acid in 1 g of test protein} \times 100}{\text{mg of the amino acid in 1 g of reference protein}}$$

The lowest score for any of the essential amino acids designates the *limiting amino acid* and gives a rough estimate of the quality of the protein tested. In practice, it is preferable to test for lysine, methionine plus cysteine, and tryptophan, because one of these amino acids is usually present in a limiting amount in most conventional and unconventional protein sources.

Table 18.2. Amino acid profile of different algae as compared with conventional protein sources and the WHO/FAO reference pattern (Becker, 1994) (g per 100 protein).

Source	Ile	Leu	Val	Lys	Phe	Tyr	Met	Cys	Try	Thr	Ala	Arg	Asp	Glu	Gly	His	Pro	Ser
Egg	6.6	8.8	7.2	5.3	5.8	4.2	3.2	2.3	1.7	5.0	—	6.2	11.0	12.6	4.2	2.4	4.2	6.9
Soybean	5.3	7.7	5.3	6.4	5.0	3.7	1.3	1.9	1.4	4.0	5.0	7.4	1.3	19.0	4.5	2.6	5.3	5.8
<i>C. vulgaris</i>	3.2	9.5	7.0	6.4	5.5	2.8	1.3	—	—	5.3	9.4	6.9	9.3	13.7	6.3	2.0	5.0	5.8
<i>D. bardawil</i>	4.2	11.0	5.8	7.0	5.8	3.7	2.3	1.2	0.7	5.4	7.3	7.3	10.4	12.7	5.5	1.8	3.3	4.6
<i>S. platensis</i>	6.7	9.8	7.1	4.8	5.3	5.3	2.5	0.9	0.3	6.2	9.5	7.3	11.8	10.3	5.7	2.2	4.2	5.1
<i>Aphanizomenon flos-aquae</i>	2.9	5.2	3.2	3.2	2.5	—	0.7	0.2	0.7	3.3	4.7	3.8	4.7	7.8	2.9	0.9	2.9	2.9

The EAA index is based on the assumption that the BV of a protein is a function of the levels of the ten essential amino acids lysine, tryptophan, valine, isoleucine, leucine, threonine, phenylalanine, methionine, and cystine (as one), arginine, and histidine in comparison to their content in a reference protein (egg).

$$\text{EAA} = \frac{\text{lys}_{\text{test}}}{\text{lys}_{\text{ref}}} \times \frac{\text{try}_{\text{test}}}{\text{try}_{\text{ref}}} \times \frac{\text{val}_{\text{test}}}{\text{val}_{\text{ref}}} \times \dots \times \frac{\text{his}_{\text{test}}}{\text{his}_{\text{ref}}}$$

The EAA index results in figures close to the value determined in feeding tests. The deficiencies in certain amino acids can be compensated for by supplementing the protein directly either with the limiting amino acid or with proteins from other sources rich in the limiting amino acid.

It was observed that the EAA index gives figures more closely related to the value as determined in animal feeding tests.

18.2.3 Carbohydrates

In most of the cases the whole algal biomass is used as food or food supplement. This means that in addition to the protein other components of the algal biomass such as carbohydrates, fibers, etc. will affect the overall value and digestibility of the algal product. Carbohydrates of algae can be found in the form of starch, cellulose, sugars, and other polysaccharides.

As the overall digestibility of the carbohydrates of the tested algae is good, there seems to be no limitation in using dried microalgae as a whole. Whether carbohydrates are likely to cause any other problems like gastro-intestinal disturbance, flatulence or fluid retention, can be established in *in vivo* experiments only.

18.2.4 Lipids

Lipids and fatty acids are constituents of all plant cells, where they function as membrane components, as storage products, as metabolites, and as sources of energy. Lipids extracted with lipophilic organic solvents (ether, petroleum ether, chloroform, etc.) are called commonly *total lipids*. Lipids can be classified according to their polarity, which depends on the non-polar (lipophilic) carbon chains (fatty acids) and the polar (hydrophilic) moieties (carboxylic groups, alcohols, sugars, etc.). The major part of the non-polar lipids (neutral lipids) of microalgae are triglycerides and free fatty acids, whereas the polar lipids are essentially glycerides in which one or more of the fatty acids has been replaced with a polar group, for instance phospholipids and glycolipids. The average lipid content varies between 1 and 40%, and under certain conditions it may be as high as 85% of the dry weight. Algal lipids are typically composed of glycerol, sugars or bases esterified to fatty acids having carbon numbers in the range of C₁₂–C₂₂. They may be either saturated or unsaturated; some cyanobacteria, especially the filamentous species, tend to have larger amounts of polyunsaturated fatty acids (25–60% of the total). On the other hand, species showing facultative anoxygenic CO₂ photoassimilation with sulfite as electron donor lack polyunsaturated fatty

acids. Eukaryotic algae predominantly contain saturated and monosaturated fatty acids, triglycerides are the most common storage lipids constituting up to 80% of the total lipids fraction. Other major algal lipids are sulfoquinovosyl diglyceride, mono and digalactosyl diglyceride, lecithin, phosphatidyl glycerol, and inositol (Table 18.3).

Characteristic for Chlorophyceae, the unsaturated double bindings are in $\omega 3$ position, while in cyanobacteria they are preferably in $\omega 6$ position. Nutritional and environmental factors can affect both the relative proportions of fatty acids as well as the total amount. Many microalgae growing under nitrogen limitation show enhanced lipid contents. Already in the late forties, it was noted that nitrogen starvation is most influential on lipid storage and lipid fractions as high as 70–85% of dry weight were reported. Some algal species such as *Dunaliella* sp. or *Tetraselmis suecica*, however, respond with decreasing lipid contents and produce carbohydrates rather than lipids under such conditions. Besides nitrogen, other nutrient deficiencies may also lead to an increase in the lipid content. In diatoms, for instance, the amount of lipids can be increased during silicon starvation. The effect of different nitrogen regimes on lipid content and other growth parameters of different algae (*S. obliquus*, *C. vulgaris*, *Anacystis nidulans*, *Microcystis aeruginosa*, *Oscillatoria rubescens*, and *S. platensis*) was studied by Piorreck *et al.* (1984).

Cyanobacteria do not show any significant changes in their fatty acid and lipid composition in response to the nitrogen supply, indicating that Chlorophyceae rather than cyanobacteria can be manipulated in mass cultures to yield a biomass with desired lipid composition.

Among the various fatty acids, the commercially important ones are the essential polyunsaturated fatty acids, namely linoleic acid (18:2 $\omega 9, 12$),

Table 18.3. Analytical data on fatty acid composition of lipids of different algae.

Fatty acid	<i>S. platensis</i>	<i>S. obliquus</i>	<i>C. vulgaris</i>	<i>D. bardawil</i>
Lauric acid (12:0)	0.04	0.3	–	–
Myristic acid (14:0)	0.7	0.6	0.9	–
Pentadecanoic acid (15:0)	traces	–	1.6	–
Palmitic acid (16:0)	45.5	16.0	20.4	41.7
Palmitoleic acid (16:1)	9.6	8.0	5.8	7.3
Hexadecatetraenoic acid (16:4)	–	26.0	–	3.7
Heptadecanoic acid (17:0)	0.3	–	15.3	2.9
Stearic acid (18:0)	1.3	0.3	15.3	2.9
Oleic acid (18:1)	3.8	8.0	6.6	8.8
Linoleic acid (18:2)	14.5	6.0	1.5	15.1
α -Linolenic acid (18:3)	0.3	28.0	–	20.5
γ -Linolenic acid (18:3)	21.1	–	–	–
Eicosadienoic acid (20:2)	–	–	1.5	–
Eicosanotrienoic acid (20:3)	0.4	–	20.8	–
Others	–	2.5	19.6	–
Reference	Hudson & Karis (1974)	Kenyon <i>et al.</i> (1972)	Spoehr & Milner (1949)	Fried <i>et al.</i> (1982)

β -linolenic acid (18:3 ω 6, 9, 12), dihomo-linoleic acid (20:3 ω 8, 11, 14), arachidonic acid (20:4 ω 5, 8, 11, 14) and eicosapentaenoic acid (20:5 ω 5, 8, 11, 14, 17). For instance, β -linolenic acid is regarded as effective in lowering the plasma cholesterol level and has been used as dietary supplement for the treatment of various diseases. Since this fatty acid is very rare in common foodstuffs, appreciable amounts are extracted from the evening primrose and added as substitute to various food and dietary preparations. It has been found that *S. platensis* may serve as a valuable source of γ -linolenic acid, since about 20–30% of its fatty acids consist of this compound.

In the same alga, poly- β -hydroxybutyrate has been identified as lipid reserve that accumulates, during exponential growth, up to 6% of dry weight. It can be assumed that in addition to different *Spirulina* species, other cyanobacteria may also synthesize and accumulate this compound in larger amounts under certain culture conditions. The red alga *Porphyridium cruentum* is one of the richest natural sources of arachidonic acid which constitutes about 36% of the total fatty acids at the usual culture temperature of 25°C. At lower temperatures (16°C) the amount may increase up to 60%.

Certain algal lipids can be used as surfactants with properties different from the ones of synthetic compounds. The advantage of the algal-derived surfactants, which are mainly phosphatidylglycerol, phosphatidylcholine and different galactosyl diglycerides, is that they are biodegradable.

Some of the essential unsaturated fatty acids found in algal lipids are of pharmaceutical importance. They are precursors of prostaglandins, prostanocyclins and leucotrienes, and as such becoming increasingly important in the pharmaceutical industry. Patents have been applied for their use as anti-hypertensive, for treatment of hyperlipidemia, for cholesterol reduction, as health food, etc. However, all these very promising sounding applications of algal lipids should always be considered realistically with regard to the current economic situation. By comparing the present world market prices for salmon oil (14% eicosapentaenoic acid, 12.4% docosahexaenoic acid, 2.8% docosapentaenoic acid) and for fish oil (18% eicosapentaenoic acid and 12% docosahexaenoic acid), one has to accept that under present conditions, algal lipids cannot compete with conventional sources of polyunsaturated fatty acids, even by considering a better price for these lipids which originate from a plant source.

18.2.5 Vitamins

Microalgae represent a valuable source of nearly all important vitamins, which improve the nutritional value of algal biomass. In addition to natural fluctuations of the vitamin content due to environmental factors, the post-harvesting treatment and methods of drying the algal biomass have a considerable effect on its vitamin content. This applies especially to the heat unstable vitamins B₁, B₂, C, and nicotinic acid, the concentration of which decrease considerably during the drying process. A compilation of data reported in the literature on the vitamin content estimated in various algae is given in Table 18.4. For comparison, the vitamin content of spinach and beef liver, both rich vitamin sources, have been added.

Table 18.4. Vitamin content of different algae in comparison with common foodstuffs and the recommended daily intake (RDI) (Becker, 1994) (values in mg kg⁻¹ dry matter).

Source	Vit A	Vit B ₁	Vit B ₂	Vit B ₆	Vit B ₁₂ *	Vit C	Vit E	Nicotinate	Biotin	Folic acid	Pantithetic acid
RDI (mg/d)	1.7	1.5	2.0	2.5	0.005	50.0	30.0	18.0	—	0.6	8.0
Liver	60.0	3.0	29.0	7.0	0.65	310.0	10.0	136.0	1.0	2.9	73.0
Spinach	130.0	0.9	1.8	1.8	—	470.0	—	5.5	0.007	0.7	2.8
Baker's yeast	trace	7.1	16.5	21.0	—	trace	112.0	4.0	5.0	53.0	
<i>S. platensis</i>	840.0	44.0	37.0	3.0	7.0	80	120.0	—	0.3	0.4	13.0
<i>Aphanizomenon flos-aquae</i>	4.8	57.3	11.1	8.0	0.7	—	0.1	0.3	1.0	6.8	
<i>C. pyrenoidosa</i>	480.0	10.0	36.0	23.0	—	—	240.0	0.15	—	20.0	
<i>S. quadrivalvis</i>	554.0	11.5	27.0	—	1.1	396.0	—	108.0	—	—	46.0

* All data in the literature concerning the content of B₁₂ in *spirulina* sp. have to be treated very carefully, since major parts of this vitamin are non-nutritive analogues.

In addition to the vitamins listed here, other vitamins such as vitamin K, isomers of tocopherol (vitamin E), etc. as well as metabolic intermediates can be found in almost all algae.

In general, the concentrations of the various vitamins are comparable between the different algae as well as other microbial sources such as bacteria and yeasts. An important exception is vitamin B₁₂. Since contradictory statements about the occurrence of this vitamin in different algal species can be found in the literature, a few explanatory remarks are necessary: plant foods are generally not considered as a source of vitamin B₁₂ and therefore data on its detection in Chlorophyceae or Rhodophyceae is rather surprising, since it is accepted that these algae are not able to synthesize this vitamin. If vitamin B₁₂ is found in samples of these algae, it has to be assumed that the vitamin was adsorbed or absorbed from bacteria closely associated with or grown on the algae. On the other hand, the true detection of this vitamin in the cyanobacterium *Spirulina* indicates the close phylogenetic link of this organism to bacteria, which are capable of synthesizing vitamin B₁₂. *Spirulina* is a practical source of vitamin B₁₂, as shown in animal experiments feeding diets deficient in vitamin B₁₂, followed by a repletion period with diets containing pure vitamin B₁₂ in the form of cyanocobalamin as well as diets fortified with *Spirulina*.

18.2.6 Pigments

One of the most obvious characteristics of the algae is their color, each phylum has its own particular combination of pigments and an individual color. In view of their phylogenetic age, it is obvious that they have developed pigments that are peculiar to them. With a few exceptions, the pigmentation of higher plants is similar to that of the Chlorophyta, from which the higher plants are thought to have evolved.

18.2.6.1 Chlorophyll

Chlorophyll-*a* is the primary photosynthetic pigment in all algae and is the only chlorophyll of the cyanobacteria and the Rhodophyta. Like all higher plants, Chlorophyta and Euglenophyta contain chlorophyll-*b* as well; additional chlorophylls of the form -*c*, -*d*, and -*e* can be found in several marine algae and freshwater diatoms. The total amount of chlorophyll in algae is in the range of 0.5–1.5% of dry weight.

The true nutritive value of chlorophyll in microalgae is quite controversial. In contrast, chlorophyll degradation products have been identified as a cause of skin irritations (see Chapter 32).

18.2.6.2 Carotenoids

In view of the commercial utilization of algae, the second group of algal pigments, the carotenoids, play an important role. Carotenoids are yellow, orange or red lipophile pigments of aliphatic or alicyclic structure, composed of eight isoprenoid units, which are linked in a manner, so that methyl groups nearest to the center of the molecule are in the 1,5 position, whereas all other

lateral methyl groups are in 1,6 position. Lycopene, synthesized by stepwise desaturation of the first 40-carbon polyene phytoene, is the precursor of all carotenoids found in algae. Two major groups of carotenoids can be distinguished: (1) pigments composed of oxygen-free hydrocarbons, i.e. the carotenes, and (2) their oxygenated derivates, the xanthophylls with epoxy-, hydroxy-, ketonic-, carboxylic-, glycosidic-, allenic-, or acetylene-groups. All algae contain carotenoids, the variety of which is greater than in higher plants. Although certain carotenoids like β -carotene, violaxanthine and neoxanthine occur in most algal classes, other carotenoids are restricted to only a few algal classes.

The average concentration of carotenoids in algae amounts to about 0.1–0.2% of the dry weight. Under appropriate culture conditions, however, much higher concentrations of β -carotene (up to 14% per dry weight) can be found in the unicellular, wall-less Chlorophyceae *Dunaliella*, several strains of which (*D. bardawil*, *D. salina*, *D. kona*) have been reported initially.

It has been accepted that carotene isolated from algal sources would be acceptable as a food additive if it was of sufficient purity to meet the specifications for synthetic β -carotene. The carotene found in the market originate from spray dried concentrated, lyophilized or dehydrated preparations and preparations obtained as vegetable oil extract of *Dunaliella*.

β -Carotene has a wide range of commercial applications. It is used as food coloring, as an additive to enhance the color of the flesh of fish and the yolk of eggs, and to improve the health and fertility of grain fed cattle. In addition, it is used as food supplement with the promise to prevent certain forms of cancer due to its anti-oxidant properties. Until the early 1980s, commercial production of β -carotene was all synthetic. During the 1970s, researchers realized that under certain culture conditions *Dunaliella* will accumulate high amounts of dry weight as β -carotene. This discovery led to commercial derivation of natural β -carotene from this organism, which is currently a substantial and growing industry.

18.2.6.3 Phycobiliproteins

In addition to the lipophilic pigments, cyanobacteria, Rhodophyta and Cryptophyta contain phycobiliproteins, i.e. deep-colored water-soluble proteinaceous accessory pigments, which are components of a complex assemblage, the phycobilisomes. The phycobiliproteins are divided into two prosthetic groups, forming phycoerythrobilins and phycocyanobilins. Among the phycoerythrins, three different types can be distinguished based on their absorption spectra: R-phycoerythrin and B-phycoerythrin found in Rhodophyceae, and C-phycoerythrin, present in cyanobacteria. Corresponding to that, three different phycocyanins have been isolated: R-phycocyanin from Rhodophyceae and C-phycocyanin and allophycocyanin from cyanobacteria.

18.3 Toxicological aspects

Unconventional food items such as microalgae have to undergo a series of toxicological tests to prove their harmlessness. Recommendations for the performance of such evaluations have been published by different inter-

national organizations, but it has to be assumed that additional national regulations exist, which specify the recommended evaluations.

As part of the toxicological characterization, the algal material has to be analysed for the presence of toxic compounds, either synthesized by the alga itself or accumulated from the environment, i.e. biogenic or non-biogenic toxins. The biogenic toxins include all compounds that are either synthesized by the alga or formed by decomposition of metabolic products and hence represent an intrinsic characteristic of the organism. Non-biogenic products comprise environmental contaminants and can be avoided in most cases by proper cultivation techniques, plant management and selection of cultivation sites free of pollution.

18.3.1 Biogenic toxins

18.3.1.1 Nucleic acids

One of the very few constituents present in all living organisms and hence in algae, and which under certain circumstances may be counted as toxin, are nucleic acids (RNA and DNA). Since these constituents are the sources of purines, they are now and then considered as the major limitation in the use of alga and other forms of SCP as food or feed ingredient. Therefore, some reflections may illustrate the matter. The nucleic acid content in algae varies normally between 4 and 6% (8–12% for yeast and up to 20% for bacteria) in dry matter. For humans, the ingestion of purines may lead to an increase of plasma uric acid concentration and urinary excretion, since human metabolism cannot degrade purines beyond the level of uric acid. Elevated serum levels of uric acid may increase the risk of gout, while elevated urinary levels may result in the formation of uric acid stones in the kidney and nephropathy. Because of a possible health hazard, the Protein Advisory Group has recommended that the daily intake of nucleic acid from unconventional sources should not exceed 2.0 g, with total nucleic acids from all sources not exceeding 4.0 g per day. Considering endangered latent hyperuricemic persons, the safe level should be at about 20 g of algae per day or 0.3 g of algae per kg of body weight.

18.3.1.2 Algal toxins

Poisoning of man by algal toxins, and poisoning of livestock and other animals attributed to toxic blooms of algae occur with considerable frequency – but unpredictable – in regions of South America, Europe, Asia, South Africa and Australia. These toxic algal strains are predominantly cyanobacteria (*M. aeruginosa*, *Anabaena flos-aquae* and *Aphanizomenon flos-aquae*), which are often morphologically indistinguishable from non-toxic strains. Fortunately, no such cases have ever been reported in connection to mass-cultured algae. However, there has been concern in the beginning of alga cultivation about the possibility that the algae produced may contain toxins. Therefore, detailed investigations were performed for the presence of toxins,

including chemical analyses, dermatological toxicological tests, and toxicity studies with mice, as well as investigations on antibiotic activities. All these tests gave negative results, and numerous feeding trials have not revealed so far any pathological symptoms due to algal toxins.

18.3.2 Non-biogenic toxins

Among the non-biogenic compounds, special attention has to be given for the concentration of heavy metals and polycyclic aromatic compounds in the algal biomass.

18.3.2.1 Heavy metals

It is a fact that almost all microorganisms, preferably those which are living in an aquatic biotop, are capable of accumulating heavy metals at concentrations which are several orders of magnitude higher than those present in the surrounding. Metal accumulation is a relatively rapid process; bacteria for instance can attain equilibrium distribution between the cell and its surrounding within a few minutes. In the case of algal cells, saturation with heavy metals normally will be reached within 24 h. Elevated amounts of various heavy metals in the algal biomass was and still is one of the major problems encountered in large scale production of algae. According to WHO/FAO guidelines, an adult person of 60 kg body weight should not incorporate more than 3 mg of lead, 0.5 mg of cadmium, 20 mg of arsenic, and 0.3 mg of mercury per week through food and beverages. Since children react more sensitively to heavy metals than adults, the tolerable amounts for children have to be still lower than simply calculated on body weight (Table 18.5).

No *normal* or characteristic levels exist for toxic metals in microalgae, the concentrations are very variable, mainly due to varying composition of the culture media, contaminations during processing, or even improper analytical techniques. At present, no official standards exist for the heavy metal content of microalgal products. On a voluntary basis, some algae manufacturers have established internal guidelines for metal levels in their products. To illustrate the present situation, selected analytical data found in the literature are summarized in Table 18.6 and compared with recommendations on upper limits of heavy metal contents in different food commodities

18.4 The effect of processing on the digestibility of microalgae

The cell wall represents about 10% of the algal dry matter and consists of a variety of macromolecules, the amounts and chemical composition of which are group-, species-, and even strain-specific. In general, at least two major components, one fibrillar and one mucilagenous, were identified in the algal cell wall. The microfibrils form the most inert and resistant part of the cell wall, the most common one of the skeletal components being cellulose. As already indicated, this cellulosic cell wall poses a serious problem in digesting/utilizing the algal biomass, since it is not digestible for humans and other non-ruminants.

Table 18.5. Data on heavy metal concentrations in algae compared to international recommendations (data given in ppm).

Source	Pb	Cd	Hg	As	References
Maximum weekly intake (mg/adult)	3.0	0.5	0.3	20.0	WHO (1972a)
Limits in SCP	5.0	1.0	0.1	2.0	IUPAC (1974)
Limits in drinking water	0.1	0.01	0.001	0.005	WHO (1972b)
Japan Chlorella Industry (for <i>Chlorella</i>)	2.0	0.1	0.1	0.1	Jassby (1988)
Japan Health-Food Ass. (for <i>Spirulina</i>)	20.0	(as total lead)			
<i>Spirulina</i> (Indian Standard)	2.5	1.0	0.1	1.1	Torres-Duran et al. (1998)
<i>Scenedesmus</i> (Thailand)	6.03	1.67	0.07	2.36	Payer & Runkel (1978)
<i>Scenedesmus</i> (Peru)	0.58	0.30	0.43	0.91	Becker & Venkataraman (1982)
<i>Spirulina</i> (India)	0.95	0.62	0.07	0.97	Becker & Venkataraman (1982)
<i>Spirulina</i> (Mexico)	5.1	0.5	0.5	2.9	Boudene et al. (1975)
<i>Spirulina</i> (Chad)	3.7	–	0.5	1.8	Boudene et al. (1975)

Table 18.6. Comparative data on PER values of different algae processed by different methods.

Alga	Processing	BV	PER	DC	NPU	References
Casein		87.8		95.1	83.4	Becker et al. (1976)
Egg		94.7		94.2	89.1	Becker et al. (1976)
<i>Chlorella</i> sp.	AD	52.9			31.4	Bock & Wünsche (1968/9)
<i>Chlorella</i> sp.	DD	76.6		89.0	68.0	Saleh et al. (1985)
<i>Spirulina</i> sp.	Raw	63.0		76.0	48.0	Clement et al. (1967a)
<i>Spirulina</i> sp.	Stewed	51.0		74.0	38.0	Clement et al. (1967a)
<i>Spirulina</i> sp.	SD	77.6		83.9	65.0	Becker (1994)
<i>Spirulina</i> sp.	DD	68.0		75.5	52.7	Narashima et al. (1982)
Casein	Raw		2.50			Becker et al. (1976)
<i>Chlorella</i> sp.	Autoclaved		0.84			Cheeke et al. (1977)
<i>Chlorella</i> sp.	DD		1.31			Cheeke et al. (1977)
<i>Chlorella</i> sp.	FD		2.0			Saleh et al. (1985)
<i>Chlorella</i> sp.	SD		1.66			Lubitz (1963)
<i>Chlorella</i> sp.	SD		0.68			Erchul & Isenberg (1968)
<i>Spirulina</i> sp.	DD		2.10			Contreras et al. (1979)
<i>Dunaliella bardawil</i>			0.77			Mokady & Cogan (1988)

DD, drum dried; SD, sun dried; FD, freeze dried.

Effective treatments are therefore necessary to disrupt the cell wall, making the algal protein and other constituents accessible for digestive enzymes.

With the exception of the cyanobacteria *Spirulina* sp. and *Aphanizomenon flos-aquae*, most of all other algae of commercial importance (Chlorophyceae, Rhodophyceae) have that rigid indigestible cell wall, which mandates that the algal cell be ruptured. This can be achieved by either physical methods like boiling, various types of high temperature drying, to a certain extent even sun drying (freeze drying *per se* will not break the cellulosic cell wall), or by chemical methods like autolysis or breaking the hydrogen bonds by phenol, formic acid or urea.

The major problem encountered with the latter methods is the necessity of recovering the solvent and ensuring that the product is not toxic, by residues of the chemicals used.

Analyses of the cell walls of cyanobacteria revealed the absence of cellulosic material and a close relationship to the structure of Gram negative bacteria. Therefore, the cell wall of *Spirulina* does not represent a barrier to proteolytic enzymes as demonstrated by the fact that this alga, in general, can be digested by monogastric vertebrates like humans without previous physical or chemical rupture of the cell wall.

In order to demonstrate the necessity of algal cell wall rupture (of non-cyanobacterial microalgae) and to compare the efficiency of the various methods tested, a short summary of representative studies shall be included here. One should always keep in mind that proper processing of the algal biomass is the key process for almost all applications of the algal biomass.

Several authors have studied the effect of different post-harvesting treatments on the digestibility of various algal species by evaluating the PER of the treated biomass. To provide a general idea of the major findings, out of the numerous reports found in the literature, representative PER values are compared in Table 18.6.

18.5 Metabolic studies

The results of the PER studies with different algae described above have already demonstrated the importance of the drying step on the nutritive value. These findings are supported by the results of metabolic studies – the principles of which are given before – summarized in Table 18.6.

The data for *Spirulina* confirm that this alga, with its thin and fragile cell wall, does not present any problems in protein utilization and even simple sun drying is sufficient to obtain acceptable values.

To complete the spectrum of nutritional studies, another method follows, i.e. examination of the protein quality by digestibility studies, in which the intestinal enzyme system pepsin/pancreatin/trypsin is simulated in *in vitro* experiments. Processing had significant effects on the digestibility of the Chlorophyceae. Protein digestibility studies with *Scenedesmus* and *Spirulina* were reported by Becker & Venkataraman (1982) who determined the digestibility by first incubating the algae with pepsin, followed by pancreatin treatment. For studies with *Spirulina*, fresh, freeze-dried and sun-dried material was used. All these methods did not alter digestibility significantly, confirming that *Spirulina per se* has a high digestibility and does not need further processing.

In conclusion, many tests made it evident that the algal biomass shows promising qualities as a novel source of protein. Neglecting extreme values, it can be stated that after suitable processing the average quality of most of the algae examined is equal or even superior to other conventional high quality plant proteins. This has repeatedly and unequivocally been confirmed by the long series of different and independent investigations, which analysed the various metabolic parameters in different animal species.

18.6 Toxicology

18.6.1 Toxicological studies with animals

18.6.1.1 Whole algae

As already mentioned, international guidelines demand proof of the toxicological safety of new unconventional food items. In accordance with these recommendations, a series of different animal tests were performed with different algae. Most of these studies were performed at a time, when the utilization of algae was still in its infancy and serious concerns were raised about the safety of this new type of food.

Two-weeks feeding trials with *S. obliquus* on rats were reported from India (Venkataraman *et al.*, 1977, 1980) showing that feeding of drum-dried *S. obliquus* over a period of 12 weeks did not reveal any significant toxicological features in the test animals.

More detailed toxicological evaluations have been performed with *Spirulina*. Probably the most comprehensive studies were published from Mexico (Chamorro, 1980; Chamorro & Salazar, 1990; Chamorro *et al.*, 1996). These reports include tests on sub-acute and chronological toxicity, reproduction, lactation, mutagenicity, and teratogenicity. The authors tested three different concentrations (10, 20, and 30%) of spray-dried *Spirulina*, produced in Mexico, which were added to a standard diet substituting the commonly used soybean meal, the amount of which in the ratios was reduced from 44% in the control to 30, 14, and 0%, respectively.

To detect any chronic toxicological effect of the algae, reproduction and lactation studies over three generations were conducted. No adverse effects were observed.

The toxicity of *S. maxima*, after 13 weeks of feeding the alga (Salazar *et al.*, 1996, 1998) was tested: Groups of ten mice of each sex were given *S. maxima* in the diet at concentrations of 0 (control), 10, 20, or 30% (w/w) for 13 weeks. The alga ingestion had no effect on behavior, food and water intake, growth, or survival. Terminal values in hematology and clinical chemistry did not reveal differences between treated and control groups.

Bourges *et al.* (1971) report of 50 days of feeding two *Spirulina* rich diets (730 and 260 g algae/kg diet) and a casein control to different groups of animals and a switching of the groups after that time either from control to algal diet or vice versa in order to detect possible age differences in response (Bourges *et al.*, 1971). In spite of the high algal content on both the algal

diets, all animals survived with apparent health, all organs were normal macroscopically and microscopically and no differences were found between control and experimental groups. Rounding up the spectrum of toxicological evaluations, tests on possible dermal toxicity were also conducted, performed in India with drum-dried *S. obliquus* and sun-dried *S. platensis* (Krishnakumari *et al.*, 1981). Testing for allergic effects, none of the animals showed any sign of erythema or oedema on the skin, and hair growth did not differ from the controls.

18.6.1.2 β -carotene

To establish the toxicological safety of carotenes derived from algae with particular attention to its main source, the Chlorophyceae *Dunaliella*, different animal feeding experiments were performed providing the necessary biological data on both dried preparations of the alga and carotenoid extracts.

Weanling rats were fed a retinol-deficient diet for 60 days, resulting in a liver retinol content of 4–5 µg. Then, six animals each allocated to nine groups were fed the same diet supplemented with (1) retinol at 7.5 mg kg⁻¹ diet; (2–4) all-trans β -carotene at 12, 29, 48 mg kg⁻¹, respectively; (5–7) lyophilized *Dunaliella* contributing 29, 58 or 112 mg β -carotene kg⁻¹, respectively; (8) maize oil extract of *Dunaliella* providing 16 mg β -carotene kg⁻¹ and (9) no supplementation. After seven days, livers were analysed for retinol, retinol isomers and β -carotene. A comparable content of retinol was detected, which was related to the dose of carotene but was irrespective of the source. Animals fed on the algae or algal extracts accumulated 9-cis retinol in addition to the all-trans isomer. It was concluded that dried *Dunaliella* or its oil extract can serve as a dietary natural β -carotene source which serves the total requirement of retinol (Ben-Amotz *et al.*, 1988).

In another pair of experiments, two groups of egg-laying hens received a control diet containing 150 g maize meal kg⁻¹ or the same diet supplemented with 4 g lyophilized *Dunaliella*/kg (200 mg β -caroten). Eggs from these hens showed an enhanced yolk colorization caused by lutein, but no β -carotene was present in the yolk (Ben-Amotz *et al.*, 1986).

Feeding studies on rats in the US showed that the bioavailability of *Dunaliella* as a source of β -carotene and the algal oil extract was higher than that of the oleoresin and the synthetic β -carotene.

Summing up all the available information on possible toxic properties of the different algae tested so far, none of these species showed any negative effect in animal feeding experiments. Rodents, employed for these investigations, accepted the algal containing rations very well, even at high algal concentrations, which in several cases resulted in increased feed consumption and weight gain. It seems safe to state that more controlled experimental nutritional and especially toxicological evaluations were performed with microalgae, mainly *Spirulina*, than with any other conventional or unconventional food commodity.

18.6.2 Toxicological studies with humans

No systematic testing program has been developed yet, which relates to the use of algae for human consumption. The objectives aimed at by the different investigations so far ranged from studies with malnourished infants to mass feeding trials. Contradictory results are found concerning the reaction of humans to algal diets. On the one hand, there are reports stating that people have lived solely on algae for prolonged periods of time without developing any negative symptoms, while in other studies, discomfort, vomiting, nausea, and poor digestibility of even small amounts of algae were reported.

One of the first studies was performed in 1961 by Powell *et al.*, who gave a mixture of *Chlorella* sp. and *Scenedesmus* sp., cooked for 2 min and dried under vacuum, to five healthy, 18–23-year-old men who received various diets, the daily algal consumption per head amounting to 0, 10, 20, 50, 100, 200, and 500 g. All men tolerated amounts up to 100 g, the taste was reported to be strong and disagreeable. Abdominal distress associated with increased flatulence was noted at the beginning of the study and became more serious at the 200 g algal level, connected with nausea, vomiting, and hard stool. Only two persons completed the period with 500 g of algae per day but complained of abdominal cramps and malaise. Beside these gastrointestinal disorders, no other anomalies were reported by the authors based on laboratory tests and physical observations.

In view of the initially mentioned possibility of using algae as protein supplement in space flights, Russian scientist tested the effect of three weeks uptake of diets containing 50, 100, and 150 g of freeze-dried alga (a mixture of *Chlorella* sp. and *Scenedesmus* sp.) as source of protein (Kondratiev *et al.*, 1966). The authors analysed different blood parameters, urine and feces of the test persons. For the groups receiving 50 and 100 g of algae daily, only negligible changes of the metabolic parameters were observed, and, except for some indices characteristic for the lipid metabolism, all analytical data remained within the limits. However, since the daily uptake of 150 g of algae affected the state of health of the majority of the test persons, it was concluded that the maximum amount for daily consumption of algae is 100 g. This assumption was confirmed by investigations performed in Germany a few years later (Müller Wecker & Kofranyi, 1973).

A different attempt for testing algae containing diets was reported from Peru, where young naval cadets and school children were employed to evaluate their tolerance to drum-dried *S. obliquus* (Gross *et al.*, 1982). Weight changes, hematological data, urine, serum protein, uric acid concentration were measured over a four-week test period, during which adults received 10 g and children 5 g of algae daily, incorporated into their normal diet. No changes in the analysed parameters were found except a slight increase in weight.

Unique was the attempt of the same authors to test the value of this alga as food supplement in the diets of hospitalized slightly (group I) and seriously undernourished (group II) children (Gross *et al.*, 1978). The four-year-old children of the first group received 10 g of algae daily over a period of three weeks. They showed a significant increase in weight (27 g d^{-1}) compared to

other children of the same group who received a normal diet. The authors attributed this positive effect to certain therapeutic properties of the algal biomass. In addition, diarrhea was cured during the algal uptake so that a better resorption of the food can be assumed.

The most genuine use of *Spirulina* can still be found in the area, where the utilization of this alga had one of its origins, i.e. in central Africa among the Kanembu tribal people living around the lake Kossorom in the prefecture of Lac in the Republic of Chad. Here, *Spirulina* is harvested by filtration and dried in the sun on the sandy shores of the lake. The semi-dried product, called *dihe*, is then cut into small squares and taken to the villages, where the drying is completed on mats in the sun. *Dihe* is mainly used to prepare a kind of broth together with fish, meat, or vegetables. Part of the harvest is sold at different places in and outside the country. It is reported that the annual trading value of the harvested alga (about 40 t) amounts to more than US\$100 000, a considerable contribution to the economy of this area (Abdulqader *et al.*, 2000).

18.7 Algae as animal feed

Even though *Spirulina* and other algal species have been used as food or food supplement for quite some time, its use as animal feed is more recent. The large number of nutritional and toxicological evaluations that was conducted in the past demonstrates the suitability of algal biomass as a valuable feed supplement. It is estimated that about 30% of the current world algal production is sold for animal feed application (Belay *et al.*, 1996).

The objective of the following chapter is to provide an overview of published information on the effect of supplementing feed rations with microalgae on some economically important domestic animal species. Since all microalgae in question, with the exception of the cyanobacteria *Spirulina* and *Aphanizomenon*, contain cellulosic cell wall material, which may cause, as already discussed, problems in digestibility, the effects of algal supplementation have to be valued with respect to two different groups of animals: ruminants and non-ruminants (monogastric vertebrates). Ruminants (cattle, sheep) are capable of digesting cellulosic material, hence offering the at least theoretical possibility to feed native algae directly to these animals, which has not gained much attraction so far. For monogastria, including humans, the algal biomass has to be processed properly before it is made suitable as feed.

18.7.1 Poultry

Most of the studies on domestic animals were performed on poultry, mainly because the incorporation of algae into poultry rations offers the most promising prospect for their commercial use in animal feeding. Test performed in Israel with sewage-grown algae with the aim of replacing soybean meal used in broiler mash indicates that all algal species tested can successfully replace 25% of the soya protein in the diet (i.e. 5% algae); higher amounts (15% algae), however, lowered the feed conversion efficiency (Mokady *et al.*, 1980). Furthermore, it was also observed that the algae could not replace fishmeal as a source of growth factors in these studies.

About 20 years ago, it was first tried to utilize *Chlorella* strains (*C. vulgaris* A1–25) with very thin cell walls in poultry feeding, since it was assumed that these would be more easily digestible after low temperature drying (air-drying) than Chlorophyceae in general. This alga, which contained less protein (24%) than common strains of *Chlorella*, was incorporated at 16.3 and 20% levels in poultry rations. The gross protein value – with 2% methionine supplementation – was 71% and no signs of toxicity were detected in the birds (Yoshida & Hoshii, 1982).

Feeding studies with different concentrations (up to 30%) of *Spirulina* showed that both protein and energy efficiency of this alga were similar to other conventional protein carriers up to a level of 10%, but were reduced at higher concentrations. In broiler experiments with *Spirulina*, weight increase was depressed as soon as the alga completely replaced traditional proteins. The reduction was less at algal concentrations up to 5%; increase in weight, however, was reduced by 16 and 26% at algal levels of 20 and 30%, respectively.

There are only few reports in the literature on the effect of algae on the performance of laying hens. For sewage grown *Chlorella*, no differences were found in egg production rate, egg weight, food conversion efficiency between controls and birds receiving up to 12% of this alga. Other studies on effects of partial substitutions of soybean meal in layer rations by *Spirulina* showed that at algal concentrations above 10% the sensory characteristics of the eggs were affected, resulting in a *chemical flavor* of the egg. The yellow color of broiler skin and shanks as well as of the egg yolk is the most important characteristic that can be influenced by feeding algae. However, it has to be kept in mind that pigmentation may reach unacceptable high levels, or may impair the quality of the broiler in such areas where non-pigmented meat is preferred.

Venkataraman *et al.* (1994) investigated the effect of sun-dried *Spirulina* in poultry diets in a 12-weeks feeding trial by replacing either fishmeal or ground-nut cake in a commercial diet with algae at an isonitrogenous concentration of 140 g kg⁻¹ and 170 g kg⁻¹, without adding extra vitamins or minerals. Efficiency of food utilization, PER and dressing percentage indicated that the substitution of the commercial proteins by algae did not affect the performance of the birds, their weight or histopathology of various organs. Meat quality remained unchanged except for a more intensive color in the case of birds fed on the algal diet.

Significantly higher growth rates and lower non-specific mortality rate were observed in turkey poult fed with *Spirulina* at the level of 1–10 g kg⁻¹ diet. Mortality decreased from 12% with birds on basal diet to 3% at 1 g of alga kg⁻¹ diet. Summarizing, in poultry rations, algae up to a level of 5–10% can be used safely as partial replacement of conventional proteins. Higher concentrations, however, caused adverse effects on prolonged feeding.

18.7.2 Pigs

In one of the first studies performed in the late sixties by Hintz & Heitmann (1967), a mixture of *Chlorella* sp. and *Scenedesmus* sp., grown on sewage

and dried either in air or by drum drying, was incorporated at 2.5, 5.0 and 10% levels in pig rations, substituting soybean and cotton seed meal. The feed efficiency ratios (FER) estimated after a feeding period of seven weeks were 3.76, 3.85, and 3.90, respectively and almost identical with the control value of 3.85, obtained with common pig mash.

The influence of fishmeal, soybean meal and drum-dried *Scenedesmus* on the growth and the quality of the pork was also tested. No significant differences in growth could be found between the various groups fed on different protein sources (group I: fishmeal, group II: soybean meal, group III: 75% soybean meal + 25% fishmeal, group IV: 25% soybean + 75% algae).

Two studies were reported on the feasibility of replacing 33% of soy protein in a basal diet with proteins from the two cyanobacteria *S. maxima* and *A. platensis*, and *Chlorella* to pigs weaned on a dry diet four to eight days of age (Yap *et al.*, 1982). Animals fed on the basal diet up to the 26th day gained weight at a rate not significantly different from those fed on alga diets. There was no sign of diarrhea, loss of appetite, toxicity, or of gross histopathological lesions of the gastro-intestinal tract. The authors suggest that at least 50% of the protein supplied by soybean meal (33% of total) can be replaced by these algae without adverse effects.

Pigs fed with the algal diet showed an improvement in weight gain as compared to the control evaluating the complete feeding period, pigs fed with 1 g algae kg⁻¹ diet tended to have the best feed efficiency, leaving room for the suggestion that lower levels of algae may have additional specific modes of action over being solely a protein replacement. In conclusion, nearly all the pig feeding studies indicated that microalgal biomass in general is a feed ingredient of acceptable nutritional quality and suited for rearing pigs. No serious difficulties in acceptability of algae were reported. Algae can replace conventional proteins like soybean meal or fish meal to a certain extent, the upper tolerable limit, however, has not been clearly demonstrated yet.

18.7.3 Ruminants

One of the studies on the utilization of fresh untreated algae for feeding ruminants has been reported from Bulgaria (Ganowski *et al.*, 1975): 11 of concentrated native *S. obliquus* ($2\text{--}3 \times 10^8$ cells ml⁻¹) were fed to calves over a period of three weeks. This feeding increased the contribution of the intestine in the digestive process without facilitating the digestion of the feed. Only minor differences were observed for digestibility between control and experimental animals.

In a study with drum-dried algae, beef steers were used as experimental animals. The rations tested were composed of alfalfa/hay (2:8), algae/hay (2:8) and algae/hay (4:6) (Hintz & Heitmann, 1967). Since the consumption of the ration containing the higher amount of algae was poor, for better comparison the intake of the other diets was restricted to the daily intake of the algal ration. The addition of algae at both levels did not decrease the digestibility of the crude protein (74%), but the feed with the higher algal

content showed a reduction of carbohydrate digestibility from 68 to 52%, in agreement with the results obtained in similar trials with sheep.

18.8 Therapeutic applications of algae

18.8.1 In humans

In recent years, processes for the isolation of a number of high value constituents from algae have been developed, including different pigments (carotene, phycobiliproteins), enzymes and radioactive labeled compounds, etc. There are many reports on the health benefits of consuming predominantly *Chlorella*, but also other microalgae. They are frequently translated from Japanese or Chinese into English and thus give the impression of lacking accuracy. Common literature on microalgae, especially the industrial references, has adopted many of the conclusions presented in the translations but has failed to represent the information in its proper context, without controls and often without giving valid or any references. Anecdotal reports, often lacking clarity and objectivity, are presented in a form, suggesting well-documented case histories.

By screening the algal literature for publications on the various uses of microalgae, the amount of papers published on the utilization of algae as therapeutics gives the impression that this application is most important. Several of the studies on this aspect have been performed in the Far East where many algae have been, or are still used in folk remedies. The active constituents are generally unknown. It is worth noting that whereas there are numerous publications on therapeutic properties of marine algae, only a very limited information is available on similar applications of freshwater microalgae.

In the following, selected reports on therapeutic uses of algae are summarized, showing the multiplicity of their application. It should be stressed that in several cases the beneficial effects were very small and that rigorous scientific controls were seemingly not applied. In addition, it is often difficult to attribute the described therapeutic effect clearly to the action of the microalgae used. On the other hand, the scarcity of warranted information does not preclude the possibility that certain microalgal species may possess properties, which are of distinct therapeutic value.

In one of the first publication in that context, pharmaceutical preparations containing *Spirulina* (whole algae or extracts) as an active ingredient were reported to accelerate the cicatrization of wounds (Clement *et al.*, 1967b). Treatment was effected with creams, ointments, solutions, and suspensions. Other reports showed that *Spirulina* and its enzymatic hydrolysates promote skin metabolism and prevent keratinization. In Czechoslovakia the effect of ointments mixed with 20% of alcoholic extracts of *S. obliquus* was tested on 109 patients suffering from trophic and varicose ulcers, burns, non-healing wounds, or eczema (Safar, 1975): about 90% of the persons were healed and in 7% an improvement could be observed while out of 112 control persons, suffering from the same ailments and treated with placebo, only one person improved. The stimulating effect of

the algal ointment on granulation and epithel formation was attributed to the chlorophylls, the carotenoids and the B-vitamins present in the preparation.

As already mentioned, *Spirulina* is a valuable source of linolenic acid, which cannot be synthesized by animals or humans. This essential fatty acid has been connected with the stimulation of prostaglandin synthesis. In strict chemical sense, the term prostaglandin refers to the derivates of the di-homo-linoleic acid, arachidonic acid and eicosapentaenoic acid, which are the precursors of mono-, bis-, and trienoic prostaglandins. Prostaglandin E2 is formed from dietary linoleic acid, which enzymatically is converted to linolenic acid and in turn to di-homo-linolenic acid (8,11,14 eicosatrienoic acid). Arachidonic acid, which actually is a metabolic product of linoleic acid, can be converted to prostaglandin E2.

Several investigations reported that a variety of non-specific, active immunostimulants can affect the growth of either spontaneous or transplanted tumors in animals or prolong the survival of cancer patients. Immunostimulants exhibiting such effects include various synthetic products, bacteria or bacterial products. In a study, performed in Japan, an antitumor activity of *C. vulgaris* was examined (Tanaka *et al.*, 1984): the growth of methyloanthrene-induced fibrosarcomas in a syngeneic or semisyngeneic host was inhibited by injection of a hot water extract of *C. vulgaris* into the tumor or into subcutaneous tissue near the regional lymph nodes. Both T-cells and macrophages appear to participate in the antitumor effect of the algal extract.

Marine alga produce bacteria-static compounds, e.g. acrylic acid, which inhibits the growth of Gram positive and, to a lesser extent, Gram negative microorganisms. Phenol derivates, frequent in macroalgae as for instance aplysiatoxins, which are known for their antimicrobial activity, have also been identified in microalgae. Certain fatty acids have been attributed to the antibiotic activity of a compound isolated from *Chlorella* called chlorellin. Also eicosapentaenoic acid, which is synthesized by some algae, shows antibiotic properties.

Experimental studies in animal models have demonstrated an inhibitory effect of *Spirulina* on oral carcinogenesis. Mathews *et al.* evaluated the chemo preventive activity of *S. fusiformis*, an algal strain isolated in India, at a ration of 1 g day⁻¹ for 12 months to investigate any reversing effect of oral leukoplakia in patients chewing tobacco, a rather widespread habit in India (Mathew *et al.*, 1995). Complete regression of lesions was observed in 20 of the 44 (45%) subjects supplemented with alga, compared with 3 of 43 (7%) in the placebo group. When stratified by type of leukoplakia, the response was more pronounced in homogeneous lesions. Complete regression was seen in 16 of 28 (57%) subjects with homogeneous leukoplakia, 2 of 8 with erythroplakia, 2 of 4 with verrucous leukoplakia, and none with ulcerated and nodular lesions. Within one year of discontinuing supplements, 9 of 20 (45%) complete responders developed recurrent lesions. Supplementation with alga did not result in increased serum concentration of retinol or β-carotene, nor was it associated with toxicity or any other harmful side effect.

18.8.2 Therapeutic studies with algae or algal extracts in animals or cell cultures

A variety of different studies have been performed in recent years to demonstrate therapeutic potentials in a broad sense on different algae. For these *in vivo* and *in vitro* studies, whole algal cells as well as extracts or isolated components were used.

The following represents an overview on the spectrum of investigations which demonstrate modern trends. It should be stressed that several of the findings mentioned here have not been reconfirmed thus mentioning their beneficial effects does not implicate clinical application.

As part of a program of the US National Cancer Institute aiming at discovering new antitumor and antiviral agents in natural sources, extracts of various cyanobacteria were tested on human cells for their protective property against HIV-1 infections (Gustafson *et al.*, 1989). A number of extracts were found to be remarkably active in protecting human lymphoblastoid T-cells from the cytopathic effect of HIV infection. Active agents, consisting of sulfolipids with different fatty acid esters were isolated from *Lyngbya lagerheimii* and *Phormidium tenue*, which were active over a wide range ($1\text{--}100 \mu\text{g ml}^{-1}$). All of the sulfolipids tested had similar levels of activity, suggesting that acyl-chain length and degree of instauration in the tested range do not affect their potency. Structurally related acyl glycerols and complex lipids did not protect against HIV-1 infections. Such sulfonic acid containing lipids were already described by Benson *et al.* (1959).

Members of this structural class are commonly referred to as sulfoquinovosyl diacylglycerols that are structural components of chloroplast membranes, commonly found in algae and higher plants. Structurally related acyl glycerols, complex lipids, detergents and simple sulfonic acid derivates did not show any protective potential. Additional cultured cyanobacterial extracts with inhibitory properties were also found in *P. cebennse*, *Oscillatoria raciborskii*, *Scytonema burmanicum*, *Calothrix elenkinii* and *Anabaena variabilis*.

These non-nucleoside compounds are presently investigated. Some include a protein called cyanovirin-N which was initially isolated from an aqueous cellular extract from *N. ellipsosporum* and was identified as a 101 amino acid antiviral peptide. Low nM concentrations of this compound prevent the *in vitro* replication and cytopathicity of primate retroviruses, including SIV and various isolates of HIV-1 and HIV-2. Cyanovirin mediates these antiviral effects through apparently conserved interactions with the viral envelope glycoprotein. The US National Cancer Institute selected this agent for pre-clinical development as a potential prophylactic viricide (Boyd, 1997).

Serum free culture of mammalian cell lines has become an effective method in *in vitro* cellular and developmental biology and for the production of monoclonal antibodies and interferons. The cultivation of these cells requires complex culture media, containing different growth promoting factors such as hormones of animal origin. Earlier studies have indicated that at least three factors, which promote the growth of hybridoma, lymphocyte, and tumor cells in serum free media, could be isolated from the extracts of the

thermophilic cyanobacteria *Synechococcus elongatus* and *Spirulina subsalsa* (Shinohara *et al.*, 1986).

More recent studies revealed that some of these growth promoting substances in the dialyzate from *Synechococcus* are phycobiliproteins: phyco-cyanin and allophycocyanin. The activity of allophycocyanin was found to be higher than that of phyco-cyanin. In addition, commercially available phyco-cyanin from *Spirulina platensis*, which may be a mixture of both pigments, also showed growth promoting activity suggesting that the phycobiliproteins from all kinds of cyanobacteria can be a growth factor for such cell lines. The chromophore of phycobiliproteins is a tetrapyrrol, bound non-covalently to polypeptides, resembling the structures of bile pigments. If these chromophores are the active principle is not yet known, there may be also a possibility that biliverdin or another phycobiliprotein such as phycoerythrin has similar growth promoting activities (Hayashi *et al.*, 1994; Qureshi & Ali, 1996).

The effect of feeding *Spirulina* at different levels (0, 0.01, 0.1, 1, and 10 g kg⁻¹) to white leghorns and broiler chicks was investigated (Qureshi *et al.*, 1996). Chicks in the highest *Spirulina* group had a higher phytohemagglutinin (PHA)-mediated lymphocyte proliferation compared to controls. Macrophages from both strains of the 10 g group had higher phagocytic activity than the 0 g group, indicating *Spirulina* supplementation of feed may enhance the disease resistance potential in chickens.

Phagocytotic *in vitro* studies were also performed in mice, which were fed for ten weeks with *Spirulina*-supplemented (10%) diet. Peritoneal macrophages showed an increase in the percentage of phagocytotic cells (Hayashi *et al.*, 1994, 1998).

Several studies on whole cyanobacteria as well as water extracts in humans, mice, rats, cats, and chickens have reported effects on phagocytosis, NK-cell function and inflammation. Further studies suggest that cyanobacteria may inhibit mast-cell mediated type I allergic reactions and even anaphylactic reactions when applied intraperitoneally to rats. It was found that *Spirulina* extracts effected decreased anaphylactic mortality, local allergic reactions were inhibited, and serum histamine levels were decreased (Kim *et al.*, 1998).

Studies in humans demonstrated that *Aphanizomenon* was able to trigger within two hours the migration of nearly 40% of the circulating NK cells. In the same study, the same alga was also shown to stimulate the mobilization of *T*- and *B*-lymphocytes (Jensen *et al.*, 2000).

Other studies using water-soluble extracts of cyanobacteria have found a novel sulfated polysaccharide, calcium spirulan (Ca-SP), to be an antiviral agent (Hayashi *et al.*, 1996). This compound was composed of rhamnose, ribose, mannose, fructose, galactose, xylose, glucose, glucoronic acid, galacturonic acid, sulfate, and calcium, whereby a chelating action of the calcium with sulfate groups was suggested to be indispensable to the antiviral effect. It appears to selectively inhibit the penetration of enveloped viruses into host cells, thereby preventing their replication. The effect was described for many different viruses like Herpes simplex virus type 1, human cytomegalovirus, measles virus, mumps virus, influenza A virus, and even HIV-1.

A glycoprotein prepared from *C. vulgaris* culture supernatant exhibited protective activities against tumor metastasis and 5-fluorouracil-induced immunosuppression in mice. In addition, the animals were exposed to stress, under which the numbers in especially CD4⁺ and CD8⁺ populations of thymocytes decreased, whereas apoptotic cells reciprocally increased. Oral application of the glycoprotein prevented significantly the apoptosis of thymocytes and suppressed the increase in serum corticosterone level in the stressed mice, indicating the *Chlorella* extract maintains homeostasis during external environmental changes.

18.8.3 β -carotene

One of the modern attempts to popularize algae is their promotion as a valuable source of carotenoids. Carotenoids are mostly used as natural food color, as additive for animal feed to enrich the color of their products (egg yolk, poultry, and fish meat), or to enhance their health or fertility. Among the over 400 known carotenoids, only very few are used for these purposes commercially, e.g. β -carotene, lutein, zeaxanthin, astaxanthin, lycopene, and bixin. The nutritional and therapeutic relevance of certain carotenoids is their ability to act as provitamin A, i.e. be converted into vitamin A. As the demand especially for carotene is higher than the production from natural sources, synthetically produced carotenoids are offered by various companies. β -carotene is the only carotenoid, which has the potential to form two molecules of vitamin A (retinol). In humans, the conversion of carotene into vitamin A occurs in the small intestine, where both carotene and vitamin A are absorbed and found in plasma, tissue, and organs of the body.

For practical purposes it is assumed that β -carotene comprises all of the carotenoids, which can be converted to vitamin A. The average daily carotene intake is about 1.5 mg, equivalent to 250 retinol equivalents or 2500 IU of vitamin A. It is further assumed that the overall utilization of carotene as a source of vitamin A is one-sixth-of that of retinol. The unicellular halotolerant alga *D. bardawil* was shown to contain high concentrations of β -carotene, composed of the all-*trans* and the 9-*cis* isomer (see Chapter 22).

Abundant literature is available regarding the utilization, nutritional value, and toxicology of carotenoids in general, and derived from *Dunaliella* in particular, tested *in vivo* and *in vitro* with different animal species as well as humans.

Carotenoids, by their quenching action on reactive oxygen species, carry intrinsic anti-inflammatory properties. This is also true for the water-soluble phycobilin pigments found in cyanobacteria, as will be detailed later. It is accepted today that the natural, *Dunaliella*-derived, *cis*-isomer of β -carotene – due to its different physico-chemical features – is nutritionally superior to the (synthetic) all-*trans* form.

18.8.3.1 *In vivo* and *in vitro* investigations with animals

Since *Dunaliella* contains high amounts of the two β -carotene isomers, i.e. all-*trans* and 9-*cis* β -carotene, tests were performed to study the effects of

a range of synthetic and natural antioxidants on the β -carotene content and isomers formed during spray drying of the alga (Orset *et al.*, 1999). Almost no losses were observed for the untreated sample and the samples containing butylated hydroxytoluene and tert-butylhydroquinone, while tocopherol-based antioxidants resulted in degradation of about 50–70% of β -carotene during the drying process. All dried samples proved to be unstable during exposure to light and air, the *cis*-isomer being significantly more unstable. The quinone, however, was successful in reducing relative losses during storage in the dark. Therefore, a dominant photo-degradative mechanism has to be assumed for the loss of the 9-*cis* isomer.

There is still an ongoing discussion, whether synthetic or natural β -carotene, extracted from *Dunaliella*, is of higher nutritional value and more effective, if at all, on tumor prevention. In order to evaluate the practical value of natural β -carotene and to elucidate the apparent discrepancy between epidemiological observations and intervention trials on its role in tumor prevention, the genotoxicity and antigen toxicity of natural β -carotene and synthetic isomers were studied comparatively, using chromosome aberration analysis and the micronucleus test in human lymphocytes *in vitro* (Xue *et al.*, 1998). The extracted crystalline carotene consisted of 70% all-*trans* and 8% 9-*cis*, the oil preparation of 40% all-*trans* and 39% 9-*cis*. The synthetic preparation consisted of about 97% all-*trans*. Finally, a mixture was tested consisting of 74% crystals of synthetic β -carotene and 26% of natural crystals. No genotoxicity was observed at concentration of 1–30 $\mu\text{g ml}^{-1}$ natural β -carotene crystals, but this concentration inhibited significantly X-ray-induced *in vitro* micronucleus formation in human lymphocytes, while the same concentration of carotene in oil extract was effective against radiation-induced and spontaneous micronucleus formation. No influence on spontaneous chromosome aberration was found with this preparation nor with the mixture of synthetic and natural β -carotene. The same concentration of synthetic carotene crystals induced a dose-dependent increase in micronucleus frequency, and also inhibited radiation-induced micronucleus formation. The carotene mixture was more effective against mitomycin-C-induced chromosome abberation than the natural oil preparation, suggesting the 9-*cis* isomer might play a critical role in the genotoxicity and antigenotoxicity of both the synthetic preparation and the mixture of natural and synthetic. The genotoxic activity of the synthetic form might be involved in carcinogenesis, while the natural form could be of practical value in tumor prevention and supplementary treatment.

To evaluate the nutritive efficacy of the all-*trans* β -carotene versus its 9-*cis* isomer, various trials have been conducted with animals. In one trial, one-day-old chicks and seven-week-old male rats were fed with diets supplemented with synthetic all-*trans* β -carotene or dried *Dunaliella* at equivalent levels of β -carotene. Liver analysis indicated that both species showed at least tenfold higher accumulation of the algal β -carotene isomer mixture than of the synthetic all-*trans* form. The ratio was similar to or higher than that present in the alga. The preferable accumulation of the natural isomer mixture suggests that attention should be paid to the source of β -carotene when testing its efficacy.

In the search for antioxidative and anticarcinogenic substances, the effect of repeated ingestion of spray-dried *Dunaliella* on mammary growth and endocrine parameters were examined in mice and compared with a group of animals fed with normal standard diet adequate in vitamin A and another group fed with the standard diet supplemented with synthetic all-*trans* β-carotene (Ben-Amotz *et al.*, 1989). The algae-containing diet showed no deleterious side effects on mammary gland and uterin growth nor on mammotropic hormone secretion; puberty and body growth were accelerated by *Dunaliella* (i.e. natural β-carotene) compared with the synthetic β-carotene.

In another study, the antioxidative efficiency of 9-*cis* β-carotene, extracted from *Dunaliella*, all-*trans* β-carotene and a mixture of both (ratio 1:2.3) at a concentration of 200 μM were compared in an experimental system consisting of 80 mM methyl-linoleate and 4 mM azo-bis-2,2'-dimethyl-valeronitrile as a free radical generating agent, by measuring the formation of methyl lineolate hydroperoxides (Levin & Mokady, 1994). Analysis suggested the *cis*-isomer has a higher antioxidant potency than the all-*trans* form and protects the methyl lineolate, as well as the all-*trans* isomer from oxidation.

The effect of β-carotene on arteriosclerosis in hypercholesterolemic rabbits was tested by Shaish *et al.* (1995) who fed a high cholesterol diet without or with supplementation with 1% probucol, 0.01% vitamin E, 0.01% all-*trans* β-carotene or 0.01% 9-*cis* β-carotene. Probucol protected low density lipoprotein (LDL) from oxidation and inhibited lesion formation. Vitamin E modestly inhibited LDL oxidation but did not prevent arteriosclerosis. While the β-carotene was not detectable in and had no effect on LDL, the all-*trans* isomer inhibited lesion formation to the same extent as probucol, indicating that the effect of this carotene on arteriosclerosis has to be separated from an action on LDL.

To study whether simultaneous supplementation of sodium cholate and β-carotene to a diet enhanced the accumulation of β-carotene in mice, male mice were fed for two weeks with either a basal diet or a diet containing *D. bardawil* β-carotene 50 mg 100 g⁻¹ that was or was not supplemented with sodium cholate (0.25 g 100 g⁻¹) (Umegaki *et al.*, 1995). The concentrations of β-carotene in liver and plasma were approximately five and ten times higher, respectively, in the mice fed with the β-carotene diet with sodium cholate, than in those fed with the β-carotene diet without sodium cholate. No β-carotene was detectable in the liver or plasma of mice fed either basal diet. It was concluded that simultaneous supplementation of sodium cholate and β-carotene to a diet markedly enhances the accumulation of β-carotene.

18.8.3.2 In vivo and in vitro investigations with humans

The uptake of α-, β-carotene and oxy-carotenoids from a basal diet supplemented with synthetic β-carotene or dried *Dunaliella* powder (40 mg carotene d⁻¹) was studied in humans over a period of two weeks (Ben-Amotz & Levy, 1996). Serum analysis detected mainly oxy-carotenoid, and to a lesser extent all-*trans* β-carotene and β-carotene, but not the 9-*cis* isomer. The preferential serum absorption of all-*trans* carotene over the *cis* isomer

suggests that the latter acts as an *in vivo* lipophilic antioxidant more efficiently than the all-*trans* form.

Since accelerated arteriosclerosis is common in patients with diabetes mellitus, which on the other hand may be linked to increased lipid peroxidation, the effect of a dietary *Dunaliella*-derived isomer mixture of β -carotene supplementation (60 mg daily for three weeks) on the oxidation of LDL obtained from diabetic patients and normal controls were compared (Levy *et al.*, 2000). The increased susceptibility to oxidation of LDL in diabetic patients is associated with an abnormal LDL lipid composition and antioxidant content. The carotene dietary supplementation normalizes the enhanced LDL oxidation and hence may be of importance in delaying accelerated development of arteriosclerosis in these patients.

Due to controversy regarding the beneficial effect of antioxidants on asthma, the acute effects of β -carotene derived from *Dunaliella* was assessed in patients with exercise-induced asthma. In a double-blind study, the patients received a placebo or 64 mg d⁻¹ β -carotene (Neumann *et al.*, 1999). All patients given placebo showed a significant post-exercise reduction of more than 15% in their forced expiratory volume, while of the 38 patients who received the daily dose of β -carotene for one week, 20 (53%) were protected against asthma, indicating a daily dose of *Dunaliella*-carotene exerts a protective effect, most probably through an *in vivo* antioxidative effect. However, it remains open, whether one special carotene isomer, or the mixture of several, caused the beneficial effect.

Blood serum analysis for the concentration of β -carotene isomers after the ingestion of natural β -carotene obtained from *Dunaliella* was performed with children exposed to radiation from the nuclear accident at Chernobyl and who showed increased levels of conjugated dienes indicating increased levels of oxidation of blood lipids (Ben-Amotz *et al.*, 1998). A diurnal supplementation of 40 mg of an equal mixture of 9-*cis* and all-*trans* β -carotene in a capsulated form of *Dunaliella* powder was given over a period of three months. After that period, the level of oxidized dienes had decreased without changes in the overall level of carotenoids. Analysis for carotenoids in the blood after that period detected mainly oxy-carotenoids, and to a lesser extent, all-*trans* β -carotene, β -carotene, but no 9-*cis* isomer. The authors suggest that the irradiation during the accident increased the susceptibility of lipids to oxidation and that natural β -carotene may act as antioxidant or radio-protector.

Long-term administration of β -carotene preparation from *Dunaliella* (60 mg d⁻¹, all-*trans*: 9-*cis* ratio 1:1) was studied in healthy male volunteers by measuring the carotene concentrations in plasma, red blood cells, platelets and mononuclear cells (Morinobu *et al.*, 1994). For plasma, the level of all-*trans* carotene increased fourfold the baseline. The basal level of the 9-*cis* isomer was only one-tenth of the all-*trans* form and increased threefold. The bioavailability of the natural β -carotene was preferentially for the all-*trans* form, although a small amount of the 9-*cis* form was detected in the plasma.

Large-scale clinical trials tested the effects of supplemental β -carotene on the risk for cancer. The populations involved were about 18 000 high risk smokers in the US, male asbestos workers, and male and female heavy

smokers from Finland (β -carotene and retinol efficacy trial = CARET), a randomized, placebo-controlled study, the α -tocopherol β -carotene (ATBC) trial with Finnish male heavy smokers, and a study with US male physicians, 11% of whom were then current smokers (physician's health study).

The CARET study tested the effect of the combination of β -carotene (30 mg) and retinyl palmitate (25.000 IU) daily. The trials ended prematurely due to unexpected findings. All three trials concluded that β -carotene provided no protection against lung cancer, on the contrary, two trials found a 46% increased lung cancer mortality and a 26% increased cardiovascular mortality for those subjects given β -carotene compared to those that were not. Hence, it was concluded that the protective effect of antioxidants is not as great as hoped.

Summarizing, it is still too early to clearly attribute a therapeutic chemopreventive anti-cancer effect to microalgae based on their content of carotenoids or phycocyanin. We have to await the results of the ongoing clinical studies and others, which may also be commenced in the near future, before it can be decided whether the above pigments will play an important pharmaceutical role for use in large populations.

18.8.4 Phycobiliproteins

The water-soluble phycobiliproteins, highly fluorescent photoreceptor algal pigments with a linear tetrapyrrole as the prosthetic group, are gaining increasing recognition as valuable ingredients. They are composed of a bile pigment or phycobilin and an apoprotein. Phycobiliproteins are deeply colored and classified according to UV-vis absorption maxima as phycocyanins (blue pigment), phycoerythrins (red pigment), and allophycocyanins (pale blue pigment), whereas the primarily potential for commercial utilization of phycobiliproteins seems to be as natural dyes, an increasing number of investigations report on health promoting properties and a broad range of pharmaceutical applications.

The major source of phycocyanins is *Spirulina*, in which they may constitute more than 20% of dry weight. In addition to *Spirulina*, the most suitable source for the extraction of phycobiliproteins is the rhodophycea *Porphyridium*. One of the first and probably still most important application of phycocyanin is its use as food pigment, replacing currently synthetic pigments. The product is generally an odorless blue powder, dissolved in water it is brilliant with a faint reddish fluorescence. It is stable from pH 4.5 to 8.0 and up to 60°C, but exhibits poor light stability. Phycocyanin, extracted with organic solvents, denatured and finally precipitated is used as non-water-soluble pigment in cosmetics.

Additional applications of phycocyanins can be found in medical diagnostics as phycofluors highly sensitive fluorescent reagents, i.e. phycobiliproteins bound to biological active molecules as immunoglobulin, biotin, or proteins used, e.g. for the labeling of antibodies applied in clinical tests such as immunofluorescence or flow cytometry (Glazer & Stryer, 1984). Other reports describe the application of phycocyanin in clinical pharmacology as an antioxidant protector of human cells against hemolysis by peroxyxyl

radicals. Indeed, an increasing number of reports describing possible pharmaceutical applications of phycocyanin, mainly as antioxidant and anti-inflammatory agent are described in various *in vitro* and *in vivo* experimental models. In addition, a hepato-protective effect against chemical-mediated toxicity could be demonstrated in rats.

These antioxidant and scavenging properties were studied in a series of *in vitro* experiments by evaluating (among other parameters) the effect of phycocyanin on radical (OH^\cdot , O_2^\cdot and RO^\cdot)-induced chemi-luminescence, zymosan-activated leucocytes, and liver microsomal lipid peroxidation, as well as *in vivo* with inflamed mouse paws. The results showed that phycocyanin scavenges OH^\cdot and RO^\cdot radicals, inhibits liver microsomal lipid peroxidation as well as edema on mouse paws (Romay & Gonzales, 2000).

Romay and co-workers investigated the antiinflammatory effect of C-phycocyanin with acetic acid-induced colitis in rats (Romay *et al.*, 1998). *Per os* application of phycocyanin reduced the myeloperoxidase activity. In addition, histopathological studies showed an inhibition in inflammatory cell infiltration and to a certain extent reduction in colonic damage in the treated animals, perhaps due to the antioxidative and scavenging properties of phycocyanin against reactive oxygen species in the anti colitic effect (Gonzales *et al.*, 1999). In addition to these findings, the authors also reported on studies, providing evidence of the dose-dependent protective effect of phycocyanin against hemolysis induced by peroxy radicals in human erythrocytes, was about 20 times more efficient than ascorbic acid, a well-known antioxidant (Romay & Gonzales, 2000). This potential of C-phycocyanin as peroxy radical scavenger was also reported by Bhat & Madyastha (2000).

Likewise, Vadiraja *et al.* (1998) observed a hepato-protective effect of C-phycocyanin, given intraperitoneal to rats as a single dose of 200 mg kg^{-1} , prior to carbon tetrachloride-mediated hepatotoxicity. Transaminase values were almost equal to control values and losses of microsomal cytochrome P450 was significantly reduced; indicating phycocyanin provides protection to liver enzymes (Vadiraja *et al.*, 1998).

18.8.5 Chlorophyll

Decomposition products of chlorophyll (pheophytins, pyropheophytin and pheophorbide) may be hazardous, causing skin irritations. There are, however, reports which attribute positive effects potencies to chlorophyll derivatives, in that these compounds exhibit identical antimutagenic effects towards 3-methylcholanthrene, suggesting that the porphyrin nucleus may directly complex with the mutagen (Chernomorsky *et al.*, 1999). The action of N'-nitro-N'nitrosoguanidine depends upon structural differences between the chlorophyll derivatives, i.e. lower activities were found for the phytol-containing pheophytin and pyropheophytene. Testing the cytotoxicity of the derivatives against tumor cells revealed that the cellular uptake and inhibition of myeloma cell multiplicity was greater for pheophorbide than for pheophytin. Hence, the indication that food sources yielding chlorophyll derivatives may play a role in cancer prevention.

18.9 Cholesterol-lowering properties of algae

Among the prospective therapeutical potencies of microalgae, their possible cholesterol-lowering properties are repeatedly described and tested. Some of the findings, mostly obtained in animal experiments, are given in an attempt to illustrate the present state-of-art.

Heart diseases are often associated with high plasma cholesterol levels and hypertension. Some marine algae such as *Cystoseira barbata*, *Fucus gardnerii* and *Phyllophora nervosa* have been shown to lower plasma cholesterol levels, and in some cases the active compounds have been identified. Unsaponifiable sterols and unsaturated fatty acids, for instance, exhibited hypocholesterolemic activities. The effects of consuming marine green algae (*Monostroma nitidum*, *Ulva pertusa*, *Enteromorpha compressa*, and *E. intestinalis*) on cholesterol metabolism have been studied in rats. All algae were found to lower significantly the cholesterol levels, most probably due to betaines present in these algae.

During the performance of nutritional studies with *S. obliquus* on rats it was observed by Rolle & Pabst (1980a) that animals fed algae containing diets showed lower cholesterol levels than the controls. The authors tested the cholesterol-reducing activity of drum-dried *Scenedesmus* as compared with controls fed with a standard diet. In animals fed six to eight weeks with standard diet enriched with 3% cholesterol, the average concentration of blood plasma cholesterol increased from 2.0 to 3.6 mmol⁻¹. In animals, receiving the cholesterol in a diet enriched with 20% algal powder, however, the plasma cholesterol level increased only slightly to 2.4–2.8 mmol⁻¹, whereas in animals fed with solely algae, the cholesterol concentration increased to 2.2 mmol⁻¹ only. The level of plasma triglycerides in animals fed with algae, with and without the addition of cholesterol, was lower than that of the controls fed with algal-free diets; the algal-enriched diet prevented an excessive deposition of cholesterol in the liver.

In an additional trial, the same authors studied the effect of hydrophilic and lipophilic algal extracts and the remaining algal residues for their cholesterol-lowering properties (Rolle & Pabst, 1980b). The different fractions were obtained by hot water treatment and chloroform/methanol extraction. The algae extracted with water lowered plasma cholesterol levels; the content of plasma triglycerides in animals receiving the different fractions was reduced by 35–55% in nearly all groups. The cholesterol content in the liver was reduced up to 50% by untreated algal powder as well as by algal material extracted with water or organic solvents; the remaining algal residue after extraction showing the best effects. These observations indicate that the crude fibers of the algae represent the effective component which lowered the cholesterol level, an observation which is also known from marine algae. Similar experiments with *Scenedesmus* and *Spirulina* were reported from India, where also rats were employed to test a possible hypo-cholesterolemic effect of different diets, containing algae at 10% and 15% protein level and casein (10% protein) as control (Anusuya Devi & Venkataraman, 1983). After a feeding period

of six weeks, serum and liver cholesterol levels were analysed. The serum cholesterol level was highest in rats fed with casein, incorporation of *Scenedesmus* at both protein levels significantly lowered the serum and liver cholesterol levels. The cholesterol-lowering property of sun dried *Spirulina* was not as pronounced as that of drum-dried *Scenedesmus*. Since the constituents of both algae have not been evaluated separately, it is difficult to explain the lesser effect of *Spirulina*. Perhaps the higher carbohydrate and cellulose concentrations in *Scenedesmus* are the main causes of the cholesterol-lowering property observed with this alga in the animal feeding studies.

The assumption that in microalgae fibers are the major active components responsible for their cholesterol-lowering action (Nakaya *et al.*, 1988) could be substantiated by tests performed in Japan. Feeding the cyanobacterium *N. commune* or its fiber to rats, which were fed with cholesterol-enriched diets (Hori *et al.*, 1994) showed that of the dietary fibers, oxalate-oxalic acid soluble substances were most effective in lowering the cholesterol level in the serum; seemingly depressing elevation of LDL-cholesterol.

The effect of whole *Chlorella* powder in cholesterol-fed rabbits was tested in Japan: addition of 1% *Chlorella* in the diet suppressed the increase of total- and β -cholesterol in the animals compared with animals fed with algal-free diet (Sano & Tanaka, 1987). Clofibrate used as positive control did not show any inhibitory effect. There are reports from China on similar effects of *Chlorella* on serum cholesterol levels in rats. Recent studies with *Spirulina* support the earlier reports of a cholesterol-lowering principle of this alga (Torres-Duran *et al.*, 1998). It was observed that after CCl₄ treatment, liver triacylglycerols were significantly lower in rats on a diet supplemented with 5% *Spirulina* compared to the control. Furthermore, the CCl₄-induced increased liver cholesterol levels were not observed in rats fed with the *Spirulina* diet.

Although these results are still preliminary, they point in a direction, where a therapeutic application of algae might be meaningful.

The studies described revealed several novel compounds isolated from algae and a wealth of novel applications, together quite frequently with the results of basic bioactivity testing. However, more often than not, that is where the investigation ends and much of this has to do with the economics of pharmaceutical development. Considerable efforts have gone into such physico-chemical endeavors, but payoff has been absent. It is pertinent, therefore, to ask if microalgae would ever become an economically viable, commercial source of pharmaceuticals. It seems quite plausible that an algal compound identified as pharmacologically useful would be turned over to the synthetic chemists to provide an alternate route of supply.

18.10 Nutritional quality standard

Commercially produced microalgae have been marketed for several years, mostly in industrialized countries, where consumers are responsive to quality

information given with the product and where education on the nutritional properties of algae helps to overcome prejudice. The introduction of microalgae and their products in developing countries is afflicted with various complications, because it may affect conservative ethnic factors, including religious and socio-economic aspects.

The reluctance to use algal or algal products among potential consumers is also caused by public-health difficulties concerning safety of such commodities, highlighted by lack of official legislative regulations, guidelines, and standards on the production and composition of algae-based products.

There exist internationally recommended testing programs, which have to or should be performed for unconventional foodstuffs such as SCP. It seems useful to have the following specifications to become available:

- Details on technical properties such as mode of production, drying, and processing of the algae in order to assure uniformity of the product, as well as specifications on the incorporation of algae or algae-based constituents as supplement into food and feed formulations.
- Details on safety regulations should be given in order to assure freedom from hazardous physical or chemical substances, from toxicological effects, or pathogenic microorganisms, which are harmful to humans as well as to animals.
- Data on nutritional value, mainly with regard to overall chemical composition (protein, carbohydrates, lipids, vitamins, minerals, etc.). Information on compatibility, with respect to physiological, nutritional, or aesthetic factors.

There are only very few countries that have so far stipulated legislative standards for *Spirulina*. It is not known whether official regulations also exist for other types of microalgae i.e. *Chlorella*, *Dunaliella*, etc. These official requirements as well as suggested quality criteria found in the literature and elsewhere are summarized in Table 18.7.

Almost no adverse symptoms have been revealed so far in connection with the consumption of microalgae and unwanted side effects appear to be extremely rare. As long as specific regulations are absent, it should be an unwritten agreement among the producers of microalgae and relevant algae-containing products that these shall meet relevant food quality and safety standards (in the US for instance, The Natural Products Quality Assurance Alliance or The Natural Nutritional Foods Association) and also shall follow the appropriate *good manufacture practice* (GMP) guidelines that cover all aspects of food processing (Jassby, 1988). In addition to GMP, national regulations, as for instance, for milk products and infant food can be applied, which in many cases cover all essential hygienic, microbial, and toxicological aspects. In addition, producers of ready-made products should provide nutrient statements on labels based upon both bulk algal powder analysis and nutrient changes due to tableting, bottling and shelflife. In most countries, microalgae can be legally marketed as long as the product is labeled accurately and contains no contaminated or adulterated substances.

Table 18.7. Microbiological and related quality standards for algae (for heavy metals see Table 11.5).

	France (Becker, 1994)	Sweden (Jassby, 1988)	Japan (Jassby, 1988)	USA (Jassby, 1988)	India (Indian Standard, 1990)	Recommended (Chernomorsky et al., 1999)
Crude protein (%)	>45.0				>55.0	
Total ash (%)					9.0	
Acid insoluble ash (%)					0.5	
Moisture (%)					<9.0	
Pheophorbide						<7.0
Standard plate count (number $\times 10^6$ /g)	<0.1		<10.0			<1.2 mg/g
Mold (number/g)			<1000.0			<0.005
Yeast (number/g)						<100.0
Coliforms (number/g)	<10.0		<100.0			<40.0
<i>Salmonella</i> sp.	Negative		Negative			Negative
<i>Staphylococcus</i> sp. (number/g)	<100.0		<100.0			Negative
Filth (insect fragments) (number/10 g)					<30.0	<500.0
Rodent hair (number/150 g)					<1.5	<1.5

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19 Microalgae for Aquaculture

The Current Global Situation and Future Trends

Arnaud Muller-Feuga

19.1 Introduction – aquaculture – a rapidly developing enterprise

Microalgae play a vital role in the rearing of aquatic animals like mollusks, shrimp, and fish, and are of strategic interest for aquaculture. This work reviews production systems of microalgae for aquaculture (e.g. Benemann, 1992; Muller-Feuga, 2000), partially reproduced and updated here (with kind permission from Kluwer Academic Publishers). All the fisheries and aquaculture production statistics quoted were obtained from the United Nations Food and Agriculture Organization (FAO, 2000).

Wild phytoplankton is at the base of the entire aquatic food chain, supporting the production of renewable resources by some 100×10^6 t of fish per year. Overall annual ocean primary production is set at 10^{11} t DW (Longhurst *et al.*, 1995; Pauly & Christensen, 1995). Microalgae's main applications for aquaculture are related to nutrition, being used fresh – as a sole component or as a food additive to basic nutrients – for coloring the flesh of salmonids and for other biological activities. This report deals principally with the nutritional role of microalgae in aquaculture.

In 1999, world aquaculture produced 43×10^6 t of plants and animals, mainly as human foodstuffs, portraying outstanding growth rates (9.1% per year from 1989 to 1999). Over-harvesting and fishing of wild populations have reached critical thresholds, and aquaculture's contribution to human nutrition is constantly increasing, playing an ever-increasing role in relieving nutritional deprivation. Freshwater fish represents 43% of total world aquaculture production, the remainder divided among: mollusks 24%, aquatic plants 22%, diadromous fish 5%, crustaceans 4%, and marine fish 2% (Fig. 19.1).

Aquatic animals used by humans for food are rarely herbivorous at the adult stage. Only filtering mollusks and a few other animals are true plankton feeders throughout their lifetime. Other farmed animals are carnivorous from their post-larval stage, or omnivorous at best. Microalgae are required for

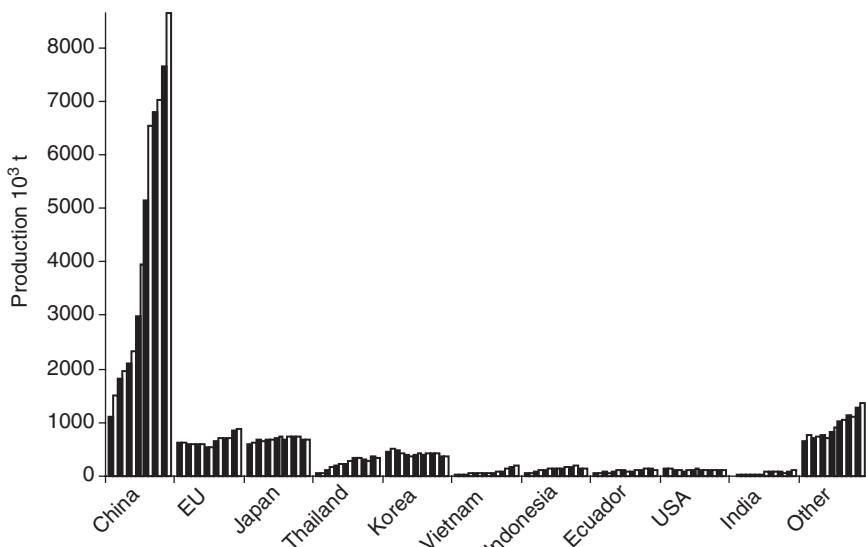


Fig. 19.1. Marine mollusks, crustaceans, and fish productions of the world's top ten producing countries (or groups of countries), and their variation from 1986 to 1999 (each vertical bar corresponds to a year). Source: FAO, 2000. Reprinted with permission from Kluwer Academic Publishers (*J. Appl. Phycol.*).

larvae nutrition during a brief period, either for direct consumption, in the case of mollusks and peneid shrimp, or indirectly as food for the live prey fed to small-larvae fish. In all these cases, the post-larvae are hatched, bred, and raised by specialized establishments known as hatcheries. The present trend is to avoid using microalgae because they require much experience to produce (Muller-Feuga *et al.*, 2003a); yet it has been established in numerous cases that microalgae are vital for the artificial reproduction of mollusks, whereas their use is less critical for the reproduction of peneid shrimp, and could be altogether avoided for some species of fish. Examples are freshwater and diadromous fish like salmonids, whose eggs have sufficient reserves to hatch large larvae capable of feeding directly on dry particles.

Marine aquaculture of filtering mollusks, crustaceans (especially peneid shrimps), and small-larvae fish (like sea breams, turbot, and other flatfish), generally require microalgae, directly or indirectly, at least for larval feeding. The production of these animals represented 12.1×10^6 t in 1999 (28% of total aquaculture). Preliminary assessment of the microalgae requirements for post-larvae production, as well as estimation of the number of post-larvae required to achieve full production in the main categories follows:

19.2 Filtering mollusks

Filtering mollusks (10.1×10^6 t in 1999) are herbivorous and consume microalgae throughout their lives. These animals, however, are often suspension feeders, taking in plankton composed of living or dead, plant or animal particles. Those filtering mollusks are mainly oysters, clams, mussels, and

pectinids. Mollusk production is by far the highest among microalgae-consuming species, with an average increase of 8.5% per year over five years (Fig. 19.2).

From post-larval and sometimes from larval stages, this production generally relies on wild phytoplankton present in the natural water masses circulating around the livestock in the open medium. Farmers can simply expose livestock to circulating water masses to take advantage of the natural resource.

When, however, larvae and then post-larvae are produced in a hatchery, i.e. in artificial conditions, most penalizing natural hazards are eliminated, but fodder microalgae produced artificially must be added to meet the food requirements of larvae, post-larvae and even broodstock. The main microalgae species used for mollusk larvae rearing in hatcheries and their relative utilization frequency is shown in Table 19.1. Since mollusk larvae rearing techniques were developed in the 1960s, microalgae have remained the only food used. New solutions, e.g. yeast, bacteria, micro particles, slurry, paste, or dried and frozen microalgae have been explored (Robert & Trintignac, 1997), none being sufficiently advanced to date to provide an alternative to live microalgae.

Microalgae requirements differ for the various mollusk species, depending on whether they are for broodstock, larval, or post-larval rearing. The larval stages require high quality algae, though in small amounts, and only for a short time. Post-larvae accept lower quality algae, but remain sensitive to the proper biochemical composition and require amounts nearly a hundred times

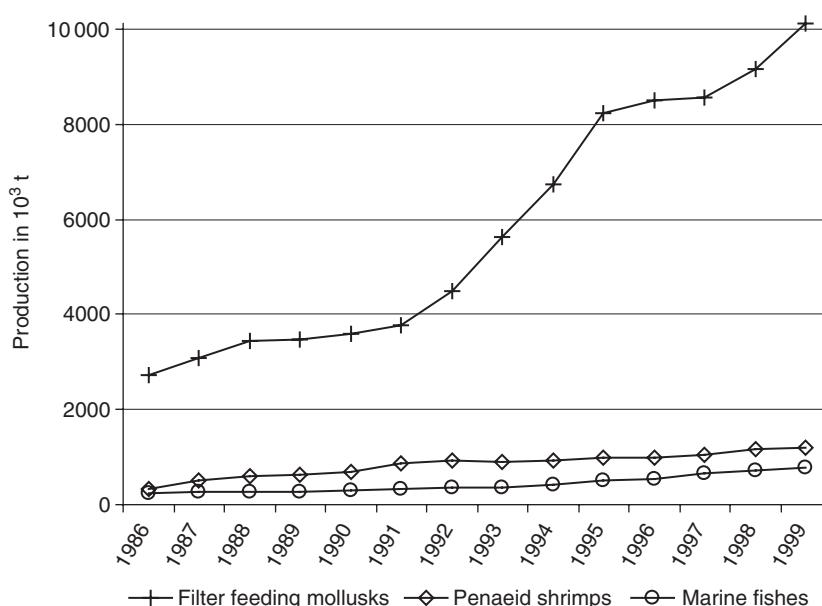


Fig. 19.2. Aquaculture productions of the main groups of marine animals that require microalgae for their post-larvae rearing, and their variation from 1986 to 1999. Source: FAO, 2000. Reprinted with permission from Kluwer Academic Publishers.

Table 19.1. Utilization frequency of microalgal species in mollusk hatcheries (from Robert & Trintignac, 1997). Reprinted with permission from Société Française de Malacologie (*Microalgues et Nutrition Larvaire en Ecloserie de Mollusques*).

Microalgal species	Class	Utilization frequency (%)		
		Walne (1970)	Lucas (1980)	Coutteau & Sorgeloos (1992)
<i>Chaetoceros calcitrans</i>	Bacillariophyceae	40	37.5	37
<i>C. gracilis</i>	Bacillariophyceae	*	*	53
<i>Skeletonema costatum</i>	Bacillariophyceae	20	12.5	14
<i>Phaeodactylum tricornutum</i>	Bacillariophyceae	50	12.5	5
<i>Thalassiosira pseudonana</i> , clone 3H	Bacillariophyceae	40	62.5	33
<i>Isochrysis galbana</i>	Prymnesiophyceae	80	75	19
<i>I. affinis galbana</i> (clone T-iso)	Prymnesiophyceae	20	0	72
<i>Pavlova lutheri</i>	Prymnesiophyceae	70	62.5	26
<i>Pyramimonas virginica</i>	Prasinophyceae	0	37.5	*
<i>Tetraselmis suecica</i>	Prasinophyceae	60	25	35
<i>Dunaliella</i> sp.	Chlorophyceae	0	25	9
<i>Nannochloropsis occulata</i>	Eustigmatophyceae	0	25	*

* No data.

greater. In contrast, the preparation of a broodstock for breeding requires both quality and quantity, but the number of animals is small. Although mass production of live microalgae in the hatchery has been mastered by some, in several cases it is subject to large quantitative and qualitative constraints.

As the animals grow larger, algae consumption increases as well, from 40 to 100 m³ of 10⁶ cells ml⁻¹ in extensive culture per 10⁶ juveniles, 6–12 mm. The quantitative microalgae requirements for the Pacific cupped oyster, *Crassostrea gigas*, and the Scallop, *Pecten maximus*, according to Robert & Gerard (1999) are as follows: One million of 0.2–0.3 mm post-larvae require about 300 g of microalgae dry weight (DW). Another report shows this requirement to amount to 14 kg (DW). The mean value of 7 kg (DW) per 10⁶ spats, and a survival rate of 20% between spats and marketable size animals (67 g) seems reasonable.

France produced 134 800 t of oysters in 1999, which theoretically required about 5000 × 10⁶ post-larvae. On the western coast of USA, 80% of post-larvae production comes from commercial hatcheries. China has a long history of aquaculture and became early the world's leading producing country. It dramatically increased its production during the 1980s, and surpassed its capture fisheries in 1988. With 7.9 × 10⁶ t, China alone produced 78% of the world's filtering mollusks in 1999. Guo *et al.* (1999) attributed 50–60% of the total oyster production to the oyster *C. plicatula*, 20–30% to the Suminoe oyster *C. rivularis* and 10–20% to the Pacific cupped oyster *C. gigas*. This information seems sufficiently accurate to attempt an estimation of world microalgae requirements.

Among the Chinese aquaculture species, Pacific oyster, colorful clam, mud cockle, bay scallop, wrinkled abalone (*Haliotis discus hannai*), and pearl oyster (*Pinctada martensii*) depend partly or totally on hatchery produced juveniles. As all of the spats came from hatcheries, and assuming a ratio

Table 19.2. Microalgae biomass requirements of *Crassostrea gigas* and *Pecten maximus* in hatchery (after Robert & Gerard, 1999).

	One breeder	10^6 larvae	10^6 post-larvae (0.2–3.0 mm)
Daily consumption (g DW)	0.08–0.16	0.32–0.64	1.6–3.2
Rearing period per batch	1–3 months	0.5–1 month	2–3 months
Average microalgae required (g DW)	8	12	200

shown in Table 19.2 is suitable for the bay scallop, this production would have required over 131 t DW of microalgae. The case of the Pacific cupped oyster, another fully hatchery-dependent production of China, is even more remarkable as this culture, which produced nearly 600 000 t in 1999, would have required 314 t DW of microalgae according to our calculation.

Wild spats are still the major source of shellfish juveniles around the world as only 11% of the mollusk production depended on the supply of hatchery juveniles worldwide in 1999. 5000 t of microalgae would have been required if hatcheries had been the sole source of juveniles for all mollusk production.

19.3 Shrimp

Shrimp farming production reached 1.2×10^6 t in 1999, with an average variation of 5% per year from 1994. This poor increase can be attributed to disease, which has destroyed this activity in some countries like China. Shrimp farming mainly takes place in subtropical regions of America – 28% with 457 hatcheries, and south east Asia – 72% with 3718 hatcheries (Rosenberry, 1998). Thailand is the main producer with 225 100 t, followed by Ecuador with 119 700 t in 1999. Algae are necessary from the second stage of larval development (zoea) and in combination with zooplankton from the third stage (myses). Although of short duration, those larval stages require microalgae culture facilities, which will vary with the size of the hatchery and the level of growth control. The larvae feed consists of a combination of microalgae and early stages of the phyllopod crustacean *Artemia* sp., as well as dry food available on the market, or manufactured locally. The main microalgae genera used are *Skeletonema*, *Chaetoceros*, *Tetraselmis*, *Chlorrella*, and *Isochrysis*, production protocols for which are given in Chapter 20. Table 19.3 indicates the microalgae species that have been investigated for penaeid shrimp larval nutrition.

Green water hatcheries may be distinguished from *clear water* ones. The former are small- and medium-sized hatcheries associated with the on-growing farms of south east Asia, where operations rely more on experience than on mastering new technologies. Naturally occurring microalgal blooms are encouraged in large ponds with low water exchange wherein the larvae are then introduced. Sometimes fertilizers and bacteria are added to induce more favorable conditions. This production system, which facilitates only a poor measure of controlling growth of microalgae, provides the better part of shrimp production. On the other hand, large-sized hatcheries require highly

Table 19.3. Main algal species tested in penaeid larval rearing (from Cahu, in Muller-Feuga et al., 2003b).

Shrimp species	Microalgal species	Observation	References
<i>Penaeus japonicus</i>	<i>Monochrysis lutheri</i> <i>Pheodactylum tricornutum</i> <i>Pseudo Isochrysis paradoxa</i> <i>Tetraselmis suecica</i>	Best growth and survival with <i>T. suecica</i>	Cahu (1979)
<i>P. monodon</i>	<i>Chaetoceros calcitrans</i> <i>T. chuii</i>	Better growth and survival with <i>C. calcitrans</i>	Tobias-Quinitio & Villegas (1982)
<i>P. vannamei</i>	<i>Isochrysis</i> sp. <i>Bacteriastrum hyalinum</i> <i>Prorocentrotum micans</i>	Best growth and survival with <i>Isochrysis</i> sp.	Sanchez (1986)
<i>P. monodon</i>	<i>T. chuii</i> <i>Dunaliella tertiolecta</i> <i>Rhodomonas baltica</i> <i>Skeletonema costatum</i>	Very poor survival with <i>D. tertiolecta</i>	Kurmaly et al. (1989)
<i>P. monodon</i> <i>P. japonicus</i> <i>P. semisulcatus</i>	<i>Chaetoceros muelleri</i> <i>T. suecica</i> <i>Dunaliella tertiolecta</i> <i>Isochrysis galbana</i> , clone T-iso	Best growth and survival with <i>C. muelleri</i> and <i>T. suecica</i>	D'Souza & Loneragan (1999)

paid technicians, multi-million-dollar investments, and highly controlled conditions. Such hatcheries are mainly located in the American continent. The observed trend is toward specialized production, particularly with the supply of post-larvae, in the hands of big, centralized hatcheries. They open a pathway to new techniques, especially genetic selection of strains with stronger immunity. Although widely used, dry formulated feeds do not work as a full replacement. Even when they are used, microalgae culture systems are kept in operation for emergencies. Nevertheless, the trend is toward reducing or even avoiding recourse to microalgae, because of ignorance in producing mass cultures of microalgae.

It takes about 1 m^3 of 3×10^6 cell ml^{-1} microalgae culture to produce 10^6 post-larvae, i.e. at the rate of 20 pg per alga, about 60 g DW (Cuzon, personal communication). This small to negligible requirement is only valid for clear water hatcheries. Since microalgae contribute to stabilizing and improving the quality of the rearing medium while providing food for the zooplankton, they are produced in far greater quantities than dictated strictly by the needs of larvae feeding in green water hatcheries. We assume here that 1 kg DW of microalgae is necessary to produce 10^6 post-larvae using the green water technique, with a 20% survival rate between post-larvae and marketable size (25 g). In this case, about 200 t DW of microalgae were required to sustain the 1999 shrimp production.

19.4 Fish

Fish are generally carnivorous (which explains their reputation as a luxury food). Some freshwater cyprinids and cichlids, nevertheless, are herbivorous and even plankton feeders. Their aquaculture has largely spread in developing

countries, contributing significantly to the protein supply of the most populous countries, especially India and China. Their rearing in Chinese freshwater aquaculture is performed within a well-integrated polyculture where cyprinids of different feeding requirements are stocked together at ratios such that food resources of the system are fully utilized. Feces from carnivorous species support phytoplankton and zooplankton blooms, which in turn are utilized by filter feeders such as silver carp (*Hypophthalmichthys molitrix*, 3.2×10^6 t in 1999) and bighead carp (*H. nobilis*, 1.6×10^6 t in 1999). Assuming that these species only feed at primary level, their requirements would have amounted some 4×10^6 t DW of microalgae in 1999.

The use of small, live, plankton feeder preys, namely the rotifers *Brachionus plicatilis*, and *B. rotundiformis*, is a prerequisite for success in hatcheries of marine small-larvae finfish like sea breams (146 000 t in 1999) and flatfish (31 400 t in 1999). These preys can be raised on yeast-based artificial feeds, but this is much less efficient than with microalgae (Table 19.4). They present an interest on three levels: (i) quick recovery of rotifer populations after collapse (7–13 days, compared to 20–35 days with yeast); (ii) improved nutritional quality of live prey; and (iii) lower bacterial contamination, especially from *Vibrio*. For numerous fresh and seawater animal species, the introduction of phytoplankton in rearing ponds leads to much better results in terms of survival, growth, and transformation index compared with clear water. For sea bream, this condition has become an economic necessity.

The reasons behind the positive role of microalgae in larvae rearing ponds of fish, as well as shrimp, have not been completely elucidated. There is wide consent that water quality is improved and stabilized by algal oxygen production, pH stabilization, etc. If, however, the algal tanks are deep and light

Table 19.4. Classes, genera, and species of major currently named microalgae grown for food in fish aquaculture, and their main utilization. Synonymous names are in brackets.

Class	Genus	Species	Main utilization
Cyanophyceae (blue-green algae)	<i>Arthrospira</i> (<i>Spirulina</i>)	<i>platensis</i> , <i>maxima</i>	OGF
Chlorophyceae (green algae)	<i>Chlorella</i> <i>Dunaliella</i> <i>Nannochloris</i> <i>Haematococcus</i>	<i>minutissima</i> , <i>virginica</i> , <i>grossii</i> <i>tertiolecta</i> , <i>salina</i> <i>atomus pluvialis</i>	FLPF, GW
Prasinophyceae (scaled green algae)	<i>Tetraselmis</i> (<i>Platymonas</i>) <i>Pyramimonas</i>	<i>suecica</i> , <i>striata</i> , <i>chuii</i> <i>virginica</i>	FLPF, GW
Eustigmatophyceae	<i>Nannochloropsis</i>	<i>oculata</i>	FLPF, GW
Prymnesiophyceae (Haptophyceae)	<i>Isochrysis</i> <i>Pavlova</i> (<i>Monochrysis</i>)	<i>galbana</i> , aff. <i>galbana</i> 'Tahiti' (T-iso) <i>lutheri</i> , <i>salina</i>	FLPF, GW
Dinophyceae (dinoflagellates)	<i>Cryptocodinium</i>	<i>cohnii</i>	SDLPF
Thraustochytriidae	<i>Schizochytrium</i>	sp.	SDLPF

OGF, On growing formulation; FLPF, Fresh live prey feed; GW, Green water; SDLPF, Spray dried live prey feed.

intensity low, algae will not increase dissolved oxygen (DO) and, during the night, algae which have not been consumed will in effect, reduce DO. The action of some excreted biochemical compounds is generally considered, along with the induction of behavioral processes like initial prey catching. Other positive functions such as regulating the bacterial population, probiotic effects, and stimulating immunity, have likewise been suggested, but they are not sufficiently understood. So far, only their action as a raw material has been considered, giving rise to what are called *green water* and *pseudo-green water* effects (Dhert *et al.*, 1998; Divanach, in Muller-Feuga *et al.*, 2000b).

The most important world marine fish production comes from China. The 1999 production was about 338 805 t, composed primarily of flounder, sea bream, mullet, and puffer fish, principally small-larvae species. We assume here that 50% of the juveniles come from hatcheries, requiring microalgae.

The second in world marine fish production is Japanese amberjack (*Seriola quinqueradiata*, *S. purpurescens*), with 140 411 t from Japan in 1999. We assume here that 15% of the juveniles come from hatcheries, where they require microalgae-fed live preys at the rate defined above.

In the case of the sea bream *Sparus aurata*, the microalgae requirement for the rearing and enrichment of rotifers is 6×10^9 cells weighing 10 pg each for a 60-day-old juvenile, which represents 60 kg (DW) per 10^6 juveniles (*Papandroulakis*, personal communication). Compared with the ratios obtained with mollusks and shrimps, this is one order of magnitude greater, which could be attributed to the more complicated food chain, with two trophic levels instead of one. This was obtained using the pseudo-green water technique, which consists of introducing algae produced elsewhere into the rearing medium. As it is particularly efficient, the use of the previous ratio gives an evaluation that rather minimizes the requirements. We also retained a hypothesis of 30% survival rate between juvenile and marketable size (500 g). If we generalize these conditions to world production of small-larvae fish, the microalgae requirements would have been 154 t (DW) per year in 1999 for this activity, alone.

19.5 Refining the products of aquaculture

Other uses of microalgae in aquaculture concern improving the product, thereby enabling the producer to obtain a higher price for the product. For example, a traditional French technique called the *greening* of oysters, consists in their acquiring a blue-green color on the gills and labial palps, which raises the product's market value by 40%. The agent responsible for this is a pigment produced by the diatom *Haslea ostrearia*, which grows naturally in ponds on the western coast of France. This refining process involves putting the oyster in contact with naturally or artificially grown algae (Barille *et al.*, 1994). A new refining technique based on producing the diatom *S. costatum* in subterranean saltwater is also appearing in some marine marshes along France's western coast: it doubles the flesh content and triples the glycogen content in 30 days at temperatures ranging from 8 to 12°C, resulting in a substantial increase in market price.

In the field of fish culture, pinkening of salmonids flesh has always been a great interest ever since the salmon culture industry started in Norway in the 1970s. Astaxanthin and canthaxanthin are the only pigments that may be fixed in the flesh of salmonids, whose pinkening requirements amount to US\$150 million per year, a rapidly expanding market (Verdelho *et al.*, 1995). Today, the biological supply sources for astaxanthin are the yeast *Phaffia rhodozyma* (Sanderson & Jolly, 1994), despite its low content (0.4%), and the fresh water chlorophycea *Haematococcus pluvialis* (Borowitzka *et al.*, 1991), containing up to 5% (see treatise on *Haematococcus*, Chapter 14).

19.6 Future developments

The world microalgae requirements for hatcheries are summarized in Table 19.5. The production of microalgae for world aquaculture, which was around 1000 t in 1999, is unequally shared between the consuming species, 62% mollusks, 21% shrimps, and 16% fish. The increased potential relies mainly on mollusks and fish, as the contributions of hatcheries in the overall recruitment are 11% and 50%, respectively. The potential microalgae requirements for mollusks, calculated as if hatcheries were the sole source of post-larvae, exceeded 5000 t in 1999 according to our calculation. This potential production is of the same order as the world production of *Spirulina*, ranging from 2000 to 4000 t DW per year. Though microalgae production for aquaculture involves several species, making for a complicated system, there is no need for harvesting the algal mass since the algae are used directly at run-time as row cultures in animal rearing tanks or ponds, simplifying post-culture processes.

Most microalgae requirements are supplied today by firms in-house, growing them in specialized units, or within the larvae rearing tanks. This is less due to a desire for independence than to the need for immediate availability of live microalgae. A supply of live and concentrated microalgae products at competitive prices would probably lead to sweeping changes in hatchery production techniques. In fact, algae culture generates high investment and running expenses, which the producers want to minimize. Recently developed techniques to produce and preserve microalgae could create a favorable situation for the rise of these new products. For example, a European program was set out to examine the conditions for substituting hatchery algae by species produced elsewhere, concentrated, and processed for storage and transportation, for larval rearing of the sea bream *Pagrus aurata*, the oyster *Crassostrea gigas* and the scallop *P. maximus* (Muller-Feuga *et al.*, 1998). Results were encouraging for the sea bream, and mostly negative for mollusks. Standards of preserved microalgae consumption for sea bream have been set, and the need for several species of live microalgae with low bacteria levels has been confirmed for mollusks. The economic stakes are attractive enough to mobilize even stronger international research efforts on larvae nutrition, mainly focusing on polyunsaturated fatty acids (PUFAs) and other essential compounds, in which microalgae would compete with formulated dry feeds.

Table 19.5. Estimates of microalgae biomass production in DW required for feeding of larvae and post-larvae of the world aquaculture in 1999, and mid-term trends of this production. These estimates are calculated by multiplying the recruitment rate by the microalgae requirements described in text, and then by the corresponding aquaculture productions (FAO, 2000).

	Average survival rate (%)	Mean market weight (g)	Recruitment rate (10^6PL t^{-1})	Microalgae requirements (kg DW per 10^6PL)	Microalgae requirements (kg DW t^{-1})	Aquaculture production (t year $^{-1}$)	Microalgae production (t DW)	Trend
Filter feeding mollusks	20	67	0.08	7.0	0.53	1 106 671	581	Sharp increase
Shrimps clear water	40	20	0.13	0.06	0.01	188 797	1	Steady state
Shrimps green water	20	25	0.20	1.0	0.20	1 005 252	201	Increase with production
Small larvae marine fishes	30	500	0.01	60	0.40	385 444	154	Sharp increase
Total						2 686 164	938	

Table 19.6. Comparison of the concentration, productivity, and cost price of some aquaculture microalgae for various types of production systems (after Muller-Feuga *et al.*, 2003a).

Type of production system	Species	Concentration (g l^{-1})	Volume productivity of dry biomass ($\text{g l}^{-1} \text{d}^{-1}$)	Order of magnitude of cost price (€ kg^{-1})	Calculation data borrowed from
Tanks	T-iso				
	<i>Skeletonema</i>	0.1	0.02	1000	Benemann (1992)
	<i>Pavlova lutheri</i>				Brown <i>et al.</i> (1993)
	<i>Nannochloropsis</i> sp.				
Photobioreactors	<i>Nannochloropsis</i> sp.	1–5	0.5–1.7	100	Borowitzka (1996) Chini Zitelli <i>et al.</i> (1999) Zou & Richmond (1999)
Fermentors	<i>Tetraselmis suecica</i>				Day <i>et al.</i> (1991)
	<i>Cyclotella cryptica</i>				Gladue (1998)
	<i>Chlorella</i> sp.	40–60	100–200	10	Barclay <i>et al.</i> (1994)
	<i>Cryptocodinium cohnii</i>				De Swaaf <i>et al.</i> (1999)
	<i>Schizochytrium</i> sp.				

Significant reductions of price seem possible by shifting to new techniques, such as supply of preserved microalgae produced in large and highly controlled facilities.

Table 19.6 summarizes the order of magnitude of cost prices to be expected from various microalgae production techniques. Heterotrophically grown microalgae are less expensive being produced in high densities by classic fermentation. In Japan, freshwater microalgae of the *Chlorella* genus are already widespread on the rotifer production market and consequently, most fish hatcheries do not include a microalgae production facility. The expected reduction of product price would make it possible to bear the additional costs brought about by preservation, storage, and delivery to hatcheries.

Because of the essential long chain PUFA requirements, fish farming is dependent on marine lipids. Formulated dry feeds for intensive fish rearing are composed of 30–60% meal and 10–20% marine fish oil, generally from clupeids. The most commonly accepted predictions for the year 2020 are for $220 \times 10^6 \text{ t}$ of aquatic products, $100 \times 10^6 \text{ t}$ of which will come from aquaculture. With this prospect, the $20\text{--}30 \times 10^6 \text{ t}$ of fish now available for reduction into meal and oil will not meet more than 5–7% of the demand for formulated dry feed for fish farming. If the specific requirements of terrestrial animals and man are also considered, the shortage of essential PUFA could amount to $10\text{--}15 \times 10^6 \text{ t}$ in 2020, if no measures are taken to greatly increase production of PUFA-rich nutrients (Divanach, personal communication). Though inconceivable today due to high production costs, the use of microalgae as a commercial source of PUFA (Apt & Behrens, 1999), and even of energy, high quality proteins, vitamins, and sterols, remains a potential solution. The combination of price increases for fish oil due to a growing shortage (stemming from distrust in animal products), and of improved cost-effectiveness of alternative sources (including genetically modified organisms) support the hope that substitution will be feasible in the future.

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20 Microalgae for Aquaculture

Microalgae Production for Aquaculture

Oded Zmora and Amos Richmond

20.1 Production in controlled closed systems

20.1.1 Freshwater chlorella in controlled reactors (see also Chapter 11)

The rapid growth of mariculture and the establishment of large hatcheries for fish and crab production have led to an enormous demand for rotifers of the species *Brachionus plicatilis* and *B. rotundiformis*. These filter feeders serve as the only live feed for larvae of numerous fish (Ito, 1960; Hirata, 1980; Nagata & Hirata, 1986; Lubzens, 1987; Lubzens *et al.*, 1989, 2001) and crab species during their first days of life (Keenan & Blackshaw, 1999). A recent calculation shows that one tonne (wet weight) of *B. plicatilis* rotifer (400 billion rotifers) and 150–240 kg of *Nannochloropsis* (dry weight) are needed for production of 10 million 1 g fry of gilthead sea bream (*Sparus aurata*) in the Mediterranean basin (Lubzens *et al.*, 2001). Ca. 216 million of gilthead sea bream fry were produced in 1998 in the Mediterranean basin and 247 million were expected to be produced in 1999, according to the Federation of European Aquaculture Producers.

Major fish species produced today requiring rotifers during early developmental stages include: Yellowtail (*Seriola quinqueradita*), red sea bream (*Pagrus major*), puffer fish (*Fugo rubripes*), turbot (*Scophthalmus maxima*), barramundi (*Lates calcarifer*), mullet (*Mugil cefalus*), gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) (FAO 1998; Lubzens & Zmora, 2003). Also, all mud crab and swimming crab production relies on rotifers for the first larval stages (zoea).

Mass production of rotifers was established in the late 1960s facilitated by a major breakthrough involving bakers' yeast, found suitable for feeding rotifers as a replacement for costly cultured microalgae (Hirata & Mori, 1967). Rotifers' cultures based on bakers' yeast, however, are unstable and cannot be intensified (Hirayama, 1987). Numerous artificial diets were,

therefore, tested as food for rotifers, accompanied by intensive efforts to reduce the cost of microalgae for rotifers' feed (Lubzens *et al.*, 2001).

The traditional industry of freshwater, single cell algae (*Chlorella*) in Japan has been investigated during the last decade developing modes of producing inexpensive and efficient diets for super-intensive production systems of rotifers. Adding vitamin B₁₂ to the algal growth medium opened up the possibility for conversion of the freshwater *Chlorella vulgaris* into a complete diet for rotifers (Maruyama & Hirayama, 1993). This application was further researched by many groups, e.g. Yoshimura *et al.* (1996), Fu *et al.* (1997), Maruyama *et al.* (1997) and Hagiwara *et al.* (2001), who accelerated rotifer production in numerous hatcheries in Japan and elsewhere.

The important advantages of using B₁₂-enriched *C. vulgaris* for production of rotifers in fish and crab hatcheries may be summarized as follows:

1. Several strains of *C. vulgaris* can be manipulated to contain a high level (4.5 µg g⁻¹ dw of algal mass) of vitamin B₁₂, essential for rotifer reproduction and culture stability (Maruyama & Hirayama, 1993).
2. *C. vulgaris* may have several modes of nutrition; autotrophic, heterotrophic, and mixotrophic and can thus be produced using different methodologies at a relatively low cost.
3. Large volumes of high density *C. vulgaris* culture may be readily concentrated up to 140 g(dw) l⁻¹. In this form *C. vulgaris* can be kept at 4°C for 30 days and be delivered anywhere in the world.
4. Concentrated *C. vulgaris* can be used as sole feed for very high-density cultures of rotifers (25 000 ind ml⁻¹), facilitating feeding without diluting the rotifers' medium.

20.1.2 Flat plate reactors

Nannochloropsis sp. may be grown satisfactorily in various photobioreactor types (see Chapter 9). A most intensive *ultrahigh* cell density culture (UHCD) in flat-plate reactors with a very short (1–2 cm) optical path had been developed (Hu *et al.*, 1998). In contrast to the open raceway, which yields a relatively dilute cell concentration of *Nannochloropsis* sp. (i.e. 1 × 10⁸ cells ml⁻¹), UHCD is usually grown under laboratory conditions, receiving strong light (up to 3000 µEm⁻² s⁻¹) and requiring frequent change of the growth medium to control growth inhibition. This culture mode is far from being cost-effective at present, but reveals, nevertheless, the potential of photoautotrophic cultivation of *Nannochloropsis* i.e. cell concentrations of a few billion cells per ml (1–5 × 10⁹ cells ml⁻¹), and culture content of eicosapentaenoic acid (EPA) reaching as high as 2000 mg per l.

Aiming to develop the methodology for cost-effective mass production of different species of microalgae in quantities required for hatcheries, a flat-panel reactor made of glass which may be readily scaled up to hold 500–1000 l of *Nannochloropsis* sp. culture was developed (Zhang *et al.*, 2001). Flat-panel reactors represent a promising photobioreactor type (Hu & Richmond, 1996; Hu *et al.*, 1998): their entire surface area is well illuminated by direct beam as well as diffused and scattered light, an essential advantage for

high phototrophic productivity and, when tilted, the front surface exposed directly to the sun receives the major thrust of solar irradiance in the fall and winter seasons, when outdoor productivity falls due to light and temperature limitations. The dissolved oxygen path (corresponding in flat plates to the height of the reactor) is only ca. 100 cm long in panel reactors, preventing buildup of dissolved oxygen (O_2), a serious problem associated with many types of tubular reactors. Flat-panel reactors may be easily cleaned, both from outside and inside, all panels being readily accessible. Wall growth inside the reactor and salt deposition outside are thus easily handled (see Chapter 9).

A newly designed (Zhang *et al.*, 2001; Zhang & Richmond, 2003) vertical flat-plate glass reactor was constructed with a 20 cm optical path (i.e. reactor width), found optimal for outdoor production of *C. muelleri* var. *subsalsum* and *Isochrysis galbana* (Fig. 20.1). It was made of 10 mm thick glass plates, measuring 160 cm long and 110 cm high (inside width 20 cm). Vertical glass supports prevent the glass plates from bulging and cracking, holding together the reactor's front and back plates.

The optimal harvesting regime was in the range of 10–15% of culture volume harvested daily and replaced with fresh growth medium. A steady state culture density of 24 and 28×10^{-6} cell ml $^{-1}$ of *C. muelleri* and *I. galbana*, respectively, was thereby maintained, yielding a steady output rate of 13–16 g cell mass m $^{-2}$ per day, per each front and back panel.

The optical path exerted a clear effect on cell content of EPA and decosa-hexaenoic acid (DHA), the former increasing progressively with the longer

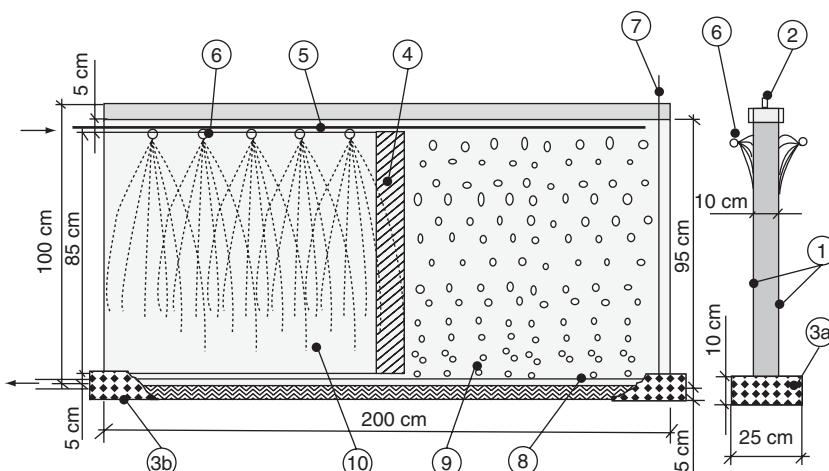


Fig. 20.1. (1) Front and back, 10 mm thick glass plates; (2) glass cover, which is placed over a sealing film with which the open end of the flat-plate reactor is sealed, leaving only a small opening for out flowing air; (3a) trough for collecting sprayed water, side view; (3b) trough for collecting sprayed water front view; (4) plates' support, connecting together the front and back glass plates; (5) spray water tube; (6) micro-sprinklers; (7) air tube; (8) perforated air tube, with 1 mm holes placed 5 cm apart; (9) air bubbles; (10) algal culture. Not shown are: water-cooling tower (providing evaporative cooling to the recycled water), not required where cool ocean water is readily available, CO_2 tank for carbon feeding and air blower providing air stream for mixing the cultures.

optical path and elevated areal density. The highest percent of EPA (on dry cell mass basis) was 3.5 and 4.8 for *C. muelleri* var. *subsalsum* and *I. galbana*, respectively. Both maxima were obtained in the longest 30 cm optical path reactor which had the highest areal density, in which the photon flux per cell (obtained by dividing the irradiance falling over a given reactor surface by the number of cells in the irradiated volume corresponding to the irradiated area) was the lowest. Highest areal output of EPA ($\text{mg m}^{-2} \text{ day}^{-1}$) for both species was ca. 500 mg, that of DHA being ca. 250 mg (Zhang & Richmond, 2003).

Economic considerations for algae production in large, flat-plate glass reactors: The economic aspects of culturing *Nannochloropsis* sp. in a flat-plate glass reactor were analysed for a postulated 2000–1 reactor (i.e. several units of the type shown in Fig. 20.1, combined). The reactor size (or two 1000–1 reactors) was judged to be of industrial scale in that it addresses the rotifer requirements of eight million gilthead sea bream fingerlings produced annually (Zhang *et al.*, 2001). Cost of production depended on the scale of production and cost of labor, ranging between \$60 and 120 kg^{-1} dry cell mass.

20.1.3 Mass cultivation in polyethylene bags

Cultivating microalgae in transparent, disposable plastic bags (Fig. 20.2) has long been a standard method by which to grow microalgal feed required for hatcheries. The bags are usually arranged vertically supported by different methods, e.g. wire mesh (in Fig. 20.2), or hung on a suitable framework (see Chapter 15). One disadvantage associated with this mode of production is that bags are not ideal for species that tend to develop wall growth. Since each bag is a separate production unit, large-scale economy mandates the units should be rather large, i.e. a large optical path (diameter) creating large 200–500 l units. The optimal cell density in the wide bags is necessarily small,



Fig. 20.2. Cultivation of *Chaetoceros calcium* in 300 l bags (courtesy of Mark Gluis). Coast seafood co., Kailua Kona, Hawaii.

resulting in low volume (cells l^{-1}) yields. To utilize this system more effectively in light-limited algal cultures, the bags should not be placed close together. If maintained in protected areas, solar light should be available from all sides, and whitewashing the floor should increase the availability of reflected light. An important advantage of bag cultures rests in the ease by which outdoor culture maintenance under very clean conditions is facilitated. The bags may be readily interconnected by proper tubing, to facilitate addition of growth medium and inoculum as well as harvesting, providing a fully automated system (Fig. 20.2).

One important use of these bags is for growing algal species required for abalone feeding. The current high market price provides a strong incentive to establish and expand abalone mariculture, and several approaches for abalone production have been tried once seeds have been obtained. In Japan, juveniles are planted directly onto coastal growing beds while raft culture is also practiced (Hahn, 1989). Land based culture facilities have been established in the USA, New Zealand, Australia, South Africa, and Japan (Elbert, 1992); and recently in Israel (Shpigel *et al.*, 1996a,b). The major impediments in the culture of abalone are slow growth and high mortality rates of the post-larvae (PL) stage which average 96% and often result in total loss during the first 8–12 weeks following larval metamorphosis and settlement (Hahn, 1989; Fallu, 1991). The high mortality of the PL can be attributed to various factors. A comprehensive review by Kawamura *et al.* (1998), however, suggests that the most likely causes are nutritional in nature and/or derived from poor digestibility (Leighton, 1985, 1989; Hahn, 1989; Kawamura *et al.*, 1995; Seki, 1997). PL abalone feed on benthic diatoms which reside on solid surfaces. In most abalone nurseries, seawater rich in natural algae (which have not been selected, and are thus of unknown nutritional value) is pumped into the settling tanks of the hatched abalone larvae. In contrast, a unique production protocol based on 150 l polyethylene bags was developed by the National Center for Mariculture (NCM) in Eilat, Israel, for production of selected species of benthic diatoms required for abalone nursery: Filtrated seawater is pumped into the abalone PL tanks but the cultured algae are introduced separately. Numerous species of benthic diatoms were isolated from the local bivalves and sedimentation ponds, were cultured, and then taken for attraction assessment and for survival and growth tests of abalone juveniles. The species *Navicula lenzi*, *N. vernuende*, *Nitzschia laveas* which exhibited the best results (Shpigel, unpublished) are cultured in 150 l polyethylene bags, provided with heavy aeration and CO_2 enrichment using a special medium developed at NCM (Zmora, unpublished). Although aggregated, the benthic algae are maintained suspended in the culture column due to a high aeration rate. When cultures reach a density of 0.6–0.8 g l⁻¹ they are harvested and distributed in the abalone tanks. The capability of controlling the quality and quantity of the bag-cultured algae in the abalone spat tanks is the major reason for obtaining an average of 25% survival rate during the first three months (Shpigel, unpublished), compared with the usual 2–5% survival rate. Similar results were recently reported from Australia (Daume, 2002): selected benthic diatom species grown in bag cultures, affected higher growth and survival rates of juvenile abalone.

20.2 Production in open systems

20.2.1 Open cylindrical tanks

Transparent culture tanks represent a suitable container for microalgal cultures and are extensively used. Tanks constructed of 1 mm polymer fiberglass, which provide 90% transparency, are commercially available in different dimensions. A suitable dimension for mass cultures would be a tank 30 cm in diameter, extending 180 cm high and holding some 150 l. Made of fiberglass, they are corrosion-proof and much lighter than glass. An efficient use of such tanks outdoors is described by Hering (1996). Hering & Wang (personal communication) observed that the main difficulty in maintaining continuous culture of microalgae for aquaculture in open production systems outdoors is centered on the difficulty to maintain monoalgal cultures, in which contamination by foreign microalgae as well as cell-grazers is minimal. They approached the problem by analysing the pertinent conditions which affect domination of an algal species in a body of water. These include the seeding level, the rate of harvesting, and culture dilution (i.e. the rate at which cells are removed from the culture), which in turn affects the standing cell density and also, the diurnal culture temperature (e.g. maximal temperature during the day and minimum temperature at night). Finally, the concentration of certain mineral nutrients and vitamins proved to be of great importance in affecting species' competition, as seen in the study of Harrison & Davis (1979) concerning the effect of mineral composition of species dominance, which illuminates this point: they used open, outdoor continuous cultures in tanks, maintaining dilution rates of 50%, 25%, and 10% of culture volume per day, imposing either nitrogen- or silicate growth-limitation. Under high nutrient flux (high rate of dilution involving large continuous addition of nutrient solution), the centric diatoms *Chaetoceros* sp. and *Skeletonema costatum* dominated. In contrast, under low nutrient flux, a mixture of centric diatoms, flagellates, and pinnate diatoms coexisted. This indicated that *Chaetoceros* and *Skeletonema costatum* would dominate the culture as long as nutrients are readily available at relatively high concentrations. In another work, Daniels & Boyd (1993) applied different ratios of nitrogen and phosphorous to marine shrimp ponds. Diatoms, 90% of which were *Chaetoceros* sp., were dominating the ponds at N:P ratios of 15:1 (cited from Hering, 1996).

In their attempt to identify culture conditions in which *Chaetoceros* sp. for aquacultural feed would dominate open, continuous cultures in a cost-effective manner, Hering (1996) identified culture conditions in which the marine *Chaetoceros* sp. reached a cell density of $7\text{--}8 \times 10^6$ cells ml⁻¹ and greater than 95% dominance in open, semi-continuous cultures in outdoor tanks. The production protocol called for maintaining critical concentrations of nitrogen, phosphorous, silicate, vitamin B₁₂, EDTA, iron, and copper. Finally, a daily harvest of up to 90% of culture volume and pH of 8.2 as set point for CO₂ injection were found optimal for *Chaetoceros* sp. dominance in open tanks. The modifications in the nutrient medium introduced by Hering (1996) to establish *Chaetoceros* sp. dominance are shown in Table 20.1.

Table 20.1 Comparison of *Chaetoceros* sp. optimal nutrient media with Guillard's f/2 media (from Hering, 1996).

Item	<i>Chaetoceros</i> sp. media	Guillard's f/2 media
NaNO ₃	3.00 mg N l ⁻¹	12.35 mg N l ⁻¹
NaH ₂ PO ₄ H ₂ O	2.75 mg P l ⁻¹	1.12 mg P l ⁻¹
Na ₂ EDTA	5.53 mg EDTA l ⁻¹	5.0 mg EDTA l ⁻¹
Na ₂ SiO ₃ 9H ₂ O	10.0 mg SiO ₂ l ⁻¹	3.0 mg SiO ₂ l ⁻¹
Thiamin HCL	None	100 µg l ⁻¹
Biotin	None	0.5 µg l ⁻¹
Vitamin B ₁₂	2.75 µg l ⁻¹	0.5 µg l ⁻¹
CuSO ₄ 5H ₂ O	8.3 µg Cu l ⁻¹	2.5 µg l ⁻¹
ZnSO ₄ 7H ₂ O	None	5.0 µg Cu l ⁻¹
CoC ₁₂ 6H ₂ O	None	2.5 µg Co l ⁻¹
MnC ₁₂ 4H ₂ O	None	5.0 µg Mn l ⁻¹
Na ₂ MoO ₄ 2H ₂ O	None	2.5 µg Mo l ⁻¹
FeC ₁₃ 6H ₂ O	0.31 mg Fe l ⁻¹	0.65 mg Fe l ⁻¹

The production system was based on maintaining seed columns, which would reach the density of up to 10⁷ cells ml⁻¹. When ready, each seed column supplies five reactor columns, which are harvested at the end of each day (Wang, 2002).

Searching for a cost-effective method for harvesting *Chaetoceros* sp., Csordas (2001) developed a system based on foam fractionation, a process involving usage of air bubbles for absorption and removal of dissolved and particulate organic carbon (Lawson, 1995) particles in the range of 5–10 µm being of concern because of the hazard of fish gill infection. An advantage of foam fractionators is seen in that it can reduce clogging of filters and pumps, increase aeration, remove proteins, and increase water clarity (Timmons, 1994). Csordas (2001) defined optimal conditions for fractionation removal of *Chaetoceros* from the culture tanks. He reported harvesting efficiencies above 95% with less than 12% culture volume removal.

According to Wang (2002), Aquaculture Technology Inc. (ATI) in Hawaii, which developed an open continuous microalgae culture system, has shown that continuous presence of *Chaetoceros* sp., in an aquaculture production system keeps it free of a large number of pathogenic bacteria.

20.2.2 Cultivation in open tanks and ponds

Cultivation of microalgal feed in open tanks represents an inexpensive and popular production mode in many hatcheries (Fig. 20.3). The open tanks differ from open raceways in their depth, which may be as high as 80 or 90 cm and in lacking, as a rule, an intensive mode of stirring. Such tanks, therefore, are suitable for low cell-density batch cultures (a few million cells per ml) that reach their optimal density in two or three growing days from the start. Continuous cultures are also possible, as long as cell density is maintained relatively low (i.e. using a high dilution rate), facilitating fast growth rates. For such low cell densities, only minimal mixing rates are required for most of the species readily grown in tanks, e.g. *Chaetoceros* sp., *Isochrysis* sp.



Fig. 20.3. Cultivation tanks of different sizes ranging from 4500 l and 14 000 l (back) to 120 000 l (Front) (courtesy Mark Gluis, Coast Seafood Company, Kailua-Kona, Hawaii).

and *Skeletonema* sp. or combinations of these species with *Thalassiosira* sp. or *Phaeodactylum* sp. The low cell density facilitates a high growth rate, and cell density may quadruple, initially, in a course of 24 h. This fast growth rate, however, would soon recede because of the tanks' long optical path coupled with the unfolding of mutual shading as cell density rises. It is essential to inoculate tanks in the evening or early morning, taking special care, particularly on bright days, not to establish a culture that is optically too thin (i.e. low cell concentrations) which may readily become photoinhibited. In extreme cases, overexposure to light could culminate in a quick culture death, due to photo-oxidation. Weissman (2002) reports on a comparison drawn between outdoor, paddle wheel-mixed ponds, and large tanks with translucent sidewalls. Both systems performed well in respect to algal productivity and culture stabilities, the tanks, however, yielding overall greater productivity (on an area basis) and stability than the paddle wheel-mixed ponds. The higher productivity of the large tanks was due to the additional light captured through the translucent sidewalls, highlighting the fact that translucent sidewalls carry obvious advantage in cultivating light-limited systems.

20.2.3 Production of *Nannochloropsis* sp. in open raceways (see Chapter 9)

The open raceway (Dodd, 1986; Richmond, 1992) developed ever since the 1950s, into a cost-effective mode by which to mass culture *Nannochloropsis* sp. for aquaculture. The method for production of *Nannochloropsis* sp. in an open raceway was developed in the NCM, Israel (Sukenik *et al.*, 1993; Lubzens *et al.*, 1995; Zmora *et al.*, 1996). It is being used successfully at a commercial aquaculture production facility in Eilat, Israel, and was recently reported by Hoffman (1999) (Fig. 20.4).



Fig. 20.4. An open raceway for production of *Nannochloropsis* sp. operated at NCM, Eilat, Israel (courtesy of Dr Angelo Colorni).

20.2.3.1 Environmental conditions

The raceway is operated throughout the year under the following conditions. Minimum temperature range in winter is 4°C and 14°C for night and day, respectively, maximum summer temperatures running 22°C and 32°C for night and day, respectively. Ambient air temperature in summer ranges from 30 to 44°C but the relative air humidity in summer is low, i.e. 15–25%, affecting sufficient evaporative cooling to prevent maximum culture temperature from rising above 32°C.

20.2.3.2 Culturing device

The raceways cover 50 or 100 m², at a depth range of 15–25 cm. Mixing is carried out by a 150 cm span paddle wheel, rotating at 15 rpm, producing a horizontal current speed of 70 cm s⁻¹. CO₂ feed: 1–2 l min⁻¹ of CO₂ is delivered automatically through ceramic or plastic diffusers, for 50 m² pond areas. CO₂ supply is adjusted to the apparent rate of consumption as reflected by the productivity and pH change. A timer regulates the CO₂ flow, which in effect stabilizes the pH range during daytime at 8.0–8.5. Incoming water is treated by UV or Ozone and filtered through a 5 µ filter.

20.2.3.3 Starting a pond culture

The nutrients (industrial grade): N as (NH₄)₂SO₄: 132 g m⁻³, P as H₃PO₄ 85%: 24 ml m⁻³, Fe as FeCl₃·6H₂O: 10 g m⁻³, NaHCO₃: 100 g m⁻³. To start a new pond 500–700 l of culture produced in the laboratory (density of 150 × 10⁶ cells ml⁻¹), or 1–2 m³ of culture from an open pond (density of 80–120 × 10⁶ cells ml⁻¹), is transferred into 10–12 m³ of fresh medium in the raceway.

20.2.3.4 Maintaining monoalgal cultures

The major difficulty in operating *Nannochloropsis* sp. outdoors cultures completely open to air-borne contaminants is that cultures become, too often, contaminated thereby becoming useless. Open cultures may be operated, therefore (either batch wise or in continuous cultures), providing a working protocol has been developed to control growth of foreign organisms. The greatest damage to these cultures is caused by grazers; most serious of which is *Paraphysomonas imperforata*, a non-specific heterotrophic flagellate, 7–12 µ, with very short generation time. Appearing as soon as temperatures become high, it may crash a dense culture of *Nannochloropsis* in 24 h. Another grazer is *Euplotes* sp., a 50–90 µ ciliate, which may graze on *Nannochloropsis* sp. cells as well as other organic matter in the culture, including bacteria. The presence of this protozoon is always associated with aggregation of the host's cells and reduction of cell numbers.

Species of algae, e.g. diatoms mostly *Amphora* sp., may also become established in an open *Nannochloropsis* culture. Although diatoms are not regarded as direct competitors to *Nannochloropsis*, their cells excrete polysaccharides which cause *Nannochloropsis* sp. cells to stick together. Relatively low numbers of diatoms can cause serious damage affecting such cell conglomerates, arresting thereby culture growth, making the culture useless. Other contaminating microorganisms are colorless microflagellates, bacteria, as well as *Uronema* sp., 20–25 µ ciliates grazing on bacteria.

20.2.3.5 Controlling contaminants

Different treatments may be applied to eliminate contamination, thereby facilitating cultivation of *Nannochloropsis* in open raceways: (a) Lowering the pH: This represents a useful tactic by which to arrest development of some contaminants. pH 6 is low enough to eliminate diatoms in a *Nannochloropsis* sp. culture; the aggregates dissolve and within a few hours the culture regains its normal appearance. Elimination of *Paraphysomonas* cells and similar contaminants requires the pH to be lowered to 2.5 for a couple of hours. *Nannochloropsis* sp. cells lose their photosynthetic capacity at this pH, which should thus be soon raised to 5.5 or 6.0 at which pH cells regain their normal functioning and after a short period of photosynthesis, the pH rises to the 8.0–8.5 at which it is maintained. (b) Chlorination: Chlorinating contaminated *Nannochloropsis* sp. cultures using concentration of 4 to 10 ppm of active chlorine is quite effective in eliminating grazers. Density of the cultured cells, the organic load (dissolved and particles) and the temperature represent the parameters to be considered in applying an adequate chlorine concentration dose for effective treatment. High doses of chlorine would be used for dense cultures of high organic loads and when culture temperatures are high, thus in summer, there is a need to chlorinate up to twice a week, whereas one application may be sufficient for two months during the cold season. Pond walls should be cleaned every three to four months in winter and one to two months in summer.

20.2.3.6 Harvesting

The desired volumes are pumped out every day either directly to the consumers or to an industrial centrifuge, creating a 40% solid paste, which is preserved for later use. After harvest, a mixture of fresh sea and tap water, including nutrients, is introduced in the ponds, bringing the culture back to its original volume.

20.2.3.7 Production quality

The harvested *Nannochloropsis* sp. is used for culturing rotifers and for maintaining the rotifers' nourishing value in the larvae rearing tanks of marine fish (*Green Water*). It is essential to focus on the algae quality, particularly the total lipid content and polyunsaturated fatty acids (PUFAs). These parameters vary during the year; much higher lipid content is found in *Nannochloropsis* sp. cells in summer than in winter. On the other hand, the percentage of EPA in fatty acids (FA) profile is much higher in winter (Sukenik *et al.*, 1993). Cell density in an open culture of *Nannochloropsis* sp. may exceed 200×10^6 cells ml^{-1} . At this cell concentration, however, productivity would be much below maximal. For maximal sustainable yield, the optimal cell concentration of $100\text{--}120 \times 10^6$ cells m^{-3} , depending on the season and cultures depth, should be maintained. The output rate (in dry weight) of cell mass varies greatly with the season. In summer, 25–30% of the culture is harvested per day, yielding an average of $16 \text{ g (dw)}/\text{m}^{-2} \text{ d}^{-1}$. In winter, due to shorter daylight and lower temperatures, only 15% of the culture is harvested daily, averaging $8 \text{ g (dw)}/\text{m}^{-2} \text{ d}^{-1}$.

20.2.3.8 Production cost

The production cost of 1 kg (dry) cell mass of *Nannochloropsis* sp. in an open raceway, on a rather small scale, is calculated to be ca. US\$40 (before harvesting). This figure is relevant for a scale of ca. 400 m^2 , and would be higher if all direct as well as indirect costs are considered. Production cost, however, may be significantly reduced upon increasing the production volume.

20.3 Integration of aquaculture with algaculture

Neori & Shpigel (2002) and Neori *et al.* (2000) have worked out a promising scheme for sustainable mariculture by integration of algae/algivores to fish/shrimp ponds. Such integration is often essential because high volume fed mariculture technologies, mainly fish cages or shrimp/fish ponds, pollute coastal waters. The cost of feed protein in fed mariculture constitutes a major expense in addition to forming the main source of pollution. Indeed, the environmental impact of this *nitrification* of coastal waters associated with mariculture, makes the growth of the industry unsustainable in many parts of the world. Integrated, sustainable mariculture offers solutions to overcome these problems by a holistic-yet-profitable approach. In essence, using algae and filter feeders, marshalling the supply of land, as well as harnessing

sunlight and work force, polluted effluents turn into profitable resources, as well as restoring water quality (Neori & Shpigel, 2002).

The technologies developed by Neori & Shpigel (2002) are generic and modular, adaptable for fish/shrimp culture at any level of intensification. Their operation is simple in principle, albeit requiring a thorough understanding of the biology of each organism and of nutrient recycling processes. Their scheme (Fig. 20.5) elucidates the principle involved, i.e. to pass or recirculate water from mariculture ponds through suitably designed algal biofilter ponds, stocked with phytoplankton or seaweed. These ponds efficiently extract dissolved nutrients, excess CO₂, and biological oxygen demand (BOD), and recharge with dissolved oxygen making the treated water suitable for reuse in mariculture or for a clean discharge. The algae are then consumed, together with other excess organic particulate matter, by high-value algivore invertebrates—bivalves (such as *Crassostrea gigas*, *Tapes philippinarum*), brine-shrimp *arthemia*, abalone or sea urchin. Certain seaweeds have value for phycocolloids or human consumption. The authors believe that using this integrated approach marginal coastal lands in many areas of the world can be turned into profitable entities with only modest investment.

A different, yet comparable concept for integration of algal systems and aquaculture was offered by Brune *et al.* (2002). Their system *partitioned aquaculture system* (PAS) technology has been under development at Clemson University, SC for the past 12 years (patented 2001). The basic concept of the PAS is to partition pond fish culture into distinct physical, chemical, and biological processes linked together by a homogenous water velocity field. The physical separation of fish culture from algal production systems allows optimization of these processes and thus, maximizes overall system performance. Algal production coupled to co-culture of filter-feeding fish and shellfish drives the increased nutrient assimilation capacity of the system. Brune *et al.* (2002) concluded the PAS to have increased fish production

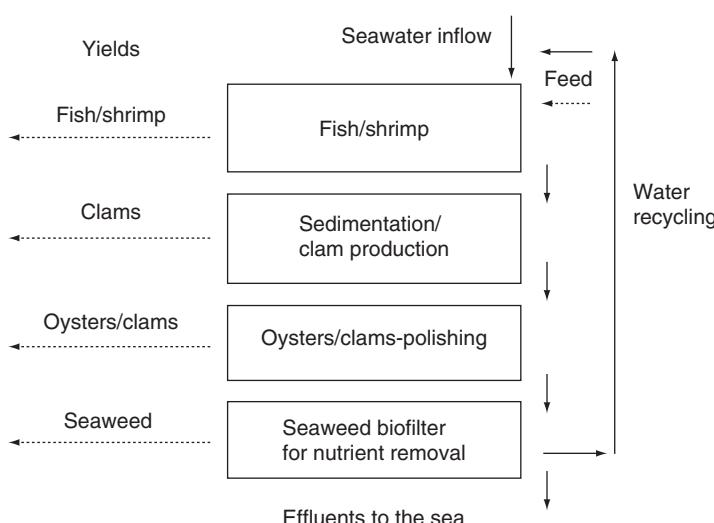


Fig. 20.5. General scheme for an integrated system (Neori & Shpigel, 2002), with permission.

nearly fourfold over that of conventional aquaculture, in excess of 20 000 kg of catfish production per hecter of water area with an additional 5000 kg ha⁻¹ of filter-feeders, simultaneously reducing feed and energy inputs in the culture. The authors believed that high rate controlled eutrophication processes such as the PAS offer the potential for integrated environmental mitigation by reducing local surface water nutrient enrichment and also by providing the potential to reduce greenhouse gas emissions from current agricultural practices. The driving force behind the suggested installations would be the profit provided by harvest of high protein aquatic biomass that may be used as animal feed, or potentially human food, bio-energy, concentrated biofertilizers, or other bioproducts (Brune *et al.*, 2002).

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21 Microalgae for Aquaculture

The Nutritional Value of Microalgae for Aquaculture

Wolfgang Becker

21.1 Introduction

There are a number of different modes in which microalgae are utilized in aquaculture, their main applications, however, being related to nutrition, the basis of the energy flow through the aquatic grazing food chain (De Pauw & Persoone, 1988). They may be used directly, i.e. as an unprocessed sole component to build up short food chains, or in the form of dried material in pelleted or otherwise processed feed preparations. The algae are used as feed to supply basic nutrients, as a source of pigments to colour the flesh (of salmonids) or the skin (of fancy fish) or for other biological purposes.

Microalgae are required for larvae nutrition during a brief period, either for direct consumption, in the case of molluscs and peneid shrimp, or indirectly, as feed for the live prey fed to small larvae fish. Although several attempts have been made to substitute inert particles for these microorganisms, which many aquaculturists find difficult to produce, concentrate and store, only shrimp and live prey for fish will, with only limited success, accept inert food. For shellfish hatcheries, multi-specific live microalgal feed remains essential. Most of the aquatic animals used by humans for food are carnivorous from their post-larval stage, or omnivorous at best, and only filtering-molluscs and a few other animals are true plankton feeders throughout their lifetime.

The positive role of microalgae in the larvae rearing tanks of fish as well as shrimp is not completely understood. One major effect is the improvement and stabilization of water quality, pH, etc. by photosynthetic oxygen production of the algae. The effect of algae on the pH, however, varies from day to night: the pH rises during the day (depending on the illumination level), CO₂ being depleted in the course of photosynthesis. The pH declines during the night due to respiratory activity in which CO₂ is released. Action of excreted algal compounds represents a positive effect, as are regulating and controlling bacterial contamination, probiotic effects and immune stimulation properties.

Various species of microalgae are known to be an essential food source in the rearing of all stages of marine bivalve molluscs (clams, oysters, scallops), and of the post-larval stages of some marine gastropods (e.g. abalone), as well as larvae of several marine fish species, penaid shrimp and zooplankton. Numerous investigations have resulted in the development of selected feed preparations, which can be classified into three different groups of live diets, commonly used in commercial larviculture of fish and shellfish (Lavens & Sorgeloos, 1996):

1. Different species of microalgae ranging between 2 and 20 µm in size for: Bivalves, Penaid shrimps, rotifers, copepods and fish;
2. The rotifers *Brachionus plicatilis* and *B. rotundiformis* (50–200 µm in size) for: Crustaceans and marine fish;
3. The brine shrimp *Artemia* sp. *nauplii* (400–800 µm in size) for: Crustaceans and fish.

In addition to these main groups, a few other live feeds are used on a more limited scale for specific larviculture practices, including *Brachionus rubens*, *Moina* spp., daphnids and decapsulated brine-shrimp cysts for freshwater fish and prawn larvae, as well as *Artemia* biomass for lobster larvae, shrimp post-larvae and broodstock, and marine fish juveniles.

More than 40 species of microalgae have been isolated and analyzed, and are cultivated as pure strains in intensive systems. The major species of algae currently used as feed for different groups of commercially important aquatic organisms, include species of diatoms, flagellated and chlorococcacean green algae as well as filamentous blue-green algae, ranging in size from about 5 µm (*Chlorella*) to more than 100 µm (*Spirulina*) (Table 21.1). The most frequently used species in commercial mariculture are the diatoms *Skeletonema costatum*, *Thalassiosira pseudonana*, *Chaetoceros gracilis*, *C. calcitrans*, the flagellates *Isochrysis galbana*, *Tetraselmis suecica*, *Monochrysis lutheri* and *Nannochloropsis* spp. (2–3 µm), and the benthic diatoms *Nitzschia paleacea* and *N. closterium*.

Several reports indicate that the growth of animals fed with diets composed of a mixture of several algal species is often superior to that obtained by feeding only one algal species, probably due to the fact that a particular alga may lack a nutrient, while another may contain that nutrient and lack a different one. A mixture of both algal species supplies the animals with a more balanced nutrition.

Studies by Renaud *et al.* (1994, 1995, 1999) and Brown *et al.* (1998) demonstrate that a large potential of local isolates is available with a promising nutritional potential for feed in aquaculture, leaving a wide field for further selection and isolation of nutritional microalgae.

21.2 Chemical composition and nutritional value of microalgae

The nutritional value of microalgae depends mainly on their chemical composition and cellular structure, which are influenced to a certain degree by culture conditions. Microalgae for use in aquaculture should possess the essential nutritive constituents, should be nontoxic, of proper size to be ingested and should have a digestible cell wall to make the nutrients available.

Table 21.1. Microalgae used as feed in aquaculture (after DePauw & Persoone, 1988; Lavens & Sorgeloos, 1996).

Bacillariophyceae	
<i>Skeletonema costatum</i>	B, B, D
<i>Thalassiospira pseudonana</i>	B, A, D
<i>Phaeodactylum tricornutum, C. muelleri</i>	B, A, D, C, F
<i>Chaetoceros affinis, C. calcitrans</i>	B, A, D, F
<i>Cylindrotheca closterium</i>	B
<i>Bellerochea polymorpha</i>	D
<i>Actinocyclus normanii</i>	D
<i>Nitzschia closterium, N. paleacea</i>	F
<i>Cyclotella nana</i>	F
Haptophyceae	
<i>Isochrysis affinis galbana, I. tahiti</i>	B, A, D, C, F
<i>Pseudoisochrysis paradoxa</i>	A, D, C
<i>Dicrateria sp.</i>	D
<i>Cricosphaera elongata</i>	D
<i>Coccolithus huxleyi</i>	D
<i>Olisthodiscus luteus</i>	I
<i>Pavlova lutheri, P. pinguis</i>	A, D, F, G
Chrysophyceae	
<i>Pyramimonas virginica</i>	A, D
<i>Micromonas pussila</i>	D
Chryptophyceae	
<i>Cryptomonas</i>	D
<i>Rhodomonas salina</i>	A, D
<i>Chroomonas salina</i>	D
Xanthophyceae	
<i>Olisthodiscus luteus</i>	D
Cyanophyceae	
<i>Spirulina (Arthrospira) platensis</i>	B, D, F, G
Chlorophyceae	
<i>Tetraselmis suecica</i>	B, A, D, E, F, G
<i>Chlorella sp.</i>	A, C, F, G, I
<i>Scenedesmus obliquus, S. quadricauda</i>	I, G, F
<i>Dunaliella tertiolecta</i>	D, F, G
<i>Chlamydomonas khaki</i>	A, D, I, G, I
<i>Chlorococcum sp.</i>	D
<i>Brachiomonas submarina</i>	D
<i>Spongiococcum excentricum</i>	A
Eustigmatophy	
<i>Nannochloris oculata, N. gaditana</i>	D, G, H

A, bivalve mollusc larvae; B, penaid shrimp larvae; C, freshwater prawn larvae; D, bivalve mollusc postlarvae; E, abalone larvae; F, brine shrimp; G, marine rotifers; H, saltwater copepods; I, freshwater zooplankton.

Due to the latter criterion, blue-green algae (cyanobacteria) may be of future importance in aquaculture because of their thin cell walls, and abundant high quality protein, essential fatty acids (FA) and vitamins.

A valuable asset in microalgae used in aquaculture is the protein content, which may amount to more than 60% of dry weight. Since fishmeal is one of

the most expensive components in conventional fish feeding, it would be of commercial relevance if this protein source could be replaced by algal protein. Experiments have shown that this approach is not always successful, e.g. algal protein having been found less effective than fish meal in promoting growth of common carp, but more effective than soya protein (Meske & Pfeffer, 1979).

With certain types of diets, raising the protein content of the feed will lower the fat content in the body to a considerable degree. Indeed, the dietary protein source may influence body fat: feeding carp with three different mixtures varying in protein level produced rather similar feed efficiency ratios and identical protein levels in the fish body, but surprisingly different fat levels. The data agree with the earlier findings that fish fed with algal-containing diets always showed a low level of body fat (Meske & Pfeffer, 1979).

The protein content per cell, considered as one of the major factors determining the nutritional value of microalgae as feed in aquaculture, was found to be more susceptible to medium-induced variation than the other cellular constituents.

21.2.1 Lipids

Lipids are highly significant at various early stages of marine fish larvae, affecting the spawning and the egg quality of many fish species. A deficiency in ($n - 3$) unsaturated fatty acids negatively affects fecundity, fertilization and hatching rates. The quality in addition to the quantity of lipids is, therefore, of prime consideration in the nutritional value of the microalgae in aquaculture.

Various polyunsaturated fatty acids (PUFAs) synthesized by algae are important for the growth of fish, shrimps and molluscs. Deficiency in these acids stems, at times, from an indiscriminate use of algal species in hatcheries and seems to be the major cause of the low survival rates often encountered.

Although there are marked differences in the compositions of the microalgal classes and species, protein is always the major organic constituent, followed usually by lipids and carbohydrates. Expressed as a percentage of dry weight, the range for the content of protein, lipids and carbohydrates are 12–35%, 7.2–23% and 4.6–23% respectively.

The content of highly unsaturated fatty acids (HUFAs), in particular eicosapentaenoic acid (EPA) (20:5 $n - 3$), arachidonic acid (AA) (20:4 $n - 6$) and docosahexaenoic acid (DHA) (22:6 $n - 3$) is of major importance in the evaluation of the nutritional composition of an algal species to be used as food for marine organisms. Significant concentrations of EPA are present in diatom species (*C. calcitrans*, *C. gracilis*, *S. costatum*, *T. pseudonana*), the Eustigmatophyte *Nannochloropsis* spp., as well as the prymnesiophyte *Platymonas lutheri*, whereas high concentrations of DHA are found in the prymnesiophytes (*Pavlova lutheri*, *Isochrysis* sp.) and *Chroomonas salina*.

Some FAs of the ($n - 6$) and ($n - 3$) family have been shown to be essential for many marine animals. The inability of these animals to synthesize long-chain PUFAs 20:5($n - 3$) and 22:6($n - 3$) limits their growth; as a consequence, ($n - 3$) FAs are required in their diet. Similar requirements exist for

the growth and metamorphosis of many larvae in aquaculture. The three FAs which are mainly involved are: 20:4(*n* – 6), 20:5(*n* – 3) and 22:6(*n* – 3) acids. Shorter chain acids, e.g. 18:3(*n* – 6) and 18:3(*n* – 3), are effective for satisfactory survival and growth of some animals, particularly bivalves. The presence of C-20 and C-22 PUFA in their feed, however, increases growth and survival rates. Hence, the quest for good algal diets in aquaculture has led to the search for algae rich in 20:5(*n* – 3) and 22:6(*n* – 3) FAs. There are also indications that at least some bivalves require dietary (*n* – 6) FAs such as the 18:2(*n* – 6) linoleic acid.

Because of the requirements for essential long chain PUFAs, fish farming depends on marine lipids. Dry fish feeds are formulated of 30–60% fishmeal and 10–20% marine fish oil, generally obtained from clupeids. Predictions for the next decade call for 10⁸ t of aquaculture production. With this figure, the 2–3 × 10⁷ t of fish now available, for reduction into meal and oil, will not meet more than 7% of the demand for formulated dry fish feed. Considering the demand by other consumers such as humans and livestock breeding, the shortage of essential PUFA could amount to more than 10⁷ t. Use of microalgae as a commercial source of PUFA, high quality proteins and other nutrients, represents, therefore, a potential solution.

21.3 Astaxanthin

21.3.1 Utilization of astaxanthin in seafood

The pink carotenoid astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) has attracted tremendous commercial interest as a food colour and is primarily used as flesh colourant, added to feeds for fish and to a lesser extent to poultry. The pink flesh characteristic of wild salmon and trout occurs in farmed fish only if this pigment is added to their feed (Bernhard, 1990; Lorenz & Cysewski, 2000). Astaxanthin may also serve as Vitamin A precursor in fish, some species of which appear unable to absorb β-carotene (Torrisen & Christiansen, 1995).

Another algal pigment, i.e. canthaxanthin, is added to the feed and can be found in farmed fish. However, the nutritional and toxicological safety of this pigment in food preparations (and pharmaceuticals, e.g. self-tanning creams) is not proven yet.

Astaxanthin and canthaxanthin, produced almost entirely by chemical synthesis, are the only pigments that can be incorporated into the flesh of fish. Consumer demand for natural products is increasing, however, making the biological sources for these pigments steadily more important. Today, natural sources for astaxanthin are the yeast *Phaffia rhodozyma* with a fairly low pigment content of about 0.4% and the Chlorophyceae *Haematococcus pluvialis* (also referred to as *Haematococcus lacustris* or *Sphaerella lacustris*) with a pigment content up to 4% (see Chapter 18).

The astaxanthin in *Haematococcus* is bound in esters to FAs, whereas it is free in the synthetic product, other differences relating to stereo-isomeric properties.

Astaxanthin supplementation has been reported to increase the amounts of vitamins A, C and E in some body tissues of Atlantic salmon (Christiansen

et al., 1995), although not consistently (Storebakken *et al.*, 1987). It has even been suggested that astaxanthin should be considered an essential vitamin for fish and crustaceans (Torrisen & Christiansen, 1995), or at least a fertilization hormone and growth enhancer (Sigurgisladottir *et al.*, 1994). The pigment also appears to stimulate the immune system in this species, although not enough to warrant inclusion as an immuno-stimulatory agent in feed formulations (Thompson *et al.*, 1995). Indeed, higher survival rates in kuruma shrimp, increased egg buoyancy and survival rate in red sea bream, enhanced liver cell structure and glycogen storage in red tilapia, and increased fertilization and survival rates of eggs, as well as higher growth rates during the early feeding period of young salmonids have all been associated with dietary astaxanthin supplementation in aquaculture (Sommer *et al.*, 1991; Kawakami *et al.*, 1998).

21.3.2 Utilization of astaxanthin in ornamental fish

Astaxanthin plays an important role not only in the cultivation of different types of aquaculture species, but also in the breeding of ornamental fish. The fledgling freshwater ornamental fish industry has experienced the problem of faded colouration in fish, especially when grown in clear water. Experiments adding top-coated algae to the diets of ornamental fish have resulted in colour enhancement. Freshwater red velvet swordtails *Xiphophorus helleri*, rainbowfish *Pseudomugil furcatus* and topaz cichlids *Cichlasoma myrneae*, all became significantly more intensely coloured when fed a diet containing 1.5–2.0% of a carotenoid-rich strain of *Spirulina platensis* and 1.0% of *Haematococcus pluvialis* for three weeks (Tamaru *et al.*, 1998; Ako & Tamaru, 1999).

Colour enhancement appeared to occur via natural carotenoid receptors. Colour intensity diminished, therefore, when fish were stressed, colouration appeared only in males in species in which only the males are normally coloured. Rosy barbs and topaz cichlids colour enhancement was found to be environment-sensitive, topaz cichlid colour developing only after the aquaria were divided into territories, and rosy barb colour intensified when a floating substrate was present.

Traditionally, manufacturers of fish feed use complex formulations, which may result in reduced palatability when introducing carotenoids into their feeds. In the studies of Ako & Tamaru (1999), a mixture of algae supplying natural stereoisomers of β -carotene, zeaxanthin, lutein, canthaxanthin and astaxanthin was used to mimic the absorption of carotenoids which occur in the wild. These formulations should be more acceptable to consumers concerned about the use of chemicals or hormones to enhance colour. The cocktail approach was taken because fish sometimes seem to metabolize carotenoids before depositing them, in a species dependent mode, onto natural receptors in the skin. This was observed for the blue-green fluorescent colours in discus fish *Syphodus* var., which seemed to be enhanced by feeding sources rich in β -carotene. Red colours seemed to be enhanced by feeding algae rich in canthaxanthin and astaxanthin (Katsuyama *et al.*, 1987).

Astaxanthin is presently exempted from certification under the US 21 CFR part 73.35 as a colour additive in fish feed, and *Haematococcus* algae meal is currently in the approval process by the Food and Drug Administration as a colour additive for aquaculture feed. *Haematococcus* algae meal has been approved in Japan as a natural food colour and as a pigment for fish feed. No toxicity associated with *Haematococcus* has ever been reported (Lorenz, 1999).

21.4 Preparation of processed microalgae

21.4.1 As diet in aquaculture

On-site production of microalga in commercial hatcheries is carried out concurrently with larval and post-larval rearing as well as broodstock management.

A possible alternative to on-site algal culture could be the distribution of preserved algae produced at relatively low cost in a large specialized cultivation facility under optimal conditions, using cost-effective production systems. For this purpose, the algae must be concentrated, and preserved or processed. The crucial step is to develop preservation methods which do not alter the nutritional values characteristic of live algae.

Centrifugation of algae into a paste form and subsequent processing until required is used at several places. The limited shelf-life and/or the high prices of the presently available algal pastes have, however, discouraged many growers from using them. Recently, the development of different preservation techniques has extended the shelf-life of microalgal concentrates, which makes it possible to utilize excess and off-season algal production. Outdoor pond production on a large scale has led to the bulk availability of a limited number of algal meals, such as spray-dried or freeze-dried products.

Various products have been tested in the attempt to provide cost-effective alternative diets by substituting live microalgae and simplifying hatchery-nursery procedures. For bivalves, the necessary requisites of an alternative diet are appropriate particle size, stability in the culture system, ease of digestibility, absence of toxicity, and a chemical composition that covers the requirements of the target animal. Costs of algal production are high for rearing bivalves under nursery conditions and may represent about 30% of total bivalve seed production costs. Hence, it is desirable to replace live microalgae with long-term preserved algae that provide good culture performance, facilitating future diversification and a higher level of specialization in the aquaculture industry.

Successful partial replacement of live algal diets has been reported in studies using dried heterotrophically grown *Tetraselmis suecica* (Laing & Millican, 1992), spray-dried *Spirulina* (Zhou *et al.*, 1991), yeast (Coutteau & Sorgeloos, 1993), or artificial diets. However, a complete replacement of live algal diets by any of these feeds has not been achieved, despite intensive research efforts. Reasons for the frequently poor nutritional value of processed diets are low acceptability and poor digestibility, deterioration of the water in the culture due to proliferation of bacteria caused by the diets, lower

stability of the culture system due to higher sedimentation produced by fresh microalgae.

Among the processed diets, concentrated microalgae have so far appeared to be the best alternative products to live algae, especially for bivalves. It is essential that the industrial product be pathogen/bacteria/virus and disease-free. Some of the common methods for algal processing steps follow.

21.4.1.1 Preservation by low temperature

One method for preserving live algae for an extended period of time is to maintain them at a low temperature (+4°C).

McCausland *et al.* (1999) reported on the successful use of cooled microalgal paste composed of *Skeletonema costatum* and *Chaetoceros calcitrans* as supplementary food for the Pacific oyster (*Crassostrea gigas*), which were effective as live algal supplementary diets.

21.4.1.2 Preservation by spray-drying

This is probably the most common technique used to preserve microalgae as sole or supplementary diet in aquaculture.

The utilization of a spray-dried *Schizochytrium*-based feed as a partial substitute for living algae in diets for juvenile clams (*Tapes semidecussata*) and Pacific oysters (*Crassostrea gigas*) was reported by Boeing (1997). Up to 40% of a mixed algal diet (*Tetraselmis suecica* and *Chaetoceros* sp.) could be replaced with *Schizochytrium* without significant reduction in growth rate.

Growth of mussels fed with solely spray-dried *Schizochytrium* was satisfactory, though less than that of mussels fed with live microalgae. It was, nevertheless, possible to improve growth by mixing *Schizochytrium* with 30–50% *Spirulina* and/or *H. pluvialis*, indicating that it was possible to completely replace a living algal diet with spray-dried algae. One of the beneficial effects may be the high protein content of *Spirulina*.

21.4.3 Preservation by freeze-drying

A good method for producing an algal feed with long shelf-life is freeze-drying, resulting in diets that are basically almost identical to fresh diets regarding size, shape and biochemical composition.

In a comparative study using *Isochrysis galbana*, *Tetraselmis suecica* and *Phaeodactylum tricornutum* as diet for a seed culture of the little neck clam (*Ruditapes decussatus*), different means of preservation of microalgae were examined, i.e. fresh, concentrated, frozen and freeze-dried. Growth rates of the seed fed on these algal preparations decreased respectively, according to the above mentioned series and were significantly lower than those achieved with the fresh algal diets. The efficiency of absorption and digestibility diminished (up to 30%) depending on whether the microalgae were administered as concentrate, frozen or freeze-dried (Albentosa *et al.*, 1997). This effect appears to be related to the lower digestibility of the algal cell mass after freezing. Substitution of a proportion of the freeze-dried algae (20%) by

fresh algal material resulted in a significant increase in seed growth rates, which was, nevertheless, still lower than those obtained with a completely fresh diet. These results are in accordance with previous studies using fresh and dried *Tetraselmis suecica* as feed for larvae of the Manila clam *Tapes philippinarum* (Laing & Millican, 1992; Curatalo *et al.*, 1993).

In contrast, the total replacement of fresh *Nannochloropsis gaditana* and *Isochrysis galbana* by freeze-dried preparations of these algae in the mass rearing of larval sea bream (*Sparus aurata*) produced similar growth and survival rates (Canavate & Fernandez-Diaz, 2001). Similar results were obtained with freeze-dried *Nannochloropsis oculata* in rearing seabream larvae. These studies indicate a potential for complete replacement of live microalgae by freeze-dried (albeit not for creating a green water effect) throughout the entire process of mass rearing sea bream larvae. Indeed, there already exists a large industry (e.g. Reed Instant Algae and others), which ships algal paste to many countries. Another technique used for distributing algae is distribution in the form of concentrated algae with glycerol, to avoid freezing.

21.4.2 Usage and potential of *Spirulina (Arthrospira) in aquaculture*

Among all the algae employed in commercial aquaculture, the cyanobacterium *Spirulina (Arthrospira)* probably has the broadest range of applications (for details see Chapter 18). Examples of the use of this pluripotent algae in aquaculture (Belay *et al.*, 1996) follow:

Reports on the utilization of *Spirulina* in aquaculture date to seventies, when the commercial mass cultivation of this microalga was still in its early stages. Already in 1976, studies were performed on the effect of feeding *Spirulina* to bigmouth buffalo (*Ictiobus cyprinellus*) and blue tilapia (*Tilapia aurea*) (Stanley & Jones, 1996). At a daily feeding rate of 29 g of alga kg⁻¹ body weight, the authors obtained a daily growth rate of 14 g kg⁻¹ body weight and a feed conversion ratio of 2.0, both of which were believed to be quite promising for a single-ingredient diet. In contrast, feeding *Spirogyra* to grass carp (*Ctenopharyngodon idella*) resulted in very poor growth and a feed conversion ratio of about 10. These findings led to the conclusion that an aquaculture system based on filamentous green algae and grass carp is less efficient than the one based on *Spirulina* and tilapia or big mouth buffalo. In similar studies with the nile tilapia (*Tilapia nilotica*), methionine-supplemented *Spirulina* replaced fishmeal without any negative effects (Belay *et al.*, 1996).

Successful substitution of formulated diets with up to 50% *Spirulina* was reported by Santiago *et al.* (1989) for the growth of milkfish fry (*Chanos chanos*).

The potential of *Spirulina* as feed for carp fry was tested, in rural development in central India, on six different carp species including silver carp (*Hypophthalmichthys molitrix*), grass carp (*Ctenopharyngodon idella*) and the common carp (*Cyprinus carpio*). The fish were fed with either a control diet composed of a mixture of groundnut oil cake and rice bran, or with the addition of 10% *Spirulina* to this mixture; grass carp and common carp in ponds were also studied by feeding them live algae. In almost all

tests, addition of *Spirulina* to this diet resulted in a better performance of the fish (Ayyappan, 1992).

Feeding the common carp *Cyprinus carpio* with diets in which different amounts of fish meal protein were replaced by *Spirulina platensis* (25%, 50%, 75%, 100%) demonstrated that even 100% replacement by the alga had no negative effects on final weight, specific growth rate, feed conversion ratio and protein efficiency ratio, nor on any other parameter including organoleptic evaluations (Nandeesha *et al.*, 1998). Comparing the nutritive potential of soybean meal, *Spirulina* meal and chicken offal meal as replacement of fishmeal in the rations for the silver sea bream (*Rhabdosargus sarba*) *Spirulina* was utilized by sea bream more effectively than the other two diets. *Spirulina* was employed as a supplement in regular diets, not only in fish rearing, but also in prawn cultivation. The production of the giant freshwater prawn (*Macrobrachium rosenbergii*), significantly improved by *Spirulina* meal. Survival and feed utilization up to a supplementation level of 20% in the feed were improved, probably due to enhancement of protein utilization (Nakagawa & Gomez-Diaz, 1995).

Studies on the suitability of five protein-rich ingredients, i.e. casein prepared with fishmeal, soya oil cake, torula yeast and *Spirulina* sp. (all diets contained 30% protein and 5% fat), as inclusion in formulated diets for the abalone (*Haliotis midea*), displayed significantly higher growth rates for fishmeal and *Spirulina* as compared to the other protein sources. Protein efficiency ratios ranged from 3.3 for the yeast up to 6.5 for *Spirulina* and were higher than those obtained from the control diet (Britz, 1996).

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22 N₂-fixing Cyanobacteria as Biofertilizers in Rice Fields

Pierre Roger

Cyanobacteria constitute the largest, most diverse, and most widely distributed group of photosynthetic prokaryotes (Stanier & Cohen-Bazire, 1977). N₂-fixing forms contribute to maintain the fertility of natural and cultivated ecosystems. Currently, research on their agronomical use has almost exclusively focussed on wetland rice. In the 110 000 000 ha of wetland ricefields, N₂-fixing cyanobacteria occur as indigenous ubiquitous free-living organisms. Research on ricefield inoculation with cyanobacteria was initiated in Japan by Watanabe *et al.* (1951). N₂-fixing cyanobacteria also occur in symbiosis with the aquatic fern *Azolla*, which has been used as green manure for rice since the eleventh century in Vietnam and the fourteenth century in China (Lumpkin & Plucknett, 1982). *Azolla* biotechnology by recombination and sexual hybridization is recent (Wei *et al.*, 1986; Lin *et al.*, 1988).

22.1 Free-living cyanobacteria

22.1.1 Ecology in ricefields

Contrary to early beliefs, N₂-fixing cyanobacteria are ubiquitous in ricefields. Counts in 396 soils of ten countries ranged from 10 to 10⁷ g⁻¹ dry soil (median: 2 × 10⁴) (Roger *et al.*, 1987). In ricefields, cyanobacteria are subjected to large diurnal and seasonal variations in insolation (0–120 k lux), temperature (5–60°C), pH (5–10), O₂ concentration (0–30 ppm) and nutrient status. In arid tropics, high light intensities and temperatures may inhibit their growth at the beginning of the crop cycle. In wet monsoonal zones, they may develop early in the crop cycle during the dry/warm season, but can be inhibited during the wet season because of light deficiency and disturbance by heavy rain. The abundance of N₂-fixing forms is positively correlated with soil pH and available P (Roger, 1996).

The major biotic factor limiting cyanobacteria growth is grazing by invertebrates, mostly ostracods, mosquito larvae and snails. A dense ostracod population may ingest 34 kg ha⁻¹ d⁻¹ (dry weight) of cyanobacteria, ingesting about 1.7 kg N and excreting 1 kg N (Grant *et al.*, 1986). Strains forming mucilaginous colonies being less susceptible to grazing than non-colonial strains, grazing leads to the dominance of mucilaginous cyanobacteria,

especially *Nostoc* spp. (Roger *et al.*, 1987), which are often less active in biological N₂-fixation (BNF) than non-colonial forms (Grant *et al.*, 1986).

Mineral N is known to inhibit BNF by cultures of cyanobacteria. In situ inhibition is less marked and depends upon the method of fertilizer application. Nitrogen broadcasting, widely practiced by farmers, often strongly inhibits photodependent BNF and also causes N losses by ammonia volatilization. In contrast, deep placement reduces the inhibitory effect of N fertilizer on cyanobacteria and decreases N losses by volatilization (Roger, 1996). Phosphorus is a key nutrient for ricefield cyanobacteria, which are often P deficient (Roger *et al.*, 1986). Its application stimulates photodependent BNF and cyanobacteria growth, especially in acid soils.

Cyanobacteria are often more resistant to pesticides than eukaryotic algae, which leads to a selective effect by some algicides and insecticides, which promote cyanobacteria growth. Insecticides inhibit invertebrates that feed on algae (grazers), thus further promoting cyanobacteria and photodependent BNF. However, over the long term, insecticide use becomes detrimental to cyanobacteria by decreasing the diversity of aquatic invertebrates and causing grazers resistant to conventional pesticides to proliferate. Among pesticides other than algicides, herbicides are the most detrimental to cyanobacteria (Roger, 1996).

22.1.2 Free-living cyanobacteria as a source of N

Cyanobacteria can develop impressive blooms. Standing crops of 5–20 ton fresh weight per hectare are usually recorded for growth visible to the naked eye. But because of the low dry matter (0.2–14%) and high ash (31–71%) content, a bloom usually represents less than 10 kg N ha⁻¹ (Roger *et al.*, 1986).

Biological N₂-fixation by cyanobacteria has been mostly estimated from acetylene reducing activity measurements. Published data vary from a few to 80 kg N ha⁻¹ crop⁻¹ (mean: 27 kg). Crop cycle measurements in 180 experimental plots at the International Rice Research Institute (IRRI) ranged from 0.2 to 50 kg N ha⁻¹ crop⁻¹ and averaged 20 kg in no-N control plots, 8 kg in plots with broadcast urea, and 12 kg in plots with deep-placed N (Table 22.1), thus demonstrating the inhibitory effect of N-fertilizer broadcasting on cyanobacteria. A compilation of 211 balance studies in rice soils indicated that with no N-fertilizer applied, BNF averaged 31 kg N crop⁻¹, among which two-third was attributable to cyanobacteria. The theoretical maximum

Table 22.1. Nitrogen fixation during the crop cycle and rice yield under different urea management practices^a (adapted from Roger & Ladha, 1992).

Urea applied (kg N ha ⁻¹)	Estimated N fixed (kg N ha ⁻¹ crop ⁻¹)	Grain yield (t ha ⁻¹)
0 (control)	20 ± 0.14	4.1 ± 0.1
38 at transplanting +17 at panicle initiation	8 ± 0.13	4.8 ± 0.1
55 deep-placed at transplanting	12 ± 0.16	5.8 ± 0.1

^aEach value is the average of 60 measurements.

BNF by cyanobacteria in ricefields can be calculated from reported productivity measurements of the photosynthetic aquatic biomass in floodwater ($0.5\text{--}1.0\text{ g C m}^{-2}\text{ d}^{-1}$). Assuming a biomass composed exclusively of N₂-fixing cyanobacteria (C/N:7) and a primary production of $0.5\text{ t C ha}^{-1}\text{ crop}^{-1}$, cyanobacteria could theoretically provide up to $70\text{ kg N ha}^{-1}\text{ crop}^{-1}$ (Roger & Ladha, 1992).

22.1.3 Effects on rice

Most rice-N originating from cyanobacteria is made available to the crop through mineralization after their death, because N excreted during the buildup of a bloom is either re-immobilized or lost by NH₃ volatilization. Depending on the nature of the material (fresh vs dried), the method of application, and the presence or absence of soil fauna, recovery of cyanobacteria N by rice varies from 13 to 50% (Roger, 1996). Recovery was highest with fresh cyanobacteria incorporated into a soil depleted of fauna. It was lowest with dried cyanobacteria applied on the surface of a soil rich in tubificid worms, which reduce the recovery of algal N by rice by making more soil N available through mineralization. Late decomposition of the bloom during the cycle and the resultant late N-availability to rice increases N content of the grain without increasing yield (Grant *et al.*, 1986).

Other possible beneficial effects of cyanobacteria on rice include (1) competition with weeds, (2) increased soil organic matter content, (3) excretion of organic acids that increase P availability to rice, (4) inhibition of sulfide injury in sulfate-reduction-prone soils by O₂ production, (5) increased water temperature and (6) possible production of plant growth regulators (PGR) (Roger, 1996). However, despite many claims that cyanobacteria can benefit rice plants by producing PGRs, this aspect is still controversial (Pedurand & Reynaud, 1987) and no study shows the isolation and characterization of a cyanobacterial PGR (Metting & Pyne, 1986).

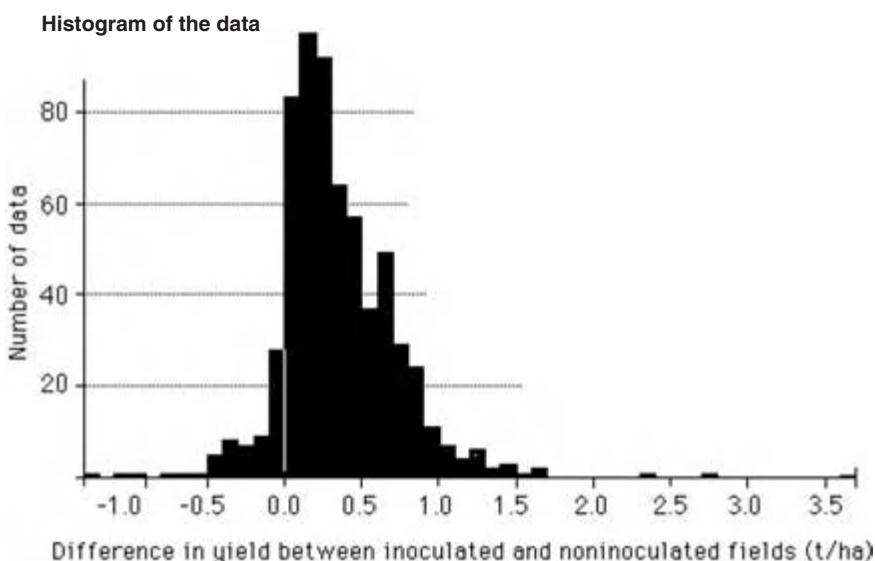
22.1.4 Inoculation of ricefields with cyanobacteria

Applied research on cyanobacteria inoculation has been conducted mostly in India, where the All-India Coordinated Project on Algae was initiated in 1977 and, to a lesser extent, in Burma, Egypt and China. Inocula are usually produced from a multistain starter inoculum produced from laboratory cultures that is propagated on the spot, in open-air trays or microplots with $5\text{--}15\text{ cm}$ water, about 4 kg soil m^{-2} , $100\text{ g superphosphate m}^{-2}$ and insecticide. When necessary, lime is added to adjust pH to $7.0\text{--}7.5$. In one to three weeks, an algal mat develops, which is allowed to dry. Algal flakes are then collected and stored for use at 10 kg ha^{-1} (Venkataraman, 1981).

A compilation of 634 field experiments reported in the literature and in technical reports (Table 22.2) showed a very large variability of the yield difference between inoculated and non-inoculated plots (CV: >100%) (Roger, 1996). Because of the strong asymmetrical data distribution, the median (257 kg ha^{-1}) was considered a better index of the average effect of inoculation than the mean (337 kg ha^{-1}). While the difference in average yield

Table 22.2. Bibliographic study of the effect of cyanobacterial inoculation on rice yield (adapted from Roger, 1996).

	Difference between control and inoculated plots	
	Absolute	Relative
Major statistics of the data		
Number of observations (<i>n</i>)	634	634
Maximum	3700 kg ha ⁻¹	+168.2%
Minimum	-1280 kg ha ⁻¹	-19.3%
Average	337 kg ha ⁻¹	11.3%
Median	257 kg ha ⁻¹	7.9%
Standard deviation	398 kg ha ⁻¹	16.0%
Coefficient of variation	118%	141%



between inoculated and non-inoculated plots was significant at $p < 0.01$, only 17% of the 634 individual observed differences were statistically significant. This indicates a small and variable response of yield to algal inoculation and also an experimental error frequently larger than the response. When interpreting data from the literature, it should also be kept in mind that unsuccessful trials were often not reported as indicated in some reports and confirmed by the asymmetrical distribution of the data. No published study reporting a significant increase in yield after algal inoculation includes BNF or cyanobacteria biomass estimates.

Reports on the adoption of algal inoculation are controversial, but even with the most optimistic evaluations, adoption seems to have been restricted to a limited area in a few states in India, in Egypt, and possibly in Burma. Currently, inoculation of ricefields with cyanobacteria is still not a technology adopted by farmers, which probably reflects the low and erratic increase in yield obtained.

22.1.5 Reconsidering cyanobacteria as a biofertilizer for rice

Methods for utilizing cyanobacteria in rice cultivation need to be reconsidered in view of studies showing that: (1) N₂-fixing forms are ubiquitous; (2) their growth is most commonly limited by low pH, P deficiency, grazing and broadcasting of N fertilizer; (3) non-indigenous strains inoculated in various soils rarely establish themselves (Reddy & Roger, 1988); and (4) indigenous N₂-fixing cyanobacteria are frequently more numerous than cyanobacteria contained in the recommended dose of 10 kg of soil-based inoculum (Roger *et al.*, 1987). More attention should be paid to cultural practices alleviating factors that limit growth and BNF by indigenous strains (liming of acidic soils, P split application, grazer control and deep placement of N-fertilizer). These practices might suffice to realize more of the potential of indigenous cyanobacteria and are a pre-requisite for establishing inoculated strains, if and when inoculation is needed.

Inoculation might be useful because the accumulation of P by the propagules of the inoculum (produced with high levels of P) gives them an initial advantage over the propagules of the indigenous cyanobacteria, which are usually P-deficient. Because spore germination is photodependent, inoculated propagules applied on the soil surface should germinate more readily than the indigenous propagules mixed with the soil. The effect of inoculation is likely to be more significant when indigenous populations have been decreased by an upland crop grown before rice or a long dry fallow. Inoculation might also permit the early establishment of a N₂-fixing bloom and thus the availability of more fixed N to rice. Available data clearly suggest that use of an inoculum produced from the soil to be inoculated should be tested whenever experiments are conducted. Local strain inoculation seems especially advisable after a long dry fallow which often results in partial sterilization in very warm climates. In that case, inoculation should be combined with either deep placement or delayed application of N-fertilizer.

One can speculate on the possibility of selecting for or designing efficient strains for inoculation. Several authors have screened for high N₂-fixing activity, but there is little reason *per se* why high N₂-fixing activity should correlate with in situ colonization potential. In fact, most fast-growing strains (doubling time of 5–12 h) belong to the genus *Anabaena*, have short filaments, and are therefore susceptible to grazing. Another approach has been the selection of nitrogenase-derepressed mutants which excrete NH₄⁺ into the medium. Such an *A. variabilis* mutant was found to provide N to rice in a N-free gnotobiotic culture more efficiently than the parent strain (Latorre *et al.*, 1986). However, studies at IRRI showed that the strain was not competitive and did not multiply when inoculated in soil (Roger *et al.*, unpublished).

Biological engineering of cyanobacteria is currently limited to unicellular strains that are morphologically, physiologically and ecologically very different from the N₂-fixing strains considered for inoculating ricefields. Probably *super N₂-fixing cyanobacteria* can be selected or designed and grown in test tubes, but the characteristics that will enable them to develop in ricefields are still largely unknown.

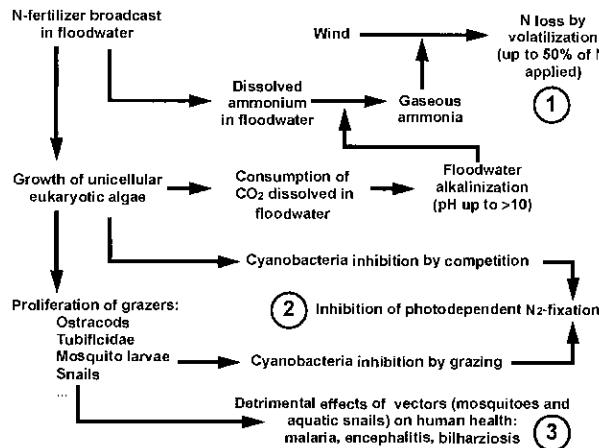


Fig. 22.1. The three major effects of N fertilizer broadcasting in ricefield floodwater.

It can be concluded that after several decades of experiments, inoculation is not a proven technology. As cyanobacteria are ubiquitous in ricefields, a specific management may insure the expression of their potential ($10\text{--}30 \text{ kg N crop cycle}^{-1}$) through grazer control combined with P application and deep placement of N-fertilizer, but this is rarely economically viable. N-fertilizer deep-placement on its own is definitely the best technology to take advantage of the potential of indigenous cyanobacteria. It avoids the proliferation of unicellular eukaryotic algae, which causes floodwater alkalization and N losses by ammonia volatilization. It avoids the early proliferation of predators of microalgae and vectors of human diseases (ostracods, mosquito larvae, etc.). It allows N₂-fixing cyanobacteria growth, otherwise inhibited by competition and grazing (Fig. 22.1). A large set of experiments in non-inoculated ricefields have demonstrated the efficiency of this technology in increasing rice yield by promoting photodependent BNF and improving N-fertilizer efficiency (Table 22.1). A better understanding of the factors that could allow inoculated non-indigenous strains to establish *in situ* is needed before cyanobacterial inoculation could be reconsidered as an agricultural practice in rice cultivation.

22.2 Symbiotic cyanobacteria: Azolla

The only symbiosis between cyanobacteria and plants of agronomic significance is that between *Anabaena azollae* and the freshwater fern *Azolla*. The endophytic *Anabaena* resides within a basal cavity of each leaf of *Azolla*. The association may proliferate without mineral N.

Azolla has been used as green manure in ricefields for centuries, in North Vietnam and South China, because of its ability to grow rapidly together with wetland rice and its high N content. It prefers areas and seasons with a cool temperature (daily average around 25°C), because at higher temperatures *Azolla* growth is reduced, and damage by insect pests is severe. Usually, *Azolla* is grown for one month and incorporated before transplanting.

Sometimes, *Azolla* is grown before and after transplanting. To a lesser extent, wide-row transplanting permits *Azolla* cultivation with rice and several incorporations during the crop cycle. This technology requires a regional network for providing farmers with fresh inoculum because *Azolla* is maintained and multiplied only vegetatively. *Azolla* biomass production by farmers then requires labor-intensive agricultural practices, including repetitive fertilization with P, K, farm-yard manure, or ash; insect control; protection against washing-out by rains; one or several incorporations, with sometimes pre-composting of part of the *Azolla* crop (Roger & Watanabe, 1986).

22.2.1 Ecology in ricefields

Azolla is widely distributed throughout the world (Lumpkin & Plucknett, 1982), but to develop significant biomass in ricefields it needs to be introduced and cultivated.

The optimum temperature for most species (20–30°C) is below the average temperature in the tropics. Cool weather is a key to successful *Azolla* cultivation in Vietnam and China. *Azolla* grows optimally at pH 4.5–7.0 and survives within a range of 3.5–10. The effect of pH is probably mostly related to nutrient deficiencies (Fe, Zn, etc.) occurring at high values, or toxicities (Al, Fe, etc.) occurring at low values (Lumpkin & Plucknett, 1982).

Phosphorus is the major limiting nutrient for *Azolla* growth in situ. Reported threshold values of P deficiency are 0.4% in *Azolla* (dw basis), 0.15 ppm in floodwater, and 20 ppm available Olsen P in soil (Watanabe & Ramirez, 1984), therefore P fertilization of *Azolla* is required in most soils.

Recorded *Azolla* pests include 30 insects, two Arachnoidea, and nine Mollusca. Yield losses of field-grown *Azolla* due to insects may reach 60% (Mochida *et al.*, 1985). Pathogenic fungi developing on plant wounds magnify the effects of insect attacks. *In vitro*, BNF by *Azolla* is more tolerant of combined N than BNF by free-living cyanobacteria. *A. caroliniana* grown in various nutrient solutions at 35 ppm N, derived more than 60% of its N from BNF (Peters *et al.*, 1981). In situ, however, N favors the growth of competing aquatic plants that may hinder *Azolla* growth.

Herbicides are toxic to *Azolla*. A beneficial effect of *Azolla* is its weedicide effect, therefore there is little interest in combining *Azolla* use with that of herbicides. Insecticides usually favor *Azolla* growth by decreasing pest incidence.

22.2.2 Azolla as a source of N

Biological N₂-fixation (BNF) by *Azolla* has usually been estimated by measuring biomass and assuming that *Azolla* N mostly originates from BNF, which was confirmed by the ¹⁵N dilution and the δ¹⁵N methods. In experimental plots, N in maximum standing crops ranged from 20 to 146 kg ha⁻¹ and averaged 70 kg ha⁻¹ (*n*: 17; cv: 58%). N₂-fixing rate ranged from 0.4 to 3.6 kg N ha⁻¹ d⁻¹ and averaged 2 kg N ha⁻¹ d⁻¹ (*n*: 15; cv: 47%) (Roger & Watanabe, 1986). In four-year field trials at 37 sites in ten countries,

productivity was lower than in experimental plots: 10–50 kg N ha⁻¹ (average 30 kg N ha⁻¹) (Watanabe, 1987).

22.2.3 Effects on rice

Azolla N becomes available to rice upon decomposition and mineralization. Nitrogen recovered by rice from ¹⁵N-labeled *Azolla* ranged from 20 to 34% and was higher when *Azolla* was incorporated into the soil (see Roger, 1996). *Azolla* has other beneficial effects. The threshold concentration of K for absorption from floodwater is lower for *Azolla* (0.85 ppm K₂O) than for rice (8 ppm) (Liu ChungChu, 1988). As irrigation water usually contains 1–5 ppm K, *Azolla* absorbs and concentrates K thus becoming a source of K for rice when incorporated. *Azolla* decreases weed incidence and water evaporation (Lumpkin & Plucknett, 1982). It improves soil structure as do most organic manures; that becomes important when an upland crop is grown after rice. Field trials for four years at 37 sites in ten countries (Watanabe, 1987) showed that incorporating one crop of *Azolla* grown before or after transplanting was equivalent to application of 30 kg N ha⁻¹, and incorporating two *Azolla* crops grown before and after transplanting was equivalent to split application of 60 kg N ha⁻¹.

22.2.4 Current use

In the late 1970s *Azolla* was probably used on more than 2 000 000 ha of ricefields in China and Vietnam (Lumpkin & Plucknett, 1982). In the 1980s, studies were conducted to improve the technology, and trials were established in Brazil, India, Pakistan, Senegal, Sri Lanka and Thailand to study the feasibility of *Azolla* adoption by rice farmers (IRRI, 1987). However, during the 1980s, the area devoted to *Azolla* in Vietnam and China decreased dramatically and *Azolla* use did not spread to other countries. In Vietnam and China this was partly attributed to (1) the advent of cheap sources of urea and potash, (2) the changing governmental economic policies and (3) a change from transplanted rice – favorable to *Azolla* growth – to direct seeded rice – where early growth of *Azolla* is detrimental. Now, *Azolla* use has become incidental in China and Vietnam.

22.2.5 Factors limiting *Azolla* use and possible methods for alleviation

Major constraints limiting *Azolla* use include: difficulties in maintaining inocula throughout the year, P deficiency, low tolerance to high temperature, damage by pests, poor water control, and economics in relation to changing agricultural practices and policies.

Phosphorus application is needed in most soils for growing *Azolla*. To be economically feasible, it requires a ratio of N fixed to P applied greater than the ratio of the prices of the N- and P-fertilizers (four in most Asian countries). As P-fertilizer is rapidly immobilized in the soil, basal application is often uneconomical while split application has an efficiency of

5–10 g N₂ fixed · g⁻¹ · P applied (Watanabe *et al.*, 1988), but requires more work.

Although commercial pesticides control *Azolla* pests, no method of field application is economical (IRRI, 1987). The possible use of insecticides is limited to inoculum production.

Azolla strains exhibit a wide range of behavior with regard to environmental factors, P requirement, BNF, productivity, etc. The ability to combine favorable characters such as resistance to high temperature and insects, low P requirement and erect growth (permitting higher productivity) would allow strains to be designed for specific agroecological conditions. For this purpose, recombination of different cyanobacterial and plant symbionts (Lin *et al.*, 1988) and sexual hybridization between species (Wei *et al.*, 1986) proved feasible. However, producing *Azolla* hybrids requires macrosporocarps and microsporocarps and no satisfactory method is yet available to induce sporocarp formation and germination at will. This also prevents using sporocarps for inoculum maintenance and distribution to farmers. A method for *Azolla* conservation as sporocarps was developed in China, but their growth was too slow to meet inoculum requirements *in situ* (Lu, 1987).

The major limiting factor remains economics, because *Azolla* technology is labor intensive. Economic studies in the Philippines concluded that under favorable conditions (soil exceptionally rich in available P) the labor cost becomes critical where wage rates approach \$2 per day (Kikuchi *et al.*, 1984) whereas in other areas *Azolla* use was uneconomical (Rosegrant & Roumasset, 1988).

22.2.6 What future for *Azolla*?

Azolla still has a future as green manure in areas where it can spread spontaneously and where pest incidence is low because of a dry climate, such as some sahelian and sub-tropical areas.

Azolla has also potential as a multipurpose biofertilizer, feed for pigs and chickens, and primary producer in rice–fish cultivation. In a rice–fish–*Azolla* system in China, average rice yield increased by 5% over the traditional system and fish production averaged 625 Kg ha⁻¹. Weed biomass and pest incidence decreased (Liu, 1988). The economics of integrated *Azolla* culture might be more favorable than green manuring alone.

The recent interest of European and American consumers in *organic* agriculture, offers another opportunity to use *Azolla* to produce *bio* or *organic* rice (produced without agrochemicals), a label that gives it a higher commercial value than rice produced with agrochemicals.

22.3 Conclusion

Among N₂-fixing microorganisms found in ricefields, cyanobacteria play a major role. Free-living indigenous forms have spontaneously achieved modest but constant yields (1–2 t ha⁻¹) to be obtained for centuries without N-fertilizer. Symbiotic forms were also used for centuries in China and Vietnam, allowing significant yields (2–5 t ha⁻¹) to be obtained through a labor-intensive and quite sophisticated green manuring technology.

Research on cyanobacteria as biofertilizers for rice reached intensive development in the 1970s and the 1980s. Since then, the number of research papers dealing with these topics has strongly declined.

Free-living cyanobacteria have a modest potential of about $30 \text{ kg N ha}^{-1} \text{ crop cycle}^{-1}$ which may translate to a yield increase of $300\text{--}450 \text{ kg ha}^{-1}$. Inoculation of ricefields with selected or transformed strains is not a proven technology. On the other hand, the knowledge acquired on the general ecology of the ricefield floodwater has allowed practical recommendations to optimize its management in a way that favors BNF by cyanobacteria, decreases N-fertilizer losses, and helps controlling detrimental invertebrates. Over the long term, biological engineering may design *super N₂-fixing cyanobacteria* but the characteristics that will enable them to survive, develop and fix N₂ in ricefields are still unknown.

Azolla has proven useful as a biofertilizer in China and Vietnam. Currently *Azolla* use has drastically diminished in these countries and has not been adopted in the countries where it was tested. Progress in strain improvement has opened ways to alleviate many environmental and nutritional limitations of *Azolla*. However, socio-economic limitations are the most important and are increasing in relation to changes in agricultural policies and practices. Some studies have shown that *Azolla* also has potential as a multipurpose biofertilizer, an animal feed and a primary producer in rice–fish–*Azolla* cultivation. The potential of *Azolla* as a multipurpose crop, may revive interest in its use.

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23 Hydrogen and Methane Production by Microalgae

John R. Benemann

23.1 Background

Microalgal production of H₂ gas, first reported over 100 years ago (Jackson & Ellms, 1896), became a subject of scientific investigation starting in the 1940s, and applied R&D was initiated in the early 1970s (Benemann & Weare, 1974). Some 30 years later and after many hundreds of publications later, advances toward practical applications in this field have been negligible (Benemann, 1997, 1998). CH₄ production through fermentation of microalgae biomass was first proposed as a method for solar energy conversion almost 50 years ago, first demonstrated in the laboratory and then conceptually developed in an initial techno-economic feasibility analysis for large-scale energy production incorporating wastewater treatment (Oswald & Golueke, 1960). Only a few, small-scale, practical applications of this concept have been achieved.

Microalgal production of liquid fuels (biodiesel) was the subject of a major (some \$50 million) R&D program in the US, carried out from about 1980 to 1994 (Sheehan *et al.*, 1998). In Japan, a major (several hundred million dollars) R&D effort for microalgae greenhouse gas mitigation and fuel production, including H₂, was carried out during the 1990s. In Germany, a major biohydrogen R&D program started in 1990 and investigated, among others, microalgal H₂ production, but was discontinued only after five years. Since the early 1990s, the U.S. Dept of Energy has supported a smaller (\$15 million funding) ongoing R&D effort in microalgal H₂ production. Research is also currently being carried out in this field in a number of laboratories in Europe. The lack of practical results in H₂ and CH₄ production can, thus, not be ascribed to limited R&D funding.

The present review critically examines the potential of microalgae for the production of gaseous fuels, H₂ and CH₄, and assesses the various proposed schemes from a technological perspective.

23.2 Efficiency of photosynthesis

For microalgae mass cultures, in particular for fuel production processes, solar energy conversion efficiency is of fundamental importance. It is generally agreed that, in theory, photosynthesis, and microalgal cultures

specifically, could achieve a 10% total light energy conversion into a primary product, such as CO₂ fixed into biomass or H₂ (Bolton, 1996). However, such extrapolations are based on theoretical considerations and on data obtained under low light conditions. Under full sunlight intensities much lower efficiencies are observed, typically only about 3% or less. As pointed out by Kok (1973), in connection with H₂ production, the rate of light capture by photosynthetic pigments (chlorophyll, etc.) is roughly ten times higher under full sunlight than the rate of electron transfer between the two photosystems (PS I and PS II) operating in series. This results in up to 90% of the solar photons captured in full sunlight by the photosynthetic apparatus (e.g. absorbed by chlorophyll and other pigments) not being used for photosynthesis and decaying as heat or fluorescence, a prodigious waste. In any microalgal bioreactor, open ponds or closed photobioreactors, not all cells are at the highest (closest to the surface) or lowest (near the bottom, where only dim light penetrates) light intensities, allowing for some effective dilution of the light. Still, this so-called *light-saturation effect*, reduces algal photosynthesis and, thus, potential fuel production, by some three- to four-fold, compared to the theoretical maximum or as seen in the laboratory at low light intensities. Various solutions to this problem were proposed already some 50 years ago: rapid mixing of the cultures, dilution of light incident on the surface of the cultures and algal mutants with reduced chlorophyll contents.

Very rapid mixing can create in the turbulent eddies surrounding the algal cells the *flashing light effect*, first demonstrated by Kok (1973). He observed that millisecond flashes of high intensity light, followed by an approximately five- to tenfold longer dark period, maximized overall photon use efficiency. The reason is that during a brief flash of high intensity light each photosynthetic unit, containing several hundred chlorophyll molecules acting as a light-capturing antenna, captures only one photon, while the subsequent longer dark phase allows for the slower transfer of electrons between the two photosystems. However, achieving the flashing light effect in algal cultures requires very high mixing power inputs, which is not practical, in particular, for low-cost fuel production processes. Some mixing is, however, required in algal mass cultures to supply nutrients (in particular CO₂), to prevent settling and maintain uniformity of the culture. In practice, mixing velocities of 30–35 cm sec⁻¹ should not be exceeded, as power inputs for mixing increase as a cube function of velocity. The effect of mixing regimes on algal mass culture productivities is somewhat controversial. Controlling confounding variables (pH, O₂ concentrations), Weissman *et al.* (1988) observed no enhancement of productivity over a wide range of mixing velocities (5–60 cm sec⁻¹) for one green algal strain. However, others view mixing and the light regime periodicities experienced by individual algal cells in mass cultures, as having a profoundly positive effect on productivity (Richmond, 1996, 2000). This topic is still a subject for active research.

The use of light attenuation devices that transfer sunlight into the depth of a dense algal culture is another approach to overcoming the light saturation effect. The simplest approach is to arrange photobioreactors into closely

packed vertical arrays, to reduce direct sunlight intensities at the culture surface. However, this arrangement increases the number (area) of photobioreactors, which is the limiting economic factor in any photobiological fuel production process. An alternative is optical fiber photobioreactors, in which light energy is collected by concentrating mirrors and piped into photobioreactors by optical fibers. This idea was first studied in the U.S. some 25 years ago by a small study of microalgal H₂ production (Manley & Pelofsky, 1978, unpublished). More recently, this concept became the centerpiece of the very large R&D effort carried out in Japan on microalgae biofixation of CO₂ and greenhouse gas mitigation, including H₂ production, even though it is clearly technically and economically completely impractical.

The more practical approach is to find or genetically construct mutants of algal cells with reduced pigment content, that is, with smaller amounts of so-called *antenna* chlorophyll or other *light harvesting* pigments (e.g. phycobiliproteins in cyanobacteria). Simply put, a photosynthetic apparatus with less light harvesting chlorophyll absorbs fewer photons at high light intensities, and, thus, also wastes fewer photons. Of course, as already pointed out by Myers (1957), we cannot expect to find such algal strains in nature, as they would be strongly selected against by evolution. This is because, in nature, as in algal mass cultures, cells are intermittently exposed to both high (at or near the surface) and low (deeper in the culture) light intensities, resulting, overall, in the evolutionary selection for strains with more light harvesting (*antenna*) pigments per photosynthetic reaction center. These are able to, on average, capture more photons per cell, thus have higher productivity than strains with fewer light harvesting pigments, even though they waste most of the captured photons when near the culture surface. In other words, in such a competitive situation each cell will strive for the largest antenna size possible, even though this results in an overall reduction in culture productivity. Although recognized over 50 years ago, the idea of increasing productivities by reducing antenna sizes remained dormant (Benemann, 1990).

This approach was revived in recent years by the author, both in Japan, as a consultant to Mitsubishi Heavy Industries, and in the U.S., through initiation of a research project at the University of California (UC) Berkeley. In Japan, this research (Nakajima & Ueda, 1999; Nakajima *et al.*, 2001) demonstrated that microalgal mutants with reduced antenna sizes exhibited a 50% increase in productivity in continuous laboratory cultures operating at high light intensities, compared to the wild type. Although not as large an increase as may be expected, this is a significant achievement and demonstration of the potential of this approach. At UC Berkeley, the initial approach was physiological, using cultures of the green alga *Dunaliella salina*. Under stress conditions, the algae exhibited both damaged PS II centers and reduced antenna sizes; when the stress was relieved the PS II centers were repaired before their antenna sizes increased. This allowed for a brief period of relatively high rates of photosynthesis under high light intensities (Melis *et al.*, 1999; Nakajima *et al.*, 2001). More recently, antenna mutants have been isolated and studied (Polle *et al.*, 2001), though efficiency increases at high light intensities were limited. An alternative approach has also been

proposed, based on algal mutants deficient in the PS I complex but that were reportedly still able to produce H₂ and fix CO₂ (Greenbaum, 1988). This so-called *PS II-only* photosynthesis, by requiring only one photon per electron transport from water to CO₂, or H₂, was proposed to be able to, in effect, double the overall solar conversion efficiencies. However, that work proved to be irreproducible (Redding *et al.*, 1999), though it was never retracted by the authors.

The goal of microalgae R&D for fuel production must be to increase productivities of outdoor cultures to the highest degree possible, which plausibly is some threefold from the current maximum of about 3% of solar energy converted into algal biomass. That would make microalgal cultures even more efficient than sugarcane cultivation in the tropics, the currently highest productivity photosynthetic process. Achieving this goal, indeed even demonstration that it would be possible to do so, still remains to be accomplished, but is required if microalgal photosynthesis is to produce fuels in practical processes. The mechanisms by which microalgal photosynthesis could yield gaseous fuels are addressed in the remainder of this review.

23.3 Microalgal hydrogen metabolism

In developing H₂ production processes a major challenge is the complexity of H₂ metabolism by microalgae. These exhibit multiple, often simultaneous, H₂ metabolizing reactions, taking place in the dark or light and involving both H₂ uptake (e.g. the oxy–hydrogen reaction, H₂-supported CO₂ fixation) and evolution, from endogenous storage materials, exogenous substrates or water (Hallenbeck & Benemann, 1979; Boichenko & Hoffman, 1994; Benemann, 1996; Schulz, 1996; Benemann, 2000a; Melis & Happe, 2002; Tamagnini *et al.*, 2002). Further complexity is due to the several distinct enzymes involved in H₂ metabolism, including several types of hydrogenases (uptake and reversible, with both Fe or Fe–Ni containing active sites) and nitrogenases (also with various metal-containing active sites). Different microalgae, or even strains of the same species, can exhibit widely differing rates of H₂ evolution under similar conditions. For example, unlike green algae, cyanobacteria do not produce H₂ in a hydrogenase-catalyzed light-driven reaction, evolving H₂ fermentatively only in the dark (Aoyama *et al.*, 1997).

Cyanobacteria do evolve H₂ in the light by action of nitrogenase. Making a virtue of necessity, most cyanobacterial H₂ production R&D has focused on nitrogenase-based H₂ evolution, despite the high ATP requirement by this enzyme. This reduces the overall efficiency of the process by at least half, compared to a reversible hydrogenase-based process, and makes this approach unacceptable from a practical perspective (Benemann, 1998). The initial justification for using nitrogenase-based systems was that these could serve as models for the future development of hydrogenase-based process (Benemann & Weare, 1974; Weissman & Benemann, 1977). However, research on H₂ production by nitrogen-fixing cyanobacteria has continued well past the point of diminishing results. In particular, most research in this field used nitrogen-fixing heterocystous cyanobacteria. However, these are even

more inefficient than non-heterocystous cyanobacteria, due to the high maintenance energy of heterocysts (Turpin *et al.*, 1985). Also, by simultaneously producing H₂ and O₂, heterocystous cyanobacteria exhibit all the drawbacks of direct biophotolysis detailed below (except for O₂ sensitivity). A further discussion of the extensive literature on cyanobacterial H₂ production would thus not be useful in the present applied R&D context.

The molecular biology of reversible hydrogenases responsible for H₂ evolution in cyanobacteria and green algae, and of the reductant transport pathways that support them, has lagged somewhat behind that of other microbes (Schulz, 1996; Hansel & Lindblad, 1998; Wuenschiers *et al.*, 2001; Tamagnini *et al.*, 2002). Still, as our basic knowledge of these systems expands, it would, at least in principle, become possible to combine in a single cell an enhanced hydrogenase-mediated H₂ evolution process coupled to a highly efficient photosynthetic process. However, even the development of such strains would be far from sufficient for practical applications, as any H₂ production process must be able to be scaled up into a plausibly economically viable process. As argued below, this is not likely achievable for most of the processes thus far proposed for microalgal H₂ production.

23.4 Direct biophotolysis

In a direct biophotolysis reaction, the reductant (reduced ferredoxin) generated by photosynthesis is directly used by a hydrogenase for proton reduction to H₂, instead of the physiological acceptor NADP, resulting in a simultaneous production of O₂ and H₂. Initially this reaction was demonstrated *in vitro* with a chloroplast–ferredoxin–hydrogenase system (Benemann *et al.*, 1973), although the existence of such a reaction in green algae had been suggested earlier (Spruit, 1958). This reaction has been studied since then by many researchers. A photon energy conversion efficiency of some 22% of visible light energy into H₂ by a direct biophotolysis process, corresponding to a solar conversion efficiency of some 10%, was demonstrated *in vivo* with the green microalga *Chlamydomonas reinhardtii* in laboratory experiments under low light intensities and very low partial pressures of O₂ (Greenbaum, 1988). These conditions avoided the light-saturation effect and inhibition by photosynthetically produced O₂. Although not specifically studied, it appears that for simultaneous H₂ and O₂ production, the O₂ partial pressures must be below 0.1%, which is less than one micromolar in the liquid phase. It would not be possible to maintain such low partial pressures in a practical direct biophotolysis process, due to the large amount of diluent gas and power inputs required for gas transfer. A direct biophotolysis process must therefore operate at a partial pressure of near one atmosphere of O₂, several thousand-fold greater than the maximum tolerated. Thus, the O₂ sensitivity of the hydrogenase enzyme reaction, and of the supporting reductant generation and transport pathways, remains the key problem, and it has been for the past 30 years in this field.

Approaches to overcome this problem include the use of O₂ absorbers, both irreversible (glucose/glucose oxidase, dithionite) and reversible (Rosenkranz &

Krasna, 1984). Use of irreversible or even regeneration of reversible O₂ absorbers would not be practical in any scaled-up process. Endogenous respiration by microalgae can reduce O₂ levels and allow a direct biophotolysis reaction, as can be deduced from a recent study with *Chlamydomonas reinhardtii* (Melis *et al.*, 2000) (further discussed below). However, endogenous respiration decreases by half the H₂ potentially evolved, as for each H₂ produced an equivalent amount of substrate is respired. This is as unacceptable a loss of efficiency as that of nitrogenase-based systems. Any practical direct biophotolysis process must be highly resistant to O₂ inhibition. However, no such reactions are known. Uptake hydrogenases have been made more O₂ tolerant through protein engineering (McTavish *et al.*, 1995), but these enzymes operate at a much higher redox potential than reversible (H₂ evolving) hydrogenases. Mutants exhibiting increased tolerance to O₂ of H₂ evolution have recently been reported (Ghirardi *et al.*, 1997; Seibert *et al.*, 1998), but likely are increased respiration phenotypes, thus providing only an appearance of enhanced O₂ resistance, and would have greatly reduced efficiency of light energy conversion. Achieving O₂ stability of H₂-evolving hydrogenase reactions is problematic and a very basic, not an applied, R&D problem.

Even if the problem of O₂ inhibition were to be somehow overcome, direct biophotolysis processes require that the entire production area be enclosed in photobioreactors, able to both produce and capture H₂ and O₂. Few cost estimates are available for large-scale closed photobioreactors. One conceptual study (Tredici *et al.*, 1998) of photobiological H₂ production, based on an inclined tubular bioreactor design with internal gas exchange, arrived at the very optimistic capital cost of only \$50 m⁻². This analysis assumed very low capital charges and minimal operating costs, extrapolating H₂ production costs of only \$15 GJ⁻¹ for a direct (or other single-stage) biophotolysis process operating at 10% solar conversion efficiency. However, in that study many cost components (contingencies, engineering, gas separation and handling) were not included, and most other costs (for materials, assembly, etc.) were speculative, lacked detail and were highly uncertain. For example, plastic tubes are not likely to retain H₂, requiring glass tubes at substantially higher costs. A more plausible cost for such photobioreactors would likely be at least \$100 m⁻², plus contingencies and engineering cost factors (Bennemann, 1998). This would make any direct biophotolysis and other single-stage processes (e.g. heterocystous cyanobacteria) uneconomical, even if a highly efficient and O₂ resistant hydrogenase-based process could be developed. Indeed, handling H₂/O₂ mixtures in large volumes over larger areas would likely be impractical. In conclusion, direct biophotolysis and similar single-stage processes (e.g. heterocystous cyanobacteria) do not warrant continued R&D.

23.5 Indirect biophotolysis processes

Indirect biophotolysis processes separate the H₂ and O₂ evolution reactions into different stages, coupled through CO₂ fixation/evolution. One technolo-

economic analysis of this concept involved four distinct steps (Benemann, 1998):

1. Production in open ponds, at 10% solar efficiency, of a nitrogen (or similar nutrient)-limited biomass that accumulates large amounts of storage carbohydrates.
2. Concentration of the biomass from the ponds in a settling pond.
3. Anaerobic dark fermentation to yield four H₂/glucose stored in the algal cells, plus two acetates.
4. A photobioreactor in which the algal cells would convert the two acetates to eight moles of H₂.

After this last step, the algal biomass would be returned to the ponds, to repeat the cycle. Required support systems include the anaerobic digestion of wasted biomass (10% per cycle), an inoculum production system to provide makeup biomass and a gas handling and separation system (to allow utilization of the H₂ and recycling of the CO₂ back to the ponds).

The cost estimates for this highly conceptual process were based on prior work on the economics of large-scale microalgal mass cultures in open ponds (Benemann, 1997), which projected total system costs at some \$7 m⁻². The photobioreactors, requiring only one-tenth the area of the ponds, were not further specified and merely assumed to cost \$135 m⁻² (including contingencies, engineering and overheads). Overall, costs of such a process were estimated at about \$10 GJ⁻¹ of H₂ (with a H₂ gas handling but without a CO₂ recycle system). The photobioreactors were about half of the total costs. Gas handling and separation was also expensive, costing as much as the pond systems.

This design was based on the assumption that less than one photon would suffice to drive evolution of one H₂ from acetate. However, this assumption was not supported on review by an expert panel, which with a minimum of two photons per H₂ produced judged a more realistic assumption (Benemann & San Pietro, 2001). This would almost triple the photobioreactor area required and double the overall costs of the H₂ produced. Clearly, such preliminary cost estimates of conceptual processes are useful mainly to identify major *show stoppers* and areas where R&D could result in significant cost-reductions. In this case, the photon requirement for light-driven H₂ evolution is the main impediment. Other key issues are the design, performance and cost of the photobioreactors. Even the Tredici-type reactor (Benemann, 1998), which was suggested as the most appropriate, that is affordable, for biological H₂ production (Szyper *et al.*, 1998), is likely to be too costly, even for indirect biophotolysis applications. Finally, the process requires both very high solar efficiencies and high carbohydrate contents, with nitrogen-limited algal cultures – both challenging goals.

Recently a novel biophotolysis process was claimed, based on the green alga *Chlamydomonas reinhardtii* (Melis *et al.*, 2000) and differing from the conceptual system, just described, mainly by eliminating the dark fermentative stage and by using sulfur, rather than nitrogen, as the limiting nutrient. Although the authors (Melis *et al.*, 2000) claimed that this was an indirect biophotolysis process, an analysis of the data (Benemann, 2000b) revealed

many experimental lapses and discrepancies, most fundamentally that this was actually a direct biophotolysis process, with respiration taking up the O₂ produced. This conclusion was subsequently confirmed (Ghirardi *et al.*, 2000; Zhang *et al.*, 2002), though not before a major, worldwide reporting on this novel *discovery*. Regardless of publicity, or the actual mechanism, the rates of H₂ production were very low, even compared to the very modest yields thus far reported with other microalgae systems. Further, the respiratory O₂ removal reaction doubles photon requirements (to at least ten photons per H₂, not five as wrongly claimed by these authors (Melis & Happe, 2002)) and makes such a process inherently impractical, as already mentioned above. In conclusion, not presently conceived, let alone experimentally demonstrated photobiological H₂ production concept can be considered suitable for practical process development, even in the long term. The more plausible approach is to produce H₂ from microalgae by dark fermentations, as discussed next.

23.6 H₂ and CH₄ fermentations by and of microalgae

One way to eliminate the photobioreactors, the major limitation in any practical biophotolysis process, would be to harness the endogenous metabolic machinery of algal cells to quantitatively convert stored carbohydrate (starch or glycogen in green algae and cyanobacteria, respectively) to H₂ in a strictly dark reaction. That would simplify the overall process to that of algal biomass production in large open ponds, under conditions inducing high content of carbohydrates, followed by a dark fermentative stage. The major issue is the feasibility of dark fermentative reaction being able to yield close to the stoichiometric 12 moles of H₂ stored in each mole of glucose. However, currently fermentations yield at most four H₂ and two acetates for each glucose, this allowing for ATP formation and, thus cell growth, which a yield of 12 moles of H₂ per glucose would not. This is why no high-yielding dark H₂ fermentations are known in microalgae, or in any other microbes for that matter.

However, metabolic pathways exist in microalgae and other microorganisms (such as the oxidative Pentose Phosphate Pathway, PPP), which can, in principle, produce essentially stoichiometric amounts of H₂ from glucose. Indeed, this was recently demonstrated with an *in vitro* system using bacterial PPP enzymes (Schulz, 1996), with stoichiometric H₂ yields from glucose obtained. However, as expected, such high yields are only achieved with very slow rates and at very low H₂ partial pressures, making such a reaction impractical. To help drive the reaction, additional metabolic energy must be supplied by dark reactions, such as by respiration, rather than photosynthetic light reactions. In this concept, respiration-driven H₂ evolution, acting through a so-called *reverse electron flow* reaction, would replace photosynthesis as the driving force for H₂ evolution from accumulated storage carbohydrates. Of course, respiration, under O₂-limited conditions, would reduce the overall H₂ yield, but plausibly to only ten moles H₂ per mole glucose, which is still a high yield, compared to what can be currently obtained from CH₄.

fermentations and sufficient for a practical process. Dark anaerobic metabolism and H₂ evolution in green algae and cyanobacteria have been studied (Stal & Moezelaar, 1979; Gibbs *et al.*, 1986; Aoyama *et al.*, 1997) and some incidental reports suggest O₂ stimulation of dark H₂ evolution (Benemann & Weare, 1974). Of course, the metabolism of algal cells would need to be fundamentally re-engineered to couple such a respiratory process to the low redox electron transport pathway required for support of hydrogenase-mediated H₂ production. However, in principle and perhaps in future practice, this approach appears to offer the best approach to overcome the practical limitations identified above for both direct and indirect biophotolysis processes.

Alternatively, if H₂ proves too difficult to obtain at high yields and low costs, or does not provide any specific advantage, CH₄/CO₂ (*biogas*) fermentations of algal biomass is already a proven technology and provides a more immediate and practical route to gaseous fuel production by microalgae. Another possibility is a mixed H₂/CH₄ fermentation, yielding up to four moles H₂ per mole of glucose, along with close to two moles of CH₄ and CO₂. Burning H₂/CH₄ mixtures can reduce air pollution compared to burning methane gas alone, and thus can provide a competitive advantage where air emissions are restricted.

23.7 Conclusions

Processes for the production of gaseous fuels, H₂ and CH₄, using microalgae cultures have been studied for several decades. The only present practical application is in the harvesting of algal biomass from wastewater treatment ponds by chemical flocculation, followed by the anaerobic digestion of the algal biomass. This is currently practiced at the City of Sunnyvale, California, wastewater treatment plant, where algal biomass is co-digested with sewage sludge. Although microalgae ponds are widely used for wastewater treatment, these are mostly small systems and this technology has long been hampered by the lack of a low-cost and efficient algal harvesting technology, such as the spontaneous settling of algal cells (*bioflocculation*) (Benemann *et al.*, 1980). One potential approach is to combine the waste oxygenation function of algal cultures (*secondary* treatment) with nutrient removal (*tertiary* treatment). This will require the addition of CO₂ to the ponds, to increase the biomass production and, thus, the nutrient removal by the algal cultures. Such a combined secondary–tertiary algal treatment process could both greatly expand the utility of microalgae systems in sewage treatment as well as increase the algal biomass available for CH₄, and potentially H₂, production. CO₂ fertilization would also allow better control over the algal pond environment, allowing for dominance and maintenance of desired strains and facilitating the use of low-cost bioflocculation for algal harvesting.

Solar energy-driven microalgae processes involving fossil CO₂ utilization and generation of renewable fuels, in particular H₂ or CH₄, are of increasing interest in the development of greenhouse gas mitigation technologies, where microalgae could make a significant contribution (Pedroni *et al.*, 2001). However, the severe economic constraints of any such solar conversion

process, even with very favorable productivity and economic assumptions (Benemann, 1997), will almost certainly require that microalgae fuel production processes be integrated with additional value generating services or products, such as wastewater treatment, and nutrient removal and recovery. Although considerable R&D is still required, the strongly favorable economic and technical fundamentals of microalgae in waste treatment applications assure the likely success of such research in the near- to midterm.

In contrast, photobiological processes for H₂ production, in which light energy drives the H₂ evolution process, are still at a very basic research stage despite 30 years of applied R&D, hundreds of publications, and, recently, extensive publicity claiming major R&D breakthroughs. One concept, pursued by several research groups around the world (Sasikala *et al.*, 1993; Zaborsky *et al.*, 1998), combines microalgae systems for CO₂ fixation with photosynthetic bacteria that produce hydrogen by a nitrogenase-mediated reaction. The largest demonstration of such a process, used two small (2 m²) algal ponds that provided biomass for a dark anaerobic fermentation that, in turn, supplied substrates to a photosynthetic bacterial H₂ production photobioreactor (Ikuta *et al.*, 1988). However, as discussed above, any H₂ production process based on nitrogenase is inherently inefficient, and with an overall efficiency of some 0.2% this process proved to be similar to that of heterocystous cyanobacteria1 (Borodin *et al.*, 2000). More fundamentally, even if high efficiencies could somehow be achieved, all photobiological processes would require large and expensive photobioreactors, making these processes economically prohibitive (Benemann & San Pietro, 2001).

A potentially plausible process for microalgal H₂ production, proposed above, would convert endogenous storage carbohydrates into H₂ under dark fermentative conditions, coupled to respiratory, rather than photosynthetic, energy generation reactions, plausibly using a metabolic engineering approach (Keasling *et al.*, 1998). Alternatively, practical bacterial CH₄ fermentation processes, using already commercial anaerobic digestion technologies (Augenstein *et al.*, 1994) can be used at present to convert microalgal biomass to CH₄ fuel. CH₄ fermentations involve intermediate H₂ production and H₂ could be derived as a by-product in the so-called two-stage fermentations (Harper & Pholand, 1986).

In conclusion, the most plausible applications of H₂ and CH₄ production by microalgae are with waste-grown biomass, in particular that derived from CO₂ fertilized systems, where nitrogen limitation would generate biomass with a high content of easily fermentable storage carbohydrates. Fundamentally, all practical microalgae processes must aim to maximize the overall solar conversion efficiency of the processes, and this is still the major challenge in this field.

23.8 Summary

For practical solar energy conversion processes, the photons-to-fuels conversion efficiency and cost per unit area are the main issues. Many factors limit photosynthetic solar conversion efficiencies in algal mass cultures, with the light-saturation effect being plausibly the major one. This limitation could be

overcome by genetically reducing the number of light-harvesting pigments in the photosynthetic apparatus, potentially allowing a 10% solar energy conversion efficiency into carbohydrates, from which hydrogen or methane fuels could be produced. Open pond cultures are of much lower cost than closed photobioreactors, although pond systems economics for fuels production with microalgae is presently speculative. Here alternative proposed mechanisms and schemes for gaseous fuels production using microalgae are reviewed.

H_2 production by *direct biophotolysis* involves the transfer of electrons from water through the photosynthetic apparatus to ferredoxin and then hydrogenase, instead of the CO_2 -fixing RUBISCO enzyme. Such a process has been known in green algae for some 50 years, but only takes place at very low levels of dissolved O_2 . Thus, direct biophotolysis processes require the presence of O_2 consuming reactions, such as respiration, or the purging of the culture with an inert gas, stratagems which make such a process impractical. Development of a hydrogenase reaction that can evolve H_2 in the presence of O_2 has been suggested, but that is speculative. Also, direct biophotolysis requires closed photobioreactors to capture the H_2 and O_2 produced, as well the separation of these gases, requirements that are economically prohibitive.

Indirect biophotolysis processes carry out the O_2 and H_2 production reactions in separate stages, the first involving CO_2 fixation into carbohydrates and the second involving carbohydrate fermentations to H_2 . The CO_2 fixation and O_2 evolving reactions of photosynthesis can be carried out in low-cost open ponds, but the fermentative step requires a light-driven H_2 evolution reaction, necessitating photobioreactors capable of capturing the H_2 gas. Even assuming a requirement of only one photon per electron transferred to H_2 , the required photobioreactors would still be cost-prohibitive, even though they would be less than half the size of those required for direct biophotolysis. Photobiological processes based on nitrogenase-mediated H_2 production, rather than on hydrogenase, have also been proposed, but these suffer from the very low efficiency of the nitrogenase reaction. A strictly dark fermentative hydrogenase reaction would be a more practical and direct mechanism to convert microalgal carbohydrates to H_2 , but a high-yielding process remains to be demonstrated. Alternatively, methane fermentations can recover as fuel most of the solar energy captured in the algal biomass, and are already commercially available for producing gaseous fuels through microalgal photosynthesis.

Regardless of mechanism, all microalgae fuel production processes require very high solar conversion efficiencies, whose achievement in practice will require long-term R&D. Combining H_2 or/and CH_4 production with wastewater treatment would relax the stringent requirements for high photosynthetic efficiencies and very low costs required for stand-alone fuel production processes and allow for near-term practical applications.

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24 Water Pollution and Bioremediation by Microalgae

Eutrophication and Water Poisoning

Susan Blackburn

24.1 The eutrophication process

Eutrophication is the process of changing the nutritional status of a given water body by increasing its nutrient sources (derived from the Greek *eu* meaning *well* and *trophe* meaning *nourishment*). It can be a natural process such as happens when nutrient-rich deep oceanic water upwells into nutrient-poor surface water. Indeed, traditionally, it was seen rather as an analogue to the fertility of terrestrial soils (Sommer, 1996). Eutrophication is often considered as an increase in primary inorganic nutrients, notably nitrogen (N) and phosphorus (P) (Richardson & Jørgensen, 1996). However, a more system-based view was suggested by Nixon (1995) based on organic carbon, namely *an increase in the rate of supply of organic carbon to an ecosystem*, where the importance of inorganic nutrients to marine ecosystems is considered to be their potential influence on the rate and form of supply of organic matter. Major concerns about eutrophication in recent decades have arisen from the dramatic increase in eutrophication from anthropogenic causes, or *cultural eutrophication*, in fresh, estuarine and coastal waters worldwide. Examples include Tolo Harbour, Hong Kong where N and P levels doubled between 1976 and 1985 (Lam & Ho, 1989) and the estimation that nitrogen inputs in the northeastern United States have increased six to eightfold since preindustrial times (Howarth, 1998).

The most immediate effect of eutrophication is increased primary production by phytoplankton and other algae, e.g. benthic and other aquatic plants. Primary production is a function of light, nutrient availability and temperature, and the net plant biomass depends on the cellular growth rate minus loss rates through dilution, sedimentation, grazing and physiological death

(Reynolds, 1984). While all factors either singly or in combination may be limiting, an increase in nutrients usually promotes an increase in plant biomass.

24.2 Impact of nutrients on eutrophication

For the phytoplankton, input of nutrients will have different effects depending on the ecosystem and the types of microalgae. For example, it is commonly considered that freshwater systems are phosphorus limited, and marine systems are more likely to be nitrogen limited. This picture may be changing in the Northern Hemisphere with a trend towards increasingly available nitrogen in estuarine, coastal and oceanic waters from atmospheric sources (Paerl & Whitall, 1999). In the brackish Baltic Sea, phosphorus input stimulates nitrogen-fixing cyanobacteria (Sellner, 1997). If silicon (Si) is limiting then diatom growth will be limited. Thus not only the nutrient levels but also the alteration of ratios such as N:P, N:Si and Si:P are important in determining phytoplankton species distribution, competition, occurrence and succession, thus affecting community structure (Smayda, 1989) including the entire food web (Zhang, 1996). Increased organic nutrient sources may also promote increased growth (Cembella *et al.*, 1984; Doblin *et al.*, 1999) with nutritional strategies such as mixotrophy and phagotrophy being important (Granéli & Carlsson, 1998). In addition, anthropogenic sources of micronutrients such as iron may be key stimulants to increased primary production (Abal *et al.*, 2001).

Eutrophication, with its increase in nutrients overall and also commonly a shift from the usual nutrient ratios, therefore affects both the scale and type of phytoplankton biomass. A major outcome may be the proliferation of phytoplankton beyond their *normal* community balance. These proliferations or significant population increases are known as algal blooms (Smayda, 1997a).

The worldwide increase in eutrophication combined with the outcome of algal blooms led Smayda (1989) to propose a global epidemic of algal blooms including unusual, harmful or exceptional phytoplankton blooms. Early evidence to support this came from the correlation of increased population in Hong Kong from 1976 to 1986 with an increase in algal blooms or *red tides* in Tolo Harbour (Fig. 24.1). In the Baltic Sea, a site of extensive cyanobacterial blooms (Sellner, 1997), there has been a doubling of primary production in the Kattegat between the late 1950s and early 1990s (Richardson & Heilmann, 1995).

24.3 Toxic effects of algal blooms

Algal blooms may be detrimental to the functioning of the ecosystem *per se*, for example by causing oxygen depletion and consequent death of fish and aquatic animals. As well, the mere physical presence of such densities of phytoplankton may cause damage to other organisms, e.g. diatoms and fish gills (Horner *et al.*, 1997). Blooms of species that produce toxins are, however, most damaging to ecosystems and can have profound food chain effects

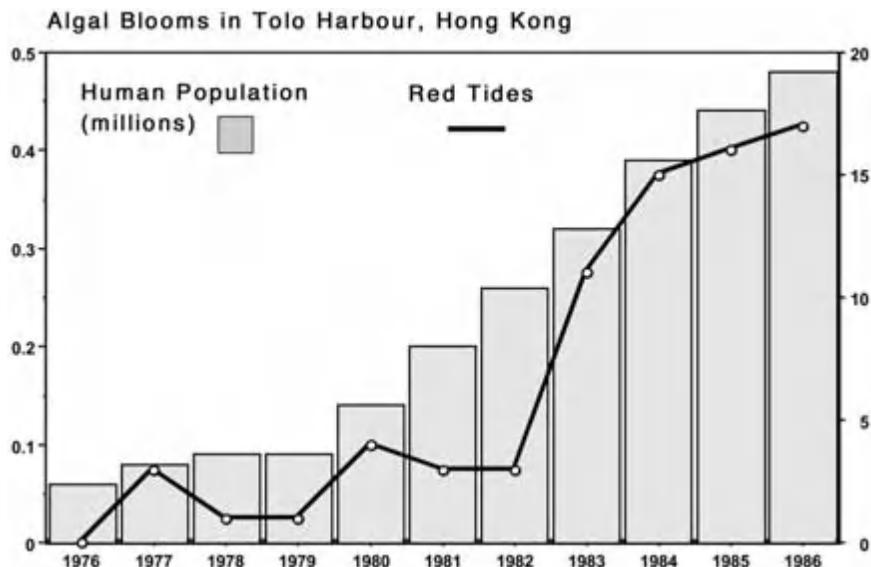


Fig. 24.1. Correlation between the number of harmful algal blooms or red tide outbreaks per year in Tolo Harbour (continuous line) and the increase of human population in Hong Kong, in the period 1976–1986 (after Lam & Ho, 1989).

including human poisonings. Both toxic and other nuisance blooms are collectively known as harmful algal blooms (HABs). Toxin-producing microalgae are found in a diversity of algal classes: the Dinophyceae (dinoflagellates), Bacillariophyceae (diatoms), Raphidophyceae (raphidophytes), Prymnesiophyceae (prymnesiophytes) and cyanobacteria (blue-green algae) (Daranas *et al.*, 2001). This diversity of microalgae is matched with a diversity in toxins (Daranas *et al.*, 2001), with some intriguing cases of convergence such as the production of the alkaloid paralytic shellfish toxins (PSTs), saxitoxin and derivatives by dinoflagellates and cyanobacteria (Oshima *et al.*, 1993; Humpage *et al.*, 1994; Negri *et al.*, 1997). Other toxins include peptides such as microcystins and other hepatotoxins, and lipopolysaccharides produced by cyanobacteria, okadaic acid and polyether toxins as found in diarrhetic shellfish poisoning (DSP)-causing dinoflagellates (Carmichael *et al.*, 1985). Poisonings and other harmful effects may be induced in humans, or affect wild or cultivated aquatic animals. Table 24.1 summarises the deleterious effects caused by harmful algae.

A characteristic of many toxin-producing microalgae is that they are very effective in concentrating biomass due to their ability to actively regulate their position in the water column. This gives them the advantage of optimising both harvesting of light and accessing nutrients, and enhances the impact of increased primary production in response to eutrophication. Some bloom-forming cyanobacteria regulate their buoyancy using gas vacuoles whereas toxic dinoflagellates and other HAB flagellates are motile. In the Huon Estuary, southeast Tasmania, blooms the toxic, chain-forming dinoflagellate *Gymnodinium catenatum* which vertically migrates 20 m on a diurnal basis

Table 24.1. Deleterious effects caused by harmful algae. Note that a single event may have several negative consequences (modified from Zingone & Enevoldsen, 2000).

Effect	Examples of causative organisms
Human health	
Paralytic shellfish poisoning (PSP)	Dinoflagellates
Diarrhetic shellfish poisoning (DSP)	Cyanobacteria
Neurotoxic shellfish poisoning (NSP)	Dinoflagellates
Amnesic shellfish poisoning (ASP)	Dinoflagellates
Azaspiracid shellfish poisoning (AZP)	Diatoms
Ciguatera fish poisoning (CFP)	Unknown
Respiratory problems and skin irritation, neurological effects	Dinoflagellates
Hepatotoxicity	Dinoflagellates
	Cyanobacteria
	Cyanobacteria
Natural and cultured aquaculture resources	
Haemolytic, hepatotoxic, osmoregulatory effects, and other unspecified toxicity	Dinoflagellates
	Raphidophytes
	Prymnesiophytes
	Cyanobacteria
	Pelagophytes
	Dinoflagellates
	Diatoms
	Prymnesiophytes
Negative effects on feeding behaviour	
Hypoxia, anoxia	
Mechanical damage	
Gill clogging and necrosis	
	Alexandrium spp., <i>Pyrodinium bahamense</i> var. <i>compressum</i> , <i>Gymnodinium catenatum</i>
	<i>Anabaena circinalis</i>
	<i>Dinophysis</i> spp., <i>Prorocentrum</i> spp.
	<i>Gymnodinium breve</i>
	<i>Pseudonitzschia</i> spp., <i>Nitzschia navis-varingica</i>
	Unknown
	<i>Gambierdiscus toxicus</i>
	<i>Gymnodinium breve</i> , <i>Pfiesteria piscicida</i>
	<i>Nodularia spumigena</i> , <i>Lyngbya</i> spp.
	<i>Microcystis aeruginosa</i> , <i>Nodularia spumigena</i>
	<i>Gymnodinium</i> spp., <i>Cochlodinium polykrikoides</i> ,
	<i>Pfiesteria piscicida</i> , <i>Gonyaulax</i> spp.
	<i>Heterosigma akashiwo</i> , <i>Chattonella</i> spp., <i>Fibrocapsa iaponica</i>
	<i>Chrysotrichulina</i> spp., <i>Phaeocystis pouchetii</i> , <i>Prymnesium</i> spp.
	<i>Microcystis aeruginosa</i> , <i>Nodularia</i> spp.
	<i>Aureococcus anophagefferens</i>
	<i>Prorocentrum micans</i> , <i>Ceratium furca</i>
	<i>Chaetoceros</i> spp.
	<i>Phaeocystis</i> spp.

Tourism and recreational activities
Production of foam, mucilage, discolouration, repellent odour

Noctiluca scintillans, <i>Prorocentrum</i> spp.
<i>Phaeocystis</i> spp.
<i>Cylindrotheaca closterium</i>
<i>Diatoms</i>
<i>Cyanobacteria</i>
<i>Noctiluca scintillans, Aphanizomenon flos-aqua,</i>
<i>Microcystis aeruginosa, Lyngbya</i> spp.

Ecosystem impacts
Hypoxia, anoxia

Negative effects on feeding behaviour, reduction of water clarity
<i>Gymnodinium breve, Alexandrium</i> spp.
<i>Pseudo-nitzchia australis</i>

Toxicity to wild fauna

Dinoflagellates
<i>Diatoms</i>
<i>Prymnesiophytes</i>
<i>Cyanobacteria</i>
<i>Pelagophytes</i>
Dinoflagellates
<i>Diatoms</i>
<i>Cyanobacteria</i>

through a picnocline, allowing access to deep nutrients (Fig. 24.2). Such species are well adapted to take advantage of the increased nutrients characteristic of eutrophied ecosystems, particularly where optimal nutrients and light may be separated in the water column. Other characteristics of HAB flagellates that influence their success are their low nutrient affinity, considerable nutritional diversity including mixotrophic tendencies, generally low growth rates, and the potential importance in allelopathy (Smayda, 1997b). The specific characteristics of the algal class or species are important and still relatively understudied despite recognition of the importance of species autecology. This need was the basis of a NATO Advanced Study Institute on the *Physiological Ecology of Harmful Algal Blooms* in 1996 (Anderson *et al.*, 1998).

Poisonings from harmful algal blooms are known from historical times. Notable examples include the 1793 record on the US west coast of paralytic shellfish poisoning and death of some of Captain George Vancouver's crew after eating mussels in Poison Cove, central British Columbia (Quayle, 1969), and the poisoning and death of cattle in Lake Alexandrina, South Australia after drinking water from a bloom of the cyanobacterium *Nodularia spumigena* (Francis, 1878). With the global epidemic of HABs (Smayda, 1989; Hallegraeff, 1993) there are both apparently new HAB phenomena and also an overall increase in the geographical spread and frequency of HABs. New types of HAB events include amnesic shellfish poisoning (ASP) by the diatoms *Pseudo-nitzchia pungens* and related species (Bates *et al.*, 1998), and the dramatic example of fish kills and human cognitive health damage by

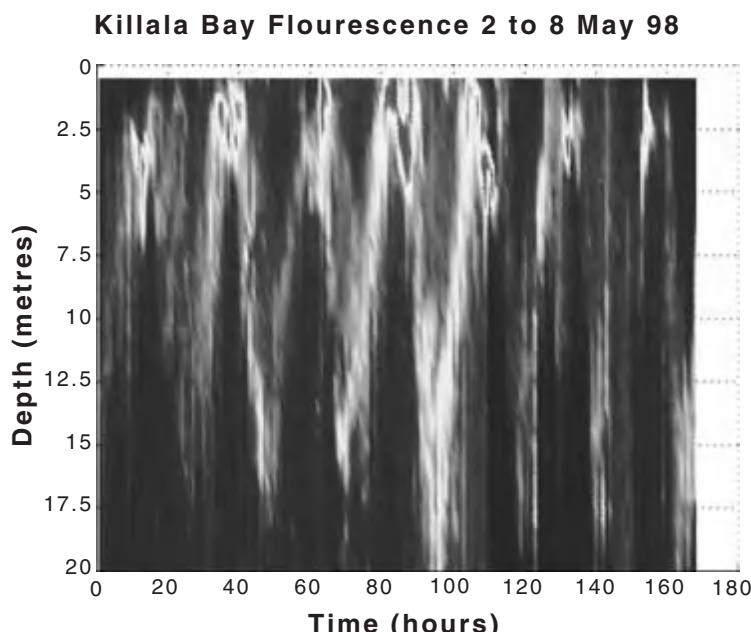


Fig. 24.2. Time-depth fluorescence showing diel vertical migration of the chainforming toxic dinoflagellate *Gymnodinium catenatum* at Killala Bay in the Huon Estuary, Tasmania.

Pfiesteria piscicida and *P. shumwayae*, phantom dinoflagellates with ambush predator tactics (Burkholder *et al.*, 1992, 2001). These apparently new phenomena have probably been undetected until recent times.

An example of an increase in spread and frequency in recent decades is that of paralytic shellfish poisoning (PSP). Fig. 24.3 shows the known global distribution of PSP in 1970 and 2000. While there is continuing debate on the relative importance of natural and anthropogenic spreading contributing to this changed distribution (Hallegraeff, 1993; Blackburn *et al.*, 2001), it is clear that local conditions including increased eutrophication are contributing to the success of PSP-forming dinoflagellates.

Poisonings are not necessarily related to the size of bloom events. A characteristic of some toxic microalgae or poisoning events is that development of visible blooms is not necessary. Both PSP and ASP may result from only thousands to tens of thousands cells per litre, levels that are not blooms in the sense of mass proliferations (Zingone & Enevoldsen, 2000). In toxic fish-killing events caused by the cryptic dinoflagellate *Pfiesteria* poisonings are associated with swarms of zoospores only in the vicinity of the fish (Burkholder *et al.*, 2001). Ciguatera finfish food poisoning from benthic dinoflagellates in coral reef areas is also cryptic in nature (Bagnis *et al.*,

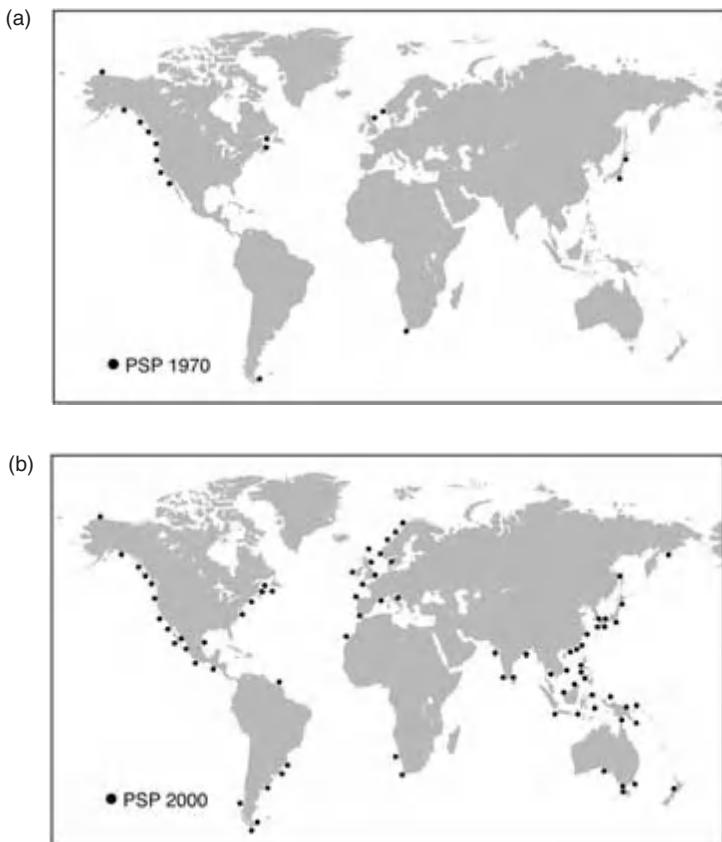


Fig. 24.3. (a & b) World Distribution of PSP in 1970 and 2000 respectively (after GEOHAB, 2001).

1985). Thus the type of toxin, the degree of toxin content in the cells, and the type of poisoning are all important in water poisonings. Toxin content will vary between algal species. For example, *Pyrodinium bahamense* var. *compressum* contains a greater proportion of the highly toxic saxitoxin than other PSP-forming dinoflagellates and is therefore toxic at low cell concentrations and highly toxic when concentrated, for example in *Sardinella* (MacLean, 1979; Oshima, 1989). As well as the inherent toxicity of the algae, environmental factors can affect the toxin content. Atypical nutrient regimes may cause species that are not normally toxic to become so. For example, phosphorus deficiency stimulates toxicity in the prymnesiophyte *Chrysochromulina polylepis* (Edvardsen *et al.*, 1990), and domoic acid production in the diatom species of *Pseudo-nitzschia* varies with availability of silicate (Bates *et al.*, 1991). For toxic cyanobacteria where species are inherently toxic, changes in nutrients will usually affect the toxicity of blooms by up to an order of magnitude (Orr & Jones, 1998).

Many of the poisonings from HABs are due to transfer through the food chain, such as consumption of shellfish which are themselves not directly affected by the toxins but can be toxic to humans. Acceptable levels of the PSTs, saxitoxin and derivatives are set by the US Food and Drug Administration at 80 µg saxitoxin equivalents per 100 g of shellfish meat (AOAC, 1990). Management strategies include closure of aquaculture operations and wild shellfish harvesting when toxins are above this level. Closures can be from months to even years. In the Philippines, where PSP causes deaths regularly (MacLean, 1993), the allowable limit is set at only 40 µg per 100 g of shellfish meat due to susceptibility by low weight Filipinos, especially children (Williams & Perez-Corral, 2001). For DSP levels of 2 µg okadaic acid and/or 1.8 µg dinophysitoxin-1 per gram of hepatopancreas are considered unfit for human consumption (Lee *et al.*, 1987). While fatalities are not known from DSP, some of the polyether toxins involved (okadaic acid, dinophysitoxin-1) may promote stomach tumours (Suganuma *et al.*, 1988), leading to chronic health problems.

Health issues are very direct in fresh waters where the water is used for human drinking water supplies. Drinking water guidelines have been set by the World Health Organisation (WHO) for consumption of microcystins (Falconer, 1999; Fitzgerald, 2001) and they have considered whether microcystin should be considered as a carcinogen. A very serious poisoning event occurred in Brazil in 1996. Water used in a haemodialysis centre was contaminated with the hepatotoxic microcystins and possibly other toxins from cyanobacteria. All 126 patients became ill and 60 eventually died, mainly from liver failure (Pournia *et al.*, 1998). Some freshwater cyanobacteria produce neurotoxins. Dramatic examples such as blooms of neurotoxic *Anabaena circinalis* over approximately 1000 km of the Darling-Barwon River system in Australia in summer 1991/92 demonstrate the potential seriousness of neurotoxins in fresh waters (Baker & Humpage, 1994). Recreational use of waters is also an issue with WHO providing recommendations for a framework of risk assessment (Fitzgerald, 2001). Shellfish and water quality guidelines help protect against poisonings but they do not affect the development and intensity of blooms, nor do they aid in prediction.

24.4 Control of eutrophication

Nutrient input quotas and other controls are one way to decrease eutrophication and the subsequent development of HABs. Such regulations have had success in systems such as the Peel-Harvey Estuary in Western Australia in helping control blooms of the toxic cyanobacterium *Nodularia spumigena* (Humphries & Robinson, 1995) and in decreasing the frequency of fish-killing blooms in Hong Kong Harbour (Okaiichi, 1989).

There is a view that algal species management, for example, to promote nontoxic diatom blooms by altering nutrient ratios, may be one way of controlling adverse effects from cultural eutrophication (Hecky & Kilham, 1988). Promoting harmless blooms could then in turn be used to promote valuable food species. This has been the premise of major ecosystem experiments, e.g. MARICULT (Sommer, 1996). Conversely, there is an increasing concern that the input of nutrients in finfish aquaculture may be stimulating algal blooms by both the increased nutrient load and the form of nutrients used in aquaculture feeds, e.g. better potential utilisation of ammonia by toxic dinoflagellates than by other phytoplankton (CSIRO Huon Estuary Study Team, 2000). Globally there is a widening gap between wild fishery production and seafood demands of the increasing world population. The United Nations Food and Agriculture Organisation estimates world aquaculture production will increase by 25% by 2010. Under such global pressure it is being increasingly realised that strict quotas, both on the level of marine farming in particular areas and also on the nutrient input by aquaculture operations, will be needed, not only because of the increased risk of HABs but also because of the more widespread ramifications of ecosystem damage.

Control of nutrient input and setting of quotas are often done in the absence of good baseline studies with long-term data sets. However there are increasing efforts to take knowledge about ecosystems and develop models to aid in prediction of algal blooms and management of ecosystems. Answers to key questions are needed. These include: would major reductions of nutrient sources (either point or non-point sources or both) help to arrest the occurrence and persistence of nuisance algal blooms (Lung & Paerl, 1988)? While there is some confidence that models for freshwater and estuarine systems can be developed that have predictive power (Harris, 1998), models for prediction of harmful algal blooms need more development (Franks, 1997).

Globally, the seriousness of eutrophication and water poisonings from harmful algal blooms is being addressed by research and management programmes at local, national, regional and international scales. Currently the Scientific Committee on Oceanic Research (SCOR) and the Intergovernmental Oceanographic Commission (UNESCO) are developing a programme on the Global Ecology and Oceanography of Harmful Algal Blooms (GEOHAB, 2001). With a ten year horizon, key objectives include determining the significance of eutrophication and nutrient transformation pathways to HAB population dynamics, defining the particular characteristics and adaptations of HAB species that determine when and where they occur and produce harmful effects, and improving detection and prediction of HABs.

by developing capabilities in observation and modelling. Other notable examples of major programmes are the US National Science Foundation and the National Oceanographic Administration ECOHAB (The Ecology and Oceanography of Harmful Algal Blooms) programme and the European Union EUROHAB programme.

24.5 Potential use of toxin-producing microalgae

While poisonings, by definition, have negative connotations within the broad context of bioactive molecules for biotechnology, there are potential benefits not only from the toxins but also by using toxin-producing microalgae as a rich source of other bioactive molecules for medical and pharmaceutical applications (Pietra, 1997; Burja *et al.*, 2001; Daranas *et al.*, 2001). To fully exploit some of these applications high biomass cultivation or *artificial bloom* formation would greatly facilitate the process. While there is much emphasis on development of high production photobioreactors for cost-effective microalgal production (Tredici, 1999; Richmond, 2000), it has been regarded that many toxic microalgae are difficult to culture and too fragile or fastidious in their requirements to be grown in high density systems. However some aspects of HAB species suggest the converse, i.e. characteristics including the capacity to concentrate and aggregate in high density localised populations, that is, HAB species have the natural capacity for high density cultivation. Recently Parker *et al.* (2002) have had success in several culture systems with high density cultivation of *Alexandrium minutum*, a relatively robust toxic dinoflagellate. Such studies pave the way for beneficial effects from microalgae that in the natural environment are often responsible for detrimental poisonings, particularly in a global environment of increased eutrophication of our waters.

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25 Water Pollution and Bioremediation by Microalgae

Water Purification: Algae in Wastewater Oxidation Ponds

Aharon Abeliovich

25.1 Introduction

The chapter on algae in wastewater oxidation ponds in the Richmond (1986) edition of the microalgal handbook was written during the height of a general enthusiasm for combined systems for wastewater treatment and algal biomass production. The algal biomass was intended, after sterilization, to replace expensive protein animal feed. Since then, the entire issue was dropped, for several reasons: one is that there happens to be some contradiction between optimal wastewater treatment and maximal algal biomass harvest, the former requiring extended retention time so that all available organic nutrients are exhausted, irrespective of algal biomass accumulation. This results in algae, which to a large extent grow heterotrophically in the oxidation pond (Abeliovich & Weisman, 1978) being kept in a state of permanent low nutrient supply. Maximal algal yield requires that organic nutrient concentration should be high, which means that wastewater treatment in the dual purpose high rate oxidation pond is incomplete, requiring further polishing steps, which in turn affects the economics of the process. Another problem is that in an open system, it is impossible to control algal species that will dominate this biotope, frustrating the goal of maintaining stable product quality: different algae have different nutritional requirements, require different harvesting conditions, and often microalgae (primarily cyanobacteria) that are resistant to harvesting by alum flocculation-flotation (see below) will overcome harvestable algae like *Chlorella* sp. or *Scenedesmus* sp. Harvesting is also an economic problem, as centrifugation of wastewater is too expensive and filtration is impractical, leaving flocculation-flotation as the method of choice. This results in the accumulation of

high concentrations of aluminum in the product (Yannai *et al.*, 1980), toxic to test animals which accumulate aluminum (Yannai & Mokady, 1985). Also, as algal species dominating the pond change frequently, separation conditions have to be optimized continuously.

Not only has the high rate oxidation pond lost its attractiveness, but the conventional oxidation pond, in many places, also fell victim to the activated sludge process in its many variations. This course has taken place because of prohibitive costs of land, as well as restrictive environmental regulations and effluent quality control restrictions, which are primarily of cosmetic value, such as BOD, COD, total suspended solids (TSS), etc.

With the advent of the concept that treated wastewater should be stored and reused for agricultural irrigation, deep wastewater reservoirs became popular. The main reason for their proliferation is that while wastewater is generated at a constant rate all year round, irrigation is restricted to specific seasons and so effluents must be stored for months to be used at a later time, during the irrigation season. As the complex biology of these water bodies was worked out, it gradually became clear that these reservoirs are not inert water bodies, and that they could be integrated to become a part of conventional treatment plants or in fact, in some cases, to replace treatment plants altogether. A comprehensive description and discussion of the operation and dynamics of wastewater reservoirs has recently been published (Juanico & Dor, 1999).

25.2 Principles of operating stabilization ponds

Wastewater treatment through stabilization ponds is considered to be a *natural process*, in contrast to all other processes that rely heavily on electro-mechanical installations. Basically, however, the stabilization pond is no more and no less *natural* than all other treatment processes, as all domestic wastewater treatment facilities depend on natural microbial populations and activities, such as growth, and on various modes of oxic respiration and anaerobic fermentation. Treatment here means that these activities turn the organic matter into a microbial biomass and metabolic end products as methane, CO₂, ammonia, etc. that are eventually disposed of one way or another. The uniqueness, or *naturalness* of the stabilization pond lies in its mode of oxygenation, which depends on algal photosynthesis rather than on the electromechanical supply of air. This dependence on algal photosynthesis for oxygenation calls for specific design parameters and a fair understanding of algal physiology and accordingly, special treatment regimes aimed at enabling the algal population to carry out optimal photosynthesis and oxygenation.

Many factors affect algal photosynthesis in stabilization ponds: first, it depends on solar energy, which means dependence on a very diffuse, unidirectional and intermittent source of energy. Maximal oxygenation calls, therefore, for a careful balance between many factors such as organic load, ratio of water depth to water surface area, seasonal fluctuations in radiation and temperature, water turbidity, algal speciation, food chain and the abundance of predators, ammonia and pH (Abeliovich & Azov, 1976; Abeliovich, 1983), to mention just a few of the obvious factors involved (see Fig. 25.1).

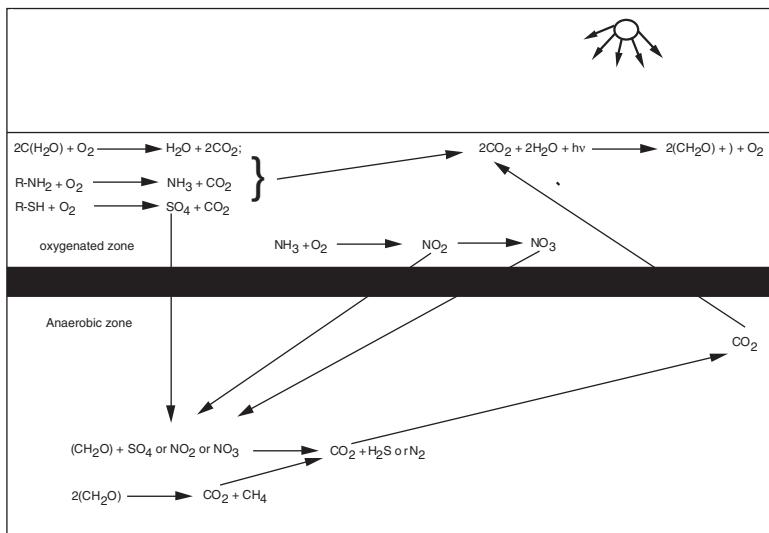


Fig. 25.1. Major recycling processes in the wastewater stabilization pond and the wastewater reservoir. Actual reaction rates for the various described reactions vary according to local conditions.

25.2.1 Biological equilibrium

A specific example of the role of water chemistry in determining the rate of photosynthesis is the case of the effect of the interplay between ammonia concentrations and pH, emphasizing the need for water chemistry to be favorable toward algal photosynthesis. The pH of the pond water is affected by respiration, carried out by all microorganisms along the entire water column as long as oxygen is present, and this tends to lower the water pH as CO₂ is released into the water in the oxic zone, while organic acids and CO₂ are discharged in the anoxic zone. On the other hand, the photosynthetic process tends to elevate water pH, due to the extraction of CO₂. This interplay between respiration and photosynthesis is responsible for quite large diurnal fluctuations in water pH, anywhere between 6.0 and 9.5, depending on sunlight, depth of the pond, inflow of organic load and hydraulic retention time. Most important in this context is the issue of ammonia concentration in domestic wastewater, which is on an average 70–80 mg l⁻¹ of ammonia, following complete urea decomposition and significant proteolysis. Ionized ammonia is harmless to algae, because its transport into the cell is controlled by specific mechanisms which do not allow accumulation of intracellular excess concentrations of ionized ammonia. Unionized ammonia, in contrast, as many other unionized small molecules, does penetrate freely through biological membranes, and since the pK of ammonia is 9.25, a very significant part of the total ammonia might be present in the pond water in an undissociated form. Experimentally, ca. 0.2 mM of unionized ammonia in wastewater inhibits 50% of the rate of oxygen release by common oxidation pond algae (*Chlorella* sp., *Scenedesmus* sp., *Micractinium* sp., *Chlamydomonas* sp., as well as various cyanobacteria). A combination of 30 mg l⁻¹ of ammonia with pH 8.2, for example, will cause such inhibition. Since this

concentration is within the range most likely to exist in waste stabilization ponds, it is rather easy to detect an inhibitory effect of ammonia on photosynthesis as it reveals itself when the pH of the surface layer of the pond is above pH 7.9–8.2 at noon on a bright sunny day. There exists, therefore, a complex interplay between ammonia concentration, respiration, photosynthesis and water pH in the stabilization pond, and this interplay is a major factor in determining the performance of the pond as it determines the rate of oxygenation of the water.

25.2.2 Health considerations

There can be no doubt that the most important aspect of domestic wastewater treatment concerns the health aspect, as raw wastewater contains excrements carrying all manner of infectious bacteria, viruses, protozoa, sporozoa, and helminthes and fungi. Official regulations require that effluent quality should conform to strict standards with regard to several parameters.

This is a serious problem as there is no practical way by which to assay all pathogens. Instead, effluent quality has to conform to specific standards with regard to concentration of marker microorganisms, such as fecal coliforms, helminthes eggs, and *Cryptosporidium* and *Giardia* in the effluents discharged into the environment or used for irrigation. Some of the standards set by governments and various international authorities (FAO, WHO), e.g. BOD and COD, total suspended solids and turbidity, however, have no real or direct public health aspect and yet prevent use of water from waste stabilization ponds which contain algae indispensable to the waste purification system, contributing a large part of the COD, BOD, TSS and turbidity, otherwise perfectly safe effluents. These parameters, therefore, are of very limited value in monitoring the health hazard that waste stabilization ponds may pose. At present, the only directly related parameter with regard to health risks is the count of fecal coliforms and specific parasites and marker viruses. In this context, it is also important to note that disinfection of effluents, as practiced, does little harm to most microbial flora except by reducing bacteria numbers by several orders of magnitude, which does not prevent a later rapid regrowth of bacteria.

The growing awareness of the health hazards associated with using domestic wastewater effluents for irrigation or hazards associated with their free discharge to the environment, resulted in intensive work being carried out in many places around the world to determine workable parameters for the safe operation and water use of waste stabilization ponds: Brissaud *et al.* (2000) studied a model for decay of fecal coliforms in ponds, and Davies-Colley *et al.* (2000) studied factors affecting survival of fecal coliforms in ponds. They concluded that sunlight is the single most important factor in waste stabilization pond (WSP) disinfection, but that the efficiency of disinfection strongly depends upon the pH, high pH values affecting a wider spectrum of light to be bactericidal.

Bouhoum *et al.* (2000) studied occurrence and removal of protozoan cysts and helminthes eggs in waste stabilization ponds in Marrakech: it can be safely stated, in general, that retention of three to four months worth of

effluents in a wastewater reservoir eliminates all human pathogens from the water through the harsh conditions (for the human flora prevailing in the reservoir), e.g. sunlight, pH, temperature, nutrients and adsorption by particles that sink to the bottom of the reservoir.

25.3 Intensive fish farming wastes

In recent years, intensive aquaculture of cold- and warm-water fish, shrimps and various mollusks has developed all over the globe, bringing with it a whole new set of environmental problems, primarily concentrated around the issue of waste disposal. The waste contains, in addition to excrement, also feed waste, as quantification of needed feed is lagging. This aspect is subject to intensive research, concentrating on development of high nutrient, low pollution diets. Prediction of aquaculture wastes using biological approaches is also being studied (Choy *et al.*, 1994). Modeling of physical dispersion of finfish aquaculture wastes was also developed by Dudley *et al.* (2000). There is an urgent need to optimize feed and reduce aquaculture waste as this industry grows, particularly by advances in recirculation technologies for land-based systems that have resulted in reduced waste production. The advent of modern recombinant DNA technologies now allows for the economic production of a variety of feed supplements, most notably microbial phytases. Others include such physiological modifications as sustained exercise and compensatory growth. More controversial biotechnological methods which may be beneficial in reduced waste management include endocrine manipulations and genetic engineering (Mayer & Mclean, 1995).

Accumulation of nitrogen in the water and wastewater, due to intensive feed regimes, is of particular importance due to the possible accumulation of ammonia and nitrite to toxic levels, requiring treatment aimed at removal of nitrogen either for recirculation purposes or for discharge of non-toxic effluents (Dvir *et al.*, 1999). A promising approach to a total treatment of water for recycling purposes is the use of a combined system using various organisms, each taking care of a specific contaminant. In a study by Neori *et al.* (2000), effluents from Japanese abalone (*Haliotis discus hawaii*) culture tanks were drained into a pellet-fed fish (*Sparus aurata*) culture tank, and the fish effluent was then drained into macroalgal (*Ulva lactuca* or *Gracilaria conferta*) culture, and biofilter tanks. Finally, the algal produce was fed to the abalone. This mode of combining several biotopes in series has great promise and is similar to the experiments carried out by Ryther (1969, 1971) using combined systems for treating domestic wastewater through a serial exposure to algae, copepods and fish.

25.4 Industrial wastewater

In contrast to domestic wastewaters, which are very similar all over the world and can be treated by similar methodologies (the only limitation being climatic), there is no *standard* industrial wastewater. Each industry requires a specifically adapted treatment process to treat unique waste streams under

specific climatic conditions. In addition, many industrial streams are toxic to microorganisms. For these reasons, treatment of industrial wastewater by stabilization ponds is considered by many as problematic as there are no universal treatment protocols, and there is no *one size fits all* solution. A main reason for this situation is that the tendency has been to develop intensive treatment processes, requiring the shortest hydraulic retention time and requiring the smallest possible facilities. With industrial wastewater this approach, more often than not, cannot work, for several reasons. The first is that in many cases, primarily in the chemical and pharmaceutical industry (and occasionally in the food industry where potent bactericidal agents used to clean production lines often find their way to the wastewater), the concentrated wastewater is toxic, and unless diluted to non-toxic levels, microbial populations will not survive. In the food industry there are cases in which strong disinfectants are used, and in the pharmaceutical industry various bactericidal or antibiotic compounds find their way into the waste streams. Therefore, physico-chemical processes must be applied to treat toxic wastes. Also, biodegradation of complex industrial waste frequently requires several cycles of alternating environmental conditions, such as shifting between anaerobic reducing conditions to aerobic oxidizing conditions so as to facilitate total biodegradation of a set of contaminants.

For all these complex situations, the use of the oxidation pond, or still better its big brother, the wastewater reservoir, is ideal, as in the various zones of a deep reservoir one finds all possible conditions for optimal degradation of various pollutants. A good example of successful application of this approach are the treatment plants devised in our laboratory for treating the wastewater discharged by two of the largest cheese manufacturing dairies in Israel. A set of deep reservoirs were built to treat very concentrated wastes (BOD up to 20 g l^{-1} , $1\text{--}2\text{ g l}^{-1}$ of emulsified fat, and ammonia at concentrations of $200\text{--}300\text{ mg l}^{-1}$). One of these, the largest dairy in Israel, located in a rural area, discharges $\sim 700\,000\text{ m}^3$ per annum of highly concentrated wastewater. By the time the study began (1990), the dairy had already been operating for about 60 years without any treatment facility for its wastewater. Because dairy wastewater contains no human pathogens, heavy metals or toxic materials, thus posing no health hazard, the prime motive behind the requirement for a treatment plant was the need to stop the dairy from being a major polluter of a local dry river bed and a generator of mass odorous nuisance to the surrounding population. Our study was designed to provide a suitable treatment process to fulfill environmental regulations, taking into account the unique local operational constraints: (1) There is no source of domestic wastewater in the vicinity to dilute the dairy's wastewater and therefore any treatment process would have to treat the undiluted wastes. (2) There are large daily, weekly and annual fluctuations in the quantity and organic load of the discharged wastewater, and the treatment process should therefore be flexible enough to tolerate large instantaneous shock loads in both quantity and quality of the wastewater. (3) The salinity of the effluents prevents their reuse for irrigation. (4) The dairy's wastewater contains significant amounts of fat, as well as detergents and bactericidal agents used several times a day to clean all facilities that are in contact with the milk.

Originally, the obvious approach by which to comply with these constraints was to build a large electromechanical plant to treat the wastes, and a deep reservoir to store the effluents for discharge at intervals to avoid damage to local fish breeders that use the dry riverbed for moving water between ponds. Instead, we decided to study the possibility of using the reservoir itself as a dual-purpose facility, for both treatment and storage of the effluents. Compared to any intensive treatment facilities, be it any variation of an activated sludge or an anaerobic digester, these dual purpose reservoirs have several operational advantages, in addition to the obvious savings in energy, manpower and cost of installations: First, they are, within wide margins, very insensitive to fluctuations in both quantity and quality of the inflowing raw water. Second, there is no need for any pretreatment for removing the large quantities of emulsified butter that is present in the raw water. As fatty acids cannot be degraded anaerobically, their removal is essential prior to any anaerobic intensive treatment. In the reservoir, as the butter separates from its emulsifiers, it floats to the surface of the water where it is exposed to atmospheric oxygen, being rapidly degraded by the microbial flora present at the air–water interface. Finally, treatment in these deep reservoirs generates insignificant amounts of sludge.

The depth of these reservoirs (three in series) is up to 13 m, and they generate effluents of the highest quality (in mg l^{-1} $\text{BOD} < 10$, ammonia 0–1.0, volatile organic suspended solids [all as algal biomass] 60–80, NO_2 0–1.0 and zero fecal coliforms). The effluents are currently discharged freely into the local dry riverbed without posing any threat to any agricultural or recreational activity in the region.

The mode of operation is simple: In the anaerobic zone, sugars are oxidized to organic acids which then undergo methanogenic fermentation, while fats and proteins are degraded in the upper aerobic layer. All this takes place in a single, fully automatic system, requiring no input of energy or manpower. The second and third reservoirs are for polishing, reducing significantly the level of contaminants at each stage.

Another example is seen in the Ramat Hovav evaporation ponds. Ramat Hovav is a large chemical industry park, south of Beer Sheba Israel, manufacturing insecticides, fungicides, herbicides, pharmaceuticals, organo-bromides, etc. The site discharges ca. $6000 \text{ m}^3 \text{ d}^{-1}$ of wastewater, but there is at present no treatment for the water and the park is not allowed to discharge it out of the park boundaries. The current solution is to store the water in large evaporation ponds. Pond water pH fluctuates between 2.5 and 10.0 and the salinity between 6 and 25% (w/v), and the water contains hundreds of organic compounds. In spite of these harsh conditions there is a large flora of microorganisms in the ponds, capable of degrading practically any compound tested (Lahav *et al.*, in press, 2002). These evaporation ponds act, in essence, as stabilization ponds, removing through biodegradation an entire range of chemicals from this potentially hazardous site. The multiplicity of niches present in a single biotope is a key factor enabling degradative processes to take place. Degradation of haloaliphatic and aromatic compounds, for example, often requires anoxic conditions for microorganisms capable of halo-respiration to carry the reduction of organic bromine or chlorine to

inorganic forms. Once this is accomplished, aerobic degradation of the organic residue can take place (Ronen & Abeliovich, 2000).

Removal of hydrocarbons by oxidation ponds was also reported by Abid & Boukfaoui (2001). Another example is the treatment of piggery wastes (very concentrated wastes) in waste stabilization ponds, which bring the effluents to an acceptable quality standard for irrigation (Estrada & Hernandez, 2002).

25.5 Summary

It has now been over 50 years since the idea of combining wastewater treatment with production of algal biomass for feeding fish, poultry and other livestock, using various configurations of the high rate oxidation pond, caught the imagination of many. Research into the ecophysiology and economics proved the idea not feasible, for several reasons. One is the need for a two to three times increase in the size of the treatment plant upon shift from summer to the cold winter season. Another concerns the expense of drying the biomass, which requires concentrating and evaporating all the treated wastewater without using unsafe flocculants. Another is the strict and absolute requirement for total disinfection of the product making it totally free of all possible pathogens. Also, the high rate oxidation pond is a very unstable ecosystem, operating at its limits as far as retention time, ammonia concentration, and photosynthesis are concerned, particularly during transition of summer/winter seasons, at which time the community structure changes, adapting to new temperature and light regimes. Nevertheless, the conventional oxidation pond has its place in small communities, and where land is available and cost is low.

Particularly in semiarid climates, where rain is seasonal while wastewater discharge is constant throughout the year, wastewater reservoirs are most suitable for optimizing reuse of the water. Indeed, when properly managed, they can also be used for carrying the treatment process itself, with particular advantage for treating industrial wastewater, where the great dilution volume of the reservoir, the long retention time, and the variability of an entire range of environmental conditions combine to facilitate degradation of many compounds considered non-biodegradable in conventional treatment plants. Also, the wastewater reservoir was proven to be very efficient in removing all pathogens from the water.

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26 Water Pollution and Bioremediation by Microalgae

Absorption and Adsorption of Heavy Metals by Microalgae

Drora Kaplan

26.1 Introduction

From a chemical point of view, the majority of the elements in the periodic table are metals and metalloids. Only H, B, C, N, P, O, S, halogens, and noble gases are not included in this category. Metal ions are further grouped into type-A, type-B and transition metal cations (Morgan & Stumm, 1991). From the biological point of view, metals and metal ions can be sorted according to their environmental impact or toxicity. Metallic elements of densities greater than 5 g cm^{-3} are called *heavy*. Since these elements exert toxic effects on living organisms they are termed *toxic heavy metals*. Some of the heavy metals, such as copper, nickel, and zinc are, at very low concentrations, essential for life because they play important roles in metabolic processes taking place in living cells (Gadd, 1993). However, elevated levels of these metal ions are toxic to most prokaryotic and eukaryotic organisms. Other heavy metals such as cadmium, lead, and mercury are nonessential and are known to cause severe damage in organisms even at very low concentrations. Metals occur in different forms: as ions dissolved in water, as vapors, or as salts or minerals in rocks, sand, and soils. They can also be bound in organic or inorganic molecules, or attached to particles in the air (Raspor, 1991; Wedepohl, 1991). Metal toxicity is often dependent on its chemical form (metal speciation). It is generally accepted that for most metals the free ion is the species most toxic to aquatic life (Sunda & Guillard, 1976; Anderson & Morel, 1978). Some organic forms such as methyl-mercury are taken up very efficiently by living organisms. It is more toxic than other mercury species (George, 1991). Industrial processes and intensive agricultural practices, often result in the release of various heavy metals into terrestrial and aquatic

environments. Heavy metals are stable and persistent environmental contaminants since they cannot be degraded or destroyed. Therefore, their toxicity poses major environmental and health problems and requires a constant search for efficient, cost-effective technologies for detoxification of metal contaminated sites.

26.2 Microalgae – heavy metals interactions

In the aquatic environment metallic elements may be present in a number of different chemical forms (species), distributed between the sediment and the solution (Florence & Batley, 1980; Foster & Wittman, 1981; Raspor, 1991). Metals may be present in the sediment as insoluble inorganic complexes, suspended particles or in association with organic colloids. In solution, metals may be present as free metal ions, as well as organic and inorganic complexes. The equilibrium among all these metal species is interchangeable and depends on such environmental factors as temperature, pH, alkalinity, etc., as well as on the biota thriving in the water (Canterford & Canterford, 1980; Peterson, 1982). A scheme illustrating the possible interactions among the various metal forms in an aquatic environment is presented in Fig. 26.1. Biological availability of trace metals either as required nutrient, e.g. iron and zinc, or as toxicant, e.g. cadmium and lead, is dependent on its chemical form (Barber, 1973). In most cases the free metal ions, which are the bioavailable forms, are the most toxic species (Sunda & Guillard, 1976; Anderson & Morel, 1978; Gachter *et al.*, 1978; Hudson, 1998). Thus, any process that

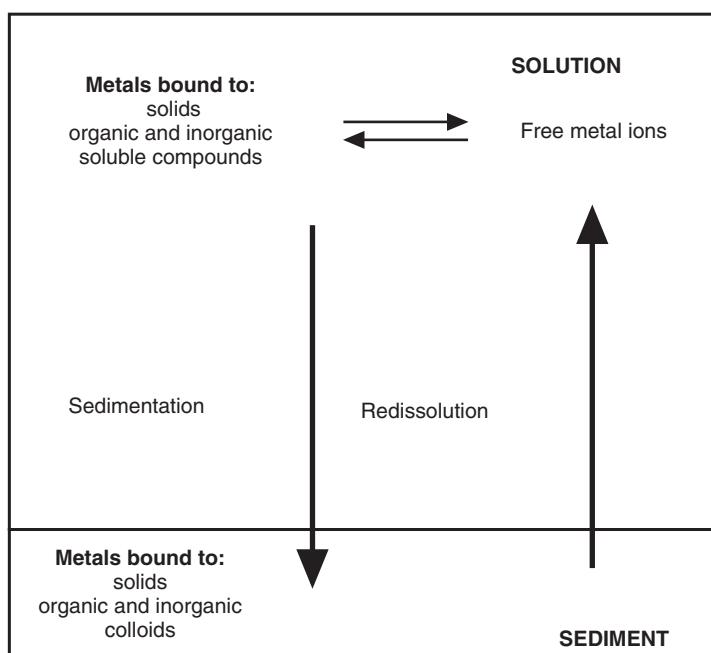


Fig. 26.1. Schematic representation of possible metallic forms in an aquatic ecosystem.

accelerates their transformation into bound forms results in a reduction of toxicity to biota (Butler *et al.*, 1980; Canterford & Canterford, 1980; Peterson, 1982).

Microalgae are very sensitive to metal toxicity and therefore are often used as biological sensors for detecting potential toxic effects of heavy metals (Torres *et al.*, 2000 and ref. therein). Toxic effects may be caused by a number of mechanisms: (a) the blocking of functional groups of biologically important molecules, e.g. enzymes and transport systems for essential nutrients and ions, (b) the displacement and/or substitution of essential metal ions from biomolecules and functional cellular units. This may result in modification and inactivation of enzymes as well as disruption of cellular and organelar membrane integrity (Mallick & Rai, 1992; Rai *et al.*, 1994). Thus toxic metals have the potential to interfere with a wide spectrum of activities in living organisms. Almost every aspect of their metabolism, growth and differentiation may be affected.

Some prokaryotic and eukaryotic organisms have developed means for rendering toxic heavy metals to innocuous forms. They possess a variety of mechanisms to maintain metal homeostasis and prevent heavy metal poisoning. Heavy metal resistance in microalgae as well as in other microorganisms may result from the ability to prevent uptake (*avoidance*). This is achieved by adsorption of toxic metal ions to cell-associated materials and/or cell wall components (Kaplan *et al.*, 1987a; Xue *et al.*, 1988), or secretion of metal-binding organic compounds to the surrounding environment (McKnight & Morel, 1979; Van der Berg *et al.*, 1979). In both cases specificity is rather low and any metal cation may interact with negatively charged residues of the organic compounds to form complexes. Metal resistance may also result from the ability to cope with high amounts of heavy metals inside tissues (*tolerance*), an active process that involves the uptake (absorption) of the metal ions. Heavy metals enter microalgae cells via micronutrient transporters (Sunda & Huntsman, 1998 and ref. therein). Once in the cell, heavy metal detoxification may be achieved by binding to specific intracellular compounds, and/or transport of the metals to specific cellular compartments (Nagano *et al.*, 1984; Heuillit *et al.*, 1986; Vymazal, 1987). A common mechanism for intracellular metal detoxification in living organisms is the formation of metal binding peptides or proteins such as metallothioneins (Hamer, 1986) and phytochelatins (Kondo *et al.*, 1984; Gekeler *et al.*, 1988). For more details concerning the various types of metal binding proteins and peptides in algae see review by Robinson, 1989. Organic compounds such as malate, citrate, and polyphosphate were also reported as intracellular chelating agents (De Filippis & Pallaghy, 1994).

Biosorption of heavy metals by microalgae is generally a bi-phasic process (Roy *et al.*, 1993). The first phase is adsorption by extracellular cell-associated materials, e.g. polysaccharides, mucilage, etc. (Kaplan, 1988; Xue *et al.*, 1988) and cell wall components, e.g. carboxy and hydroxy groups, as well as sulfate (Crist *et al.*, 1981, 1999; Volesky, 1990; Eccles, 1999). This is a non-metabolic, rapid process, that occurs in both living and non-living cells. It is dependent on a number of parameters: pH (Hassett *et al.*, 1981; Lau *et al.*, 1999), heavy metal species (Fourest & Volesky, 1997; Radway

et al., 2001), type of algae (Kaplan *et al.*, 1987a; Donmez *et al.*, 1999; Chong *et al.*, 2000), and biomass concentration (Donmez *et al.*, 1999). The second phase is absorption and accumulation inside the cell. This is a slow process involving active transport through the cell membrane into the interior and binding to proteins and other intracellular sites. It is a metabolic-dependent mechanism that is inhibited by low temperatures, absence of an energy source, metabolic inhibitors, and uncouplers, and occurs only in living cells (Wilde & Benemann, 1993). Understanding the mechanisms that convey metal resistance in various organisms can provide strategies for their removal from the environment.

26.3 Metal detoxification

Industrial activities such as mining, electroplating, electronics, etc. significantly increase the contamination of the environment by toxic heavy metals. Physico-chemical approaches are presently available for metal detoxification and removal from polluted environments. Nevertheless, during the last two decades, there is increasing interest in applying biological approaches for this purpose.

26.3.1 Physico-chemical approaches

The most common physico-chemical methods available for remediation of metal contaminated waters are: precipitation by adjusting the pH, filtration, flocculation and/or adsorption by organic compounds. These methods often lack the specificity required for treating target metals. They are also inefficient and expensive, especially when metal concentration in the wastewater is low. In addition, high cost often limits their use in large-scale *in situ* operations.

26.3.2 Biological approaches

Biological approaches are based on the use of naturally occurring processes. Many microorganisms take part in the bio-geo-chemical cycling of toxic heavy metals. Microalgae as well as other microorganisms play a significant role in the transformation of heavy metal ions in the environment. Organic compounds released from growing cells, as well as biodegradation products of various origins, may serve as complexing agents for metal ions, thereby decreasing metal toxicity (McKnight & Morel, 1979; Kaplan *et al.*, 1987a). Binding of metal ions to cell wall components of microalgae was also reported (Kaplan *et al.*, 1987b; Kaplan, 1988; Xue *et al.*, 1988). Various metabolic processes such as photosynthesis, respiration, nutrient uptake, etc. take place during the growth of microalgae. All of them influence the equilibrium between free metal ions and the bound forms, as well as that between sedimentation and re-dissolution in the aquatic environment. Microalgae thriving in metal-contaminated sites also possess intracellular mechanisms that enable them to cope with the toxic effects of metals. Such species may be used for *in situ* bioremediation of large water bodies contaminated with low

concentrations of metal ions (for more detailed comparison between physico-chemical and biological approaches for metal detoxification see Wilde & Benemann, 1993; Eccles, 1999; Volesky, 2001).

26.4 Potential applications of microalgae in heavy metal bioremediation

The last two decades witnessed increasing interest in biological processes involved in metal transformation and detoxification in the environment (Gadd & White, 1993; Kratochvil & Volesky, 1998; Eccles, 1999; Gadd, 2001; Volesky, 2001). To date, it is generally accepted that technologies based on naturally occurring biological processes have a number of advantages over presently available physico-chemical techniques for remediation of sites contaminated with toxic heavy metals. These advantages have been discussed in detail, in a number of publications (Wilde & Benemann, 1993; Eccles, 1999; Gadd, 2001; Volesky, 2001).

The potential of many organisms (algae, bacteria, cyanobacteria, and fungi) as well as dead biomass derived from them for metal bioremediation was examined (Gadd & White, 1993; Wilde & Benemann, 1993; Volesky & Holan, 1995; Kratochvil & Volesky, 1998; Donmez *et al.*, 1999).

Microalgae are very abundant in the natural environment and are well adapted to a wide range of habitats, e.g. fresh and sea water, domestic and industrial effluents, salt marshes and constructed wetlands. They have a remarkable ability to take up and accumulate heavy metals from their surrounding environment (De Filippis & Pallaghy, 1994). Their ability to sequester various metal ions such as copper, cadmium, nickel, gold and chromium is well documented (Darnall *et al.*, 1986; Harris & Ramelow, 1990; Asku *et al.*, 1992; Cho *et al.*, 1994; Chong *et al.*, 2000; Wong *et al.*, 2000; Tam *et al.*, 2001). Therefore, attempts were made to use microalgae, living cells or their dead biomass, for removing heavy metals from contaminated waters (Gadd, 1990; Volesky, 1990; Wilde & Benemann, 1993; Rehman & Shakoori 2001).

The use of living cells is most efficient for removal of metal ions from large water bodies containing low concentrations (ppb range) of metal ions. Resistant microalgal species isolated from metal-contaminated sites have a higher capacity for accumulating heavy metals compared with species isolated from non-contaminated sites (Trollope & Evans, 1976; Wong & Pak, 1992; Wong *et al.*, 2000). During algal growth, metals are removed from the surrounding environment and accumulated in the cells by both non-metabolic dependent processes (adsorption) and metabolic dependent ones (absorption). Provided that adequate environmental conditions for supporting microalgae growth, such as light, temperature and pH are present, the use of living microalgal biomass offers an efficient, simple and cost-effective method. Microalgae in consortium with other microorganisms, such as microbial mats, are also capable of removing metals and metalloids as well as other recalcitrant organic compounds from contaminated sites (Bender *et al.*, 2000 and ref. therein).

Microalgal biomass has been successfully used as sorbing material (Corder & Reeves, 1994; Donmez *et al.*, 1999; Klimmek *et al.*, 2001). Commercial algal biomass AlgaSORB® was produced based on the basic and applied work of Darnall (1991). Moreover, metals adsorbed on cell wall surfaces of algal biomass can be recovered and the sorbing material can be restored for reuse (Kotrba & Rumí, 2000 and ref. therein; Volesky, 2001 and ref. therein). Removal of metals from sites contaminated with high concentrations of metals can be achieved using nonviable biomass as biosorbents (Kotrba & Rumí, 2000). Yet, it should be noted that biomass obtained from different algal species differ largely in their binding capacity for various heavy metals (Donmez *et al.*, 1999; Chong *et al.*, 2000; Wong *et al.*, 2000; Klimmek *et al.*, 2001; Radway *et al.*, 2001). The metal-binding capacity of biosorbents depends on the cell wall composition of the organism it is derived from and on the chemical composition of the metal ion solution to be treated. Therefore, in order to choose the most adequate biosorbent for metal decontamination of a specific site it is essential to know which metals are present there and the concentration of each. Selection of the appropriate biomass is actually dictated by the metals to be removed, and the correct choice is essential for achieving efficient bioremediation.

In conclusion, many species of microalgae as well as their cell mass can be used for bioremediation of metal polluted sites. In order to bring this potential to the applicable stage on a commercial basis, the interdisciplinary cooperation among professionals from various fields, e.g. biologists, chemists, engineers, and environmentalists is needed.

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27 Water Pollution and Bioremediation by Microalgae

Impacts of Microalgae on the Quality of Drinking Water

Carl J. Soeder

27.1 Introduction

By their photosynthetic oxygen production, microalgae may provide the basis for maintenance of good water quality by means of self-purification, especially in those deeper surface waters that are still clean and healthy. Where water quality is endangered or impaired by pollution, water management can improve the situation by removal of pollutants at source in wastewater treatment plants. Any such water management is intended to eventually manage and control algal populations under natural conditions. As perhaps the most promising option for employing algal biotechnologies in water treatment, removal of heavy metals is mentioned below.

Today, most people do not have sufficient access to uncontaminated deep aquifers with high-quality fresh water. Instead, they have to rely on processed surface water taken from often eutrophicated reservoirs, lakes or rivers. Here, one is often confronted with negative influences of algae such as disturbances of water processing, off-flavours and even toxification.

27.2 Contributions of microalgae to water purification

The most prominent contribution of microalgae to improvement or maintenance of water quality is their photosynthetic oxygen production in most types of basically nutrient-limited surface waters. They thereby ensure aerobic microbial degradation of dissolved organics, oxidation of ammonia, etc., i.e. the entire complex of processes known as *self-purification* (Lampert & Sommer, 1999; Wetzel, 2001, see also Chapter 24). This capacity is essentially lost upon pronounced eutrophication and may be regained by those

eco-biotechnological measures that lead towards oligotrophication (see Section 27.4). The scale at which related strategies of water management are pursued exceeds, of course, the scale of processes in ordinary bioreactors by many orders of magnitude.

Many microalgae can take up, degrade and utilize various kinds of commonplace dissolved organics, though by no means as efficiently as heterotrophic bacteria (and perhaps some aquatic fungi). Even more negligible is their potential to degrade xenobiotic substances in general. However, at least several unicellular green algae are able to desulphonate naphthalene sulphonic acids (Soeder *et al.*, 1987), a group of otherwise very persistent and ubiquitous by-products of chemical industries. For example, illuminated suspensions of *Scenedesmus obliquus* desulphonated 1-naphthalenesulphonic acid at a rate of about 25 mg g^{-1} dry biomass d^{-1} , and somewhat less in the presence of $2 \times 10^{-4} \text{ M}$ sulphate. In mixed culture with bacteria, the latter degraded the resulting hydroxylic compounds and might also do so in nature, thereby contributing to self-purification of another mode.

The advantages of immobilization for utilizing microorganisms or cell cultures for biotechnological processes stimulated analogous approaches with microalgal biomass (a rather costly material). Most of these numerous studies are aimed at the elimination of various water pollutants. For instance, Garbisco *et al.* (1991) thoroughly investigated the removal of nitrate by *Phormidium laminosum*, immobilized in polyvinyl or polyurethane foam. Although nitrate uptake was impressively stable over weeks, the fate of assimilated nitrogen in the system remained as unclear as the competitiveness with existing technical processes (denitrification, ion absorption).

Among the many reports on the elimination of dissolved heavy metal ions (see Chapter 26) by biosorption through algal biomass (Veglio & Beolchini, 1997), the one by Bunke *et al.* (1999) is of particular technical relevance. Possessing a high metal-sorption potential, the marine cyanobacterium *Lyngbya tailorii* was immobilized in cellulose-sulphate pellets and loaded with Cd, Ni, Pb or Zn. Desorption with diluted hydrochloric acid gave a metal-ion concentrate to be precipitated. Loading and deloading cycles were repeated many hundreds of times without significant loss of capacity. The same promising results were obtained with *Lyngbya*-trichome residues after chemical extraction of antivirus compounds. In this case, an otherwise valueless by-product could be used for an important step in water treatment.

27.3 Negative impacts of microalgae on water quality

27.3.1 Disturbances of lake-water processing

Technical efforts and costs associated with the processing of surface water into safe drinking water increase with algal concentration in the raw water. The first steps of processing have to be the removal of most of the suspended matter including bacteria, phytoplankton, zooplankton, inanimate organic particles and clay minerals (Bernhardt, 1995). This is done for two reasons: removal of the greater part of potential pathogens; and a decrease of organic matter that consumes hygienizing chemicals such as chlorine or ozone in the

final steps of treatment. Moreover, formation of hazardous by-products of chemical hygienization (e.g. chloro-organic compounds in case of chlorination) is also proportional to the concentration of suspended matter (Hoyer, 1998). Disinfection by UV irradiation seems to have the advantage that no undesirable by-products are formed (Hoyer, 1998).

The most important techniques for suspensoid elimination from raw water are flocculation by polykations like polymeric Al^{3+} or Fe^{3+} hydroxides with subsequent sand filtration and/or groundwater re-infiltration (Bernhardt, 1995). Microstraining may be a useful pretreatment. Regardless of the technology employed, algal blooms will always create extra expenses: greater amounts of chemicals are consumed, filter running times are shortened, and substitute filters have to be provided. In surface water, the carbon bound in the less readily degradable dissolved organic compounds may clearly exceed the carbon content of suspended algal biomass (Wetzel, 2001). Hence, the consumption of flocculants depends not only on the concentration of negatively charged seston particles but, to a significant extent, also on extracellular algal products such as acid polysaccharides, uronic acids and lower carboxylic acids (Lüsse *et al.*, 1985). These bind flocculants and may cause seston and flocculant to break through the filters, unless the flocculant dose is increased according to the results of adequate flocculation tests (Hoyer & Schell, 1998). For example, flocculation of a bentonite suspension (10 mg l^{-1}), in the bottom water of a lake required 20 mg l^{-1} of Al^{3+} , and about 500 mg l^{-1} in the filtrate of a bloom of *Planktothrix rubescens* sampled at the surface of the same lake. But even at the highest Al^{3+} dosage the residual turbidity, i.e. the non-flocculable seston, was much higher than in the case of near-bottom water. This shows how algal blooms can lower water quality in a sometimes unmanageable way.

As substantial amounts of acid polysaccharides accumulate also in unsterile open mass cultures of planktonic freshwater algae (Lüsse *et al.*, 1985), these compounds are at least relatively resistant to degradation by adapted bacteria (see also Weiss & Simon, 1999).

In groundwater re-infiltration, the critical step is the filtration through sand filters that are, in fact, downflow biofilters by which even single microbial cell can be kept from being transported by the seepage water into the aquifer. The more phytoplankton the raw water contains, the sooner the filter surface will be clogged, not only by algal biomass but also by heterotrophic microorganisms decomposing dead algae. Another problem is secondary massive growth and eventual decay of filamentous algae on the filter surface (Müller *et al.*, 1982). Here, anaerobic decomposition of biomass may occur, resulting in the production of indole, skatole, polyamines and odourous hydroxylated fatty acids, etc. all of which can create off-taste and odour problems.

27.3.2 Phycogenic off-tastes and odours

Aerobic, massive populations of freshwater algae can produce and release quite a variety of compounds that are either odourous or cause off-flavours, or even both. This first became obvious in cases of decay of dense vegetations

of filamentous algae (e.g. *Cladophora*; Lembi *et al.*, 1994), followed by pronounced development of actinomycetes (Müller *et al.*, 1982) producing the aforementioned geosmins in shallow or littoral zones of surface waters. Putrescent amines as released by several types of algae cause problems in case of certain blooms while other algae (e.g. planktonic diatoms) are notorious for producing geranium-like, muddy, rancid or stinging odours (Müller *et al.*, 1982). Until now, only a couple of freshwater algae, definitely less than the number of bloom-forming species, have been investigated with regard to the production of olfactoriously unpleasant products, and many of those compounds separated by gas chromatography are as yet not identified.

27.3.3 Algal and cyanobacterial toxins

Toxins of eukaryotic freshwater algae, mainly flagellates like *Prymnesium parvum*, cause occasional fish kills or death of water fowl. Yet, of much greater concern are the cyanobacterial toxins such as produced by species of *Anabaena*, *Anacystis*, *Microcystis*, *Lyngbya*, *Nodularia*, etc. These toxins have repeatedly killed cattle and can cause fatal illness in humans. For further details see Chapter 24, and Chorus (2001).

27.4 Regaining ecological benefits from algae by lake restoration

When algae spoil water quality through release of undesirable products, this is usually associated with algal blooms where populations of planktonic algae have become so dense that there is a macroscopic colouration of the surface water. Above a certain threshold of nutrient input and internal loading, the basic rule is: the higher the eutrophication, the denser the phytoplankton at blooming periods are and the greater the water quality problems (Lampert & Sommer, 1999; see also Chapter 24).

Algal blooms, usually consisting of a relatively few species of worldwide distribution, occur in various shades of green, blue-green, brownish, or even red. They often oscillate between peaks and decay periods, or may be seasonal phenomena (Wetzel, 2001). Cyanobacterial blooms, however, can be more or less permanent because many planktonic blue-greens form gas vacuoles that can enable them to top virtually all competitors by shading and chemical warfare.

Eutrophic standing waters with permanent blooms of cyanobacteria pose considerable phycogenic water-quality problems (see Chapter 24). As observed by the author, this holds, for example, for the shallow Wolga reservoirs and for the vast majority of lakes and reservoirs in the more densely inhabited areas of the People's Republic of China where the rule is confirmed that in warmer climates much smaller quantities of phosphate are needed for eutrophication than in the temperate or subpolar regions (Wetzel, 2001).

Eutrophication is reversible by lake restoration, also known as oligotrophication. This is accomplished by combining the following measures: (a) efficient and complete treatment of nutrient-rich wastewaters in the entire catchment area, (b) restricted fertilization of arable land in the same area, (c) removal and safe disposal of bottom sludge (for controlling *internal loading*).

These measures are frequently termed as *entirely utopic*. Rightly so? An outstanding example of oligotrophication concerns the Wahnbach Reservoir near Bonn, Germany (storage capacity 41 million m³) that supplies part of the Rhine Valley with drinking water. After the reservoir was filled in 1962, it became so strongly eutrophicated that dense blooms of *Oscillatoria rubescens* developed soon and the hypolimnion turned anaerobic. When hypolimnic aeration failed to prevent further massive developments of phytoplankton, it was decided to remove the orthophosphate from the influent creek almost completely by precipitation with iron hydroxide. To this end, a large phosphate elimination plant has been in operation since 1978 (Bernhardt, 1995). Thereafter the annual maximum of phosphate dropped from 25–30 mg m⁻³ to present 2–3 mg m⁻³, whereas nitrate levelled off at about 20 mg m⁻³. Annual means of algal chlorophyll decreased from >20 mg l⁻¹ to <1 mg m⁻³, while Secchi depth (as a measure of vertical visibility) rose from initially <1 m to present >5 m with maxima of 15 m (O. Hoyer, personal communication). Although the initial investment was substantial, it certainly paid off by saving much extra expense for water treatment during algal blooms and by stabilizing raw-water quality in addition to substantial improvements of drinking-water quality.

For the reasons mentioned above, restoration of warm-water lakes is much more difficult than of standing waters in cooler regions. The author actually knows of no case of successful lake restoration in the subtropics or tropics.

Contrary to the immensely positive value of photosynthetic oxygen production in surface waters, microalgal biotechnologies are hitherto of rather limited practical relevance in water treatment. Detrimental impacts of algae on water quality consist in disturbances of water processing, off-flavours and toxin production by a few species. Restoration and proper management of surface waters are the appropriate countermeasures. Unfortunately, they are too rarely practised because of the costs entailed.

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Part IV

New Frontiers

28 Targeted Genetic Modification of Cyanobacteria: New Biotechnological Applications

Wim F.J. Vermaas

28.1 Introduction

A major change that truly redefined the biological potential of life on this earth was brought about by the ability of early cyanobacteria to carry out oxygenic photosynthesis and to convert a very reducing and anaerobic atmosphere to one with increasing amounts of oxygen. Oxygenic photosynthesis is the process of using light energy to oxidize water (forming molecular oxygen) and to generate electrons that eventually can be used to fix carbon dioxide to sugars. The cyanobacterial phylum may be about 3.5 billion years old (Schopf, 1993; Golubic & Lee, 1999), and this makes cyanobacteria the earliest known group of organisms that contributed greatly to the formation of the oxygen that we breathe. Cyanobacteria or their close relatives are the evolutionary ancestors to chloroplasts (reviewed by McFadden, 1999; Moreira *et al.*, 2000). Therefore, oxygenic photosynthesis that is responsible for almost all of the oxygen in the atmosphere originated from cyanobacteria, and cyanobacteria may have helped shaping the ecology of this earth more than any other group of organisms until humans developed modern technologies. Evolutionarily ancient model systems such as cyanobacteria retain much potential for change and adaptation, and therefore provide excellent targets for modification for use in biotechnological applications.

Cyanobacteria represent a diverse phylum, with several genera responsible for a large portion of the photosynthetic production in the open ocean (Geider *et al.*, 2001). Cyanobacteria can be found virtually anywhere near the surface of the earth, from Antarctica to hot springs, and the most thermophilic organism capable of oxygenic photosynthesis is a cyanobacterium.

Some of the very few habitats that may be devoid of cyanobacteria but that harbor other organisms are hyperthermal vents, which are the home to a variety of archaea, and the deep sea. The implication of this wide distribution is that many cyanobacteria can thrive in a relatively large temperature range and use many different growth modes. This flexibility is of considerable importance when selecting organisms to introduce new or altered metabolic or physiological processes for biotechnological applications.

In contrast to most eukaryotes, a number of cyanobacterial species are very suitable for genetic modification. Important reasons for this suitability are that several cyanobacteria, including *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002, are spontaneously transformable and efficiently integrate foreign DNA into their genome by double homologous recombination (Fig. 28.1) (see Williams, 1988; Vermaas, 1996, 1998). Double homologous recombination not only allows targeted knock-out mutagenesis (deletion of specific genes), but also facilitates targeted gene replacements and insertions as well as introduction of genes with specific mutations. In contrast, in most eukaryotes introduced genes are integrated into the genome at virtually random positions, which essentially eliminates the possibility of targeted gene deletion and makes the expression level of introduced genes rather unpredictable.

Several other important advantages of using cyanobacteria are: (1) some cyanobacteria grow well in the absence of physiologically important pathways such as photosynthesis or respiration, as long as suitable substrates are provided, (2) the genomic sequence for a number of cyanobacteria is known, and in general the number of microbes with a sequenced genome far exceeds that of eukaryotes, and (3) several unrelated genes can be deleted and/or introduced into the same strain as multiple antibiotic-resistance cassettes can be used.

In this chapter the status of genetic modification of cyanobacteria will be reviewed, and procedures and accomplishments to delete or modify native genes, or to express foreign genes in cyanobacterial systems will be highlighted. In view of size constraints, this chapter will present primarily new avenues that happened to result from work in my own group.

28.2 Sequence information

Table 28.1 summarizes the current status of genomic sequencing projects involving cyanobacteria. The genome size between different cyanobacteria varies by more than fivefold, presumably reflecting the metabolic complexity that the various strains are required to maintain for survival. The cyanobacterium with the smallest genome sequenced thus far, *Prochlorococcus marinus* MED4, is a marine organism that may have specialized in growing photoautotrophically in an aqueous, marine environment, in which light and fixed-nitrogen sources are available. *Nostoc punctiforme*, in contrast, can fix atmospheric N₂ and can occur in different developmental states (as heterocysts, motile cells, or spore-like cells, and free-living or in symbiosis) depending on environmental conditions.

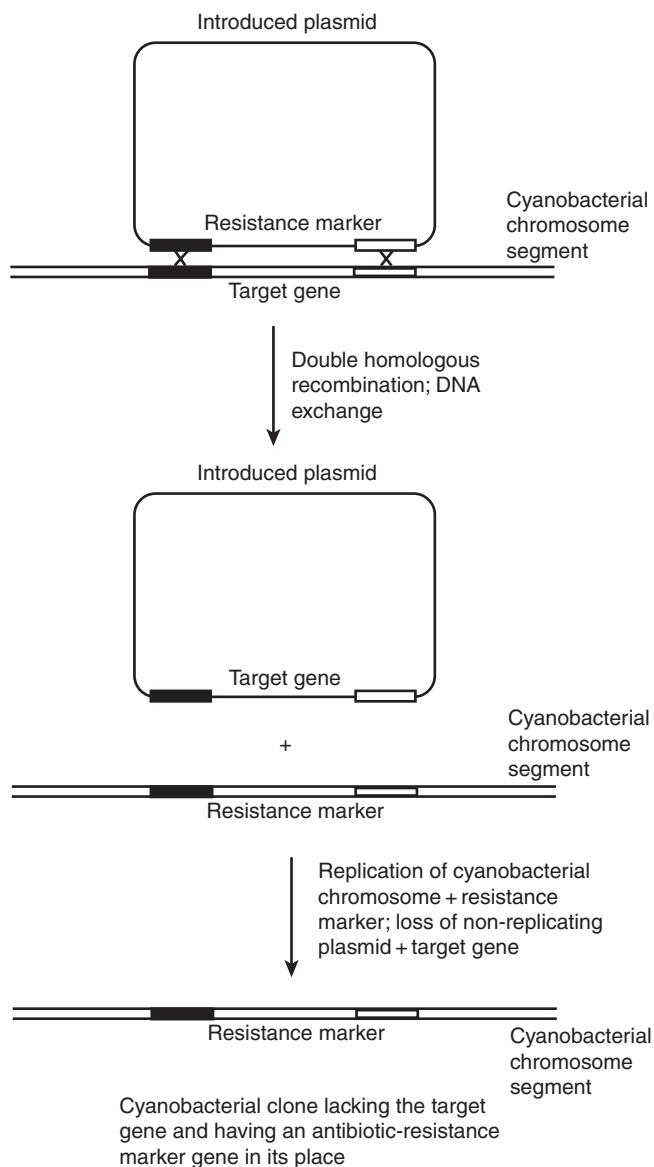


Fig. 28.1. Schematic representation of double-homologous recombination between the cyanobacterial genome and an introduced plasmid that carries *Synechocystis* sp. PCC 6803 sequences that are identical to those up- and downstream of a cyanobacterial target gene (up- and downstream sequences are indicated by open and closed rectangles). Suitable plasmids are those used for cloning in *Escherichia coli* (pUC18/19, etc.) with appropriate *Synechocystis* sequences and an antibiotic-resistance marker gene inserted. Upon double homologous recombination, DNA exchange occurs between the plasmid and the cyanobacterial genome, and the cyanobacterial genome loses its target gene and gains an antibiotic-resistance marker. In the presence of the appropriate antibiotic, the cyanobacterial cell carrying the transformed genome copy can propagate normally. The plasmid does not replicate in *Synechocystis* sp. PCC 6803, and is lost.

Table 28.1. Cyanobacteria with partially or completely sequenced genomes.

Strain	Genome (Mb)	Sequencing group	Year of completion	Web address
<i>Anabaena</i> sp. PCC 7120	7.2	Kazusa	2000	www.kazusa.or.jp/cyano/
<i>Arthrosphaera platensis</i>	?	Beijing	In progress	
<i>Gloeobacter violaceus</i> PCC 7421	4.6	Kazusa	2003	www.kazusa.or.jp/cyano/
<i>Microcystis aeruginosa</i>	4.8	Pasteur	In progress	
<i>Nostoc punctiforme</i> ATCC 29133	9.2	JGI	In progress	www.jgi.doe.gov/
<i>Prochlorococcus marinus</i> MED4	1.7	JGI	2001	www.jgi.doe.gov/
<i>P. marinus</i> MIT9313	2.4	JGI	2001	www.jgi.doe.gov/
<i>P. marinus</i> SS120	1.8	Genoscope	In progress	www.genoscope.cns.fr
<i>Synechococcus</i> sp. PCC 6301	2.7	Nagoya	In progress	
<i>Synechococcus</i> sp. PCC 7002	2.7	Beijing	In progress	
<i>Synechococcus</i> sp. PCC 7942	2.7	Texas A&M	In progress	
<i>Synechococcus</i> sp. WH 8102	2.7	JGI	2001	www.jgi.doe.gov/
<i>Synechocystis</i> sp. PCC 6803	3.7	Kazusa	1996	www.kazusa.or.jp/cyano/
<i>Thermosynechococcus elongatus</i>	2.6	Kazusa	2002	www.kazusa.or.jp/cyano/

The first cyanobacterium with a sequenced genome was *Synechocystis* sp. PCC 6803 (Kaneko *et al.*, 1996). Because of its ease of transformation and handling, this cyanobacterium has been the focus of many studies for the past five years. CyanoBase (<http://www.kazusa.or.jp/cyano>) is a useful website with searchable genomic information on cyanobacteria sequenced at Kazusa. Table 28.1 lists websites with information on several other cyanobacteria for which considerable genomic sequence is available. Bioinformatics tools to predict physiological potential based on comprehensive sequence analysis are being developed and refined, and tools to use genomic information for functional or physiological purposes are expected to become increasingly complete, accurate, and useful in the coming years. Examples of databases with useful metabolic information include MetaCyc (<http://ecocyc.org/ecocyc/metacyc.html>) and KEGG (<http://www.genome.ad.jp/kegg/metabolism.html>).

28.3 Transformation

Several cyanobacteria show spontaneous DNA uptake, without requiring a specific pretreatment of the cells. These cyanobacteria include *Synechococcus* sp. PCC 7002 and 7942, and *Synechocystis* sp. PCC 6803. Single-stranded DNA is taken up by the cell. Homologous recombination is likely to involve the Rec system; inactivation of *recJ*, coding for a nuclease that is likely to degrade DNA that has been taken up, boosts the transformation efficiency of *Synechocystis* sp. PCC 6803 by about two orders of magnitude (Kufryk *et al.*, 2002), suggesting that degradation of DNA that has been taken up may limit the frequency of transformation.

If cyanobacteria do not show spontaneous DNA uptake, they may be transformed by means of conjugation or electroporation (Thiel, 1994). Generally, introducing genetic material into cyanobacteria is not a major

limitation. A more important factor that helps to determine the usefulness of an organism for biotechnological purposes is whether the organism routinely integrates foreign DNA into its genome, and if so, whether this occurs by single- or double homologous recombination. The latter type of recombination allows for both facile deletion and introduction of genetic information (see Fig. 28.1). As indicated in Fig. 28.2, single homologous recombination results in incorporation of the entire plasmid into the genome. A single homologous recombination event will result in functional gene interruption only when the plasmid carries just an internal part of a gene and lacks both the 5' and the 3' end (Fig. 28.2). Otherwise upon integration one or both of the gene ends will be restored to a complete gene. Fortunately, double homologous recombination is the rule for several spontaneously transforming cyanobacteria including *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7002.

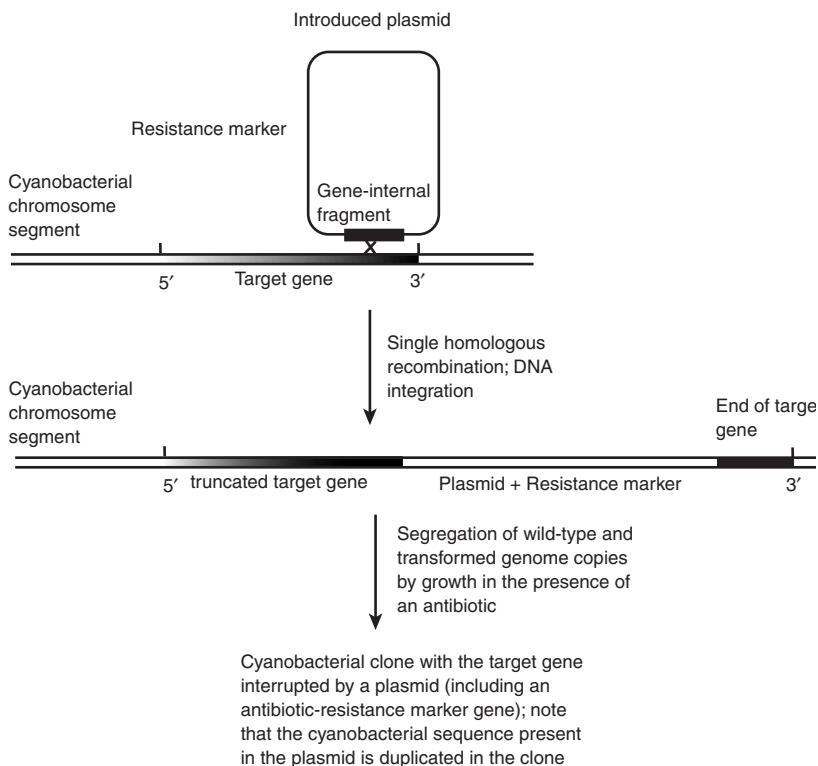


Fig. 28.2. Schematic representation of single homologous recombination between the cyanobacterial genome and an introduced plasmid that carries a gene-internal *Synechocystis* sp. PCC 6803 sequence (i.e. lacking both the transcription start and stop codons of the gene). Upon single homologous recombination, the plasmid is integrated into the cyanobacterial genome, and the *Synechocystis* gene of which part was carried in the plasmid is interrupted. If plasmid constructs are made carrying either the transcription start site region or translation stop codon of the cyanobacterial gene, an intact copy of the gene will remain in the transformed genome, and a phenotype is unlikely.

28.4 Metabolic flexibility

In addition to transformation and recombination characteristics, another important property to consider when selecting a cyanobacterium for biotechnological purposes is the ability to utilize added organic compounds for cell functions. If the organism is able to utilize external fixed-carbon sources, the organism can be grown in the absence of photosynthesis and – depending on the compound added – specific metabolic pathways may be turned on. Examples of cyanobacteria that can utilize external carbon sources and that grow very well in the presence of these compounds even if photosynthesis has been inhibited include *Synechocystis* sp. PCC 6803 (growing on glucose or organic acids) and *Synechococcus* sp. PCC 7002 (growing on glycerol). Other cyanobacteria (such as *Synechococcus* sp. PCC 7942) are obligate photoautotrophs and cannot be grown in the absence of a complete photosynthetic electron transport chain.

Another physiological parameter that is different for the various groups of cyanobacteria is their dependence on fixed-nitrogen sources. Many filamentous cyanobacteria carry specialized cells, heterocysts, that carry out N₂ fixation. As nitrogenase, the enzyme complex responsible for N₂ fixation, is O₂ sensitive, heterocysts do not carry out oxygenic photosynthesis and depend on neighboring vegetative cells for their fixed-carbon supply. Among unicellular cyanobacteria, N₂ fixation is rare. *Cyanothece* sp. ATCC 51142 is one of the few unicellular N₂-fixing cyanobacteria, and this organism temporally separates oxygenic photosynthesis and O₂-sensitive N₂ fixation (Sherman *et al.*, 1998). Organisms that fix their own carbon and nitrogen require only small quantities of phosphate and other ions for growth. However, their growth rate under these conditions tends to be slow as N₂ fixation is a very energy-intensive process. Therefore, if access to fixed nitrogen is available and cyanobacterial cultures are grown in facilities that required major capital investment, it is usually economical to make use of faster-growing species or growth modes rather than depending on N₂ fixation for growth.

Perhaps the main surprise that resulted from the completed genome sequence of *Synechocystis* sp. PCC 6803 and other cyanobacteria with a sizeable genome size was the organisms' apparent flexibility in terms of metabolism. In hindsight, this is not surprising in view of the long evolutionary history of cyanobacteria as a group of primarily free-living photosynthetic organisms that are found virtually in all habitats. For example, the *Synechocystis* sp. PCC 6803 genome sequence suggests that the organism has the ability to metabolize glucose both by glycolysis and by the oxidative pentose phosphate pathway. Physiological characterization indicated that this cyanobacterium utilizes a modified citric acid cycle even though a traditional 2-oxoglutarate dehydrogenase complex is absent (Cooley *et al.*, 2000). The organism contains genes for three terminal oxidases, and the activity of both a cytochrome oxidase and a quinol oxidase has been demonstrated (Howitt & Vermaas, 1998). The bottom line is that *Synechocystis* sp. PCC 6803 has a large variety of parallel physiological pathways, which probably enable the organism to survive and thrive in a large spectrum of physiological conditions.

28.5 Use of mutants

Over the past decade, the analysis of targeted cyanobacterial mutants has yielded detailed insight into the requirements of photosynthesis, respiration and other physiological processes. A listing of some of the genes that have been modified in *Synechocystis* sp. PCC 6803 is available on a website named CyanoGenes reachable via (<http://www.kazusa.or.jp/cyano/Synechocystis>). Mutant analysis using other systems (algae, plants and purple bacteria) has also been successful and useful, but progress using the cyanobacterial system has been particularly noteworthy; this is probably due to the ease and elegance of the experimental system. Whereas in plants and algae identification of nuclear knock-out mutants is fairly tedious (involving Polymerase Chain Reaction (PCR) screening through an extensive T-DNA collection), in cyanobacterial systems with efficient double homologous recombination such knock-outs are very easy to generate (Fig. 28.1).

In *Synechocystis* sp. PCC 6803 and other cyanobacteria, several types of mutants have been developed that are of use for basic science as well as for more applied research. These mutants include: (1) interruption/deletion mutants, in which an antibiotic-resistance cassette or other selectable marker has been inserted into a gene, or replaces this gene (Fig. 28.1), (2) mutants with random or targeted single-base mutations in a specific gene, (3) combinatorial mutants selected from a collection of mutants with random sequences in a specific region of a gene of interest, (4) mutants with a foreign gene functionally inserted, and (5) mutants with a combination of mutations at different loci. These different types of mutants will be explained in subsequent sections.

28.5.1 Insertion/deletion mutants

Reverse genetics (gene knock-outs) followed by phenotypic mutant characterization remains a popular approach to determine the function of specific genes. The simplest and most straightforward knock-out mutagenesis strategy is to insert an antibiotic-resistance cassette somewhere in the gene, or to replace part of the gene by this cassette. Whereas in the case of gene interruptions or small deletions a partial gene activity remains possible (for example, see He *et al.*, 1998; Tichy & Vermaas, 1999), remaining gene activity can be virtually excluded if most or all of the coding region has been deleted from the genome. A partial listing of *Synechocystis* sp. PCC 6803 genes that have been inactivated can be found at CyanoMutants (<http://www.kazusa.or.jp/cyano/Synechocystis/mutants/index.html>).

It is striking that in a large number of cases interruption or deletion of a specific gene does not lead to a reported phenotype (see CyanoMutants). There are several possible reasons for this: (1) several genes may code for proteins of overlapping function, and only if all genes contributing to a particular function have been deleted a phenotype will result, (2) if one does not know the function of a particular gene product, it is difficult to design the appropriate experiment to screen for a phenotype and (3) the gene plays a fully dispensable role under the environmental conditions that were tested.

28.5.2 Mutants with single-base changes in specific genes

Site-directed mutagenesis has been applied to *Synechocystis* sp. PCC 6803 proteins for more than a decade. In most cases, first a gene deletion is introduced (Fig. 28.1), the gene deletion is segregated, and then the gene (now carrying a site-directed mutation) is re-introduced. Analysis of the resulting site-directed mutant can help identify the function of specific residues in the protein.

The site-directed mutagenesis approach is useful if one wishes to test the function of specific residues in a protein. However, in many cases it is unknown which domains are functionally important, and then more random introduction of changes in a particular gene, followed by phenotypic analysis of resulting mutants, is more appropriate. For this “targeted random” mutagenesis approach (random mutations in a specific gene or gene region and not elsewhere), two methods are available. One method employs the property of sodium bisulfite to introduce mutations specifically in single-stranded regions: by hybridization of a heat-denatured plasmid with the complementary strand of a plasmid that is identical except that it carries a specific deletion, a hybrid is obtained that is double-stranded except in the region of the deletion. Upon treatment of this hybrid with bisulfite, mutations are introduced in the single-stranded region. This approach has been used for introducing mutations in the gene coding for the D2 reaction center protein in PS II of *Synechocystis* sp. PCC 6803 (Ermakova-Gerdes *et al.*, 1996, 2001). The second method employs PCR amplification under suboptimal conditions, leading to increased mutation rates (Narusaka *et al.*, 1998, 1999). As reported by Ermakova-Gerdes *et al.* (2001), frequent recombinations may occur between similar but non-identical genome copies, and further permutations may be generated. In either method, mutants with interesting phenotypes are selected and analyzed in terms of the site of the mutation as well as of the functional consequences of the alteration.

28.5.3 Combinatorial mutagenesis

Combinatorial mutagenesis is a powerful approach to modify larger protein regions randomly and select for clones with desired properties. In this approach, six to eight residues are selected for simultaneous modification, and oligonucleotide mixes with random nucleotide combinations at the corresponding codon locations are used for mutagenesis. The resulting molecules are introduced into the cyanobacterium, and mutants with desired properties are selected. As more than a billion (20^8) different amino acid combinations can result from combinatorial mutagenesis involving eight amino acid residues, a powerful selection screen is required. This selection screen may include the potential to grow under specific conditions (Kless & Vermaas, 1995, 1996; Keilty *et al.*, 2000, 2001), or the capability to convert a specific substrate to a readily identifiable product.

The use of combinatorial mutagenesis in cyanobacteria thus far has involved only PS II components, but results are quite far-reaching. For example, using combinatorial mutagenesis a residue necessary for variable chlorophyll fluorescence was discovered, providing a new mechanism for the nature of

non-photochemical quenching in photosynthetic systems (Vavilin *et al.*, 1999). Further use of such mutagenesis techniques may lead to modification of the substrate specificity of enzymes, leading to cyanobacterial strains making novel compounds, etc.

28.5.4 Insertion of a foreign gene

The codon usage of *Synechocystis* sp. PCC 6803 is such that essentially all codons can be recognized (Table 28.2), and therefore expression of a foreign gene is feasible as long as the transcript is stable and translatable. Start codons used in *Synechocystis* sp. PCC 6803 are AUG and GUG (Table 28.2). The best approach to express foreign genes is to incorporate the coding region into a plasmid that contains sequences up- and downstream of a highly expressed but dispensable *Synechocystis* gene. Examples of such highly expressed but dispensable genes are *psbA2* and *psbA3*, both coding for the D1 protein of PS II. Replacing the translated region of either of these genes with a foreign or native gene, high levels of expression of the inserted gene have been obtained (He *et al.*, 1999; Lagarde *et al.*, 2000).

28.5.5 Introduction of mutations at different loci

A critical issue in all mutant generation is the availability of suitable selectable markers that are linked to the mutation to be introduced, so that transformants with the desired mutation can be readily identified and selected. Many of the selectable markers are antibiotic-resistance “cassettes” (genes coding for a protein metabolizing a particular antibiotic) obtained from transposons or plasmids. In many cases, the bacterial promoter and transcription terminator sequences in the cassette are recognized sufficiently by the cyanobacterial transcriptional apparatus so that cassettes from other bacteria generally can be used in cyanobacteria without further modification. Different cassettes can be used in the same cyanobacterial strain, and therefore mutants with deletions at multiple independent loci can be generated. Antibiotic-resistance cassettes used in *Synechocystis* sp. PCC 6803 include those leading to resistance to kanamycin, chloramphenicol, spectinomycin, and erythromycin (see Vermaas, 1996), zeocin (Howitt *et al.*, 1999), gentamicin (He *et al.*, 1999), and streptomycin (Howitt *et al.*, 2001). Note that the cassette routinely used for spectinomycin resistance also leads to streptomycin resistance, and therefore the streptomycin-resistance cassette needs to be introduced before the spectinomycin resistance one.

Even though this number of antibiotic-resistance cassettes is sufficient for virtually all applications as with these cassettes one can modify or delete up to seven genes in the same strain, there may be applications where the presence of an antibiotic cassette in the final strain is not desired (for example, if the strain is used for industrial applications, full inactivation of the strain before release into the waste stream is impractical). In this case, antibiotic-resistance cassettes can be removed by coupling them to a cassette with the *sacB* gene. The *sacB* gene codes for levan sucrase, which if

Table 28.2. Codon usage frequency in *Synechocystis* sp. PCC 6803. Data were calculated from 3166 open reading frames using the *Synechocystis* codon usage table available at <http://www.genome.ad.jp/kegg>.

Amino acid	Total codons	Relative occurrence of each codon (numbers may not add up to 100.0% due to rounding) (%)
A (Ala)	87723	GCA 12.4
C (Cys)	10354	GCC 45.6
D (Asp)	51937	UGC 37.8
E (Glu)	62448	GAC 35.8
F (Phe)	41460	GAA 74.2
G (Gly)	76223	UUC 26.5
H (His)	19318	GGC 31.2
I (Ile)	64945	CAC 38.4
K (Lys)	43232	AUC 28.5
L (Leu)	117880	CUC 12.3
M (Met)	17605	CUG 17.9
N (Asn)	41827	AAC 37.5
P (Pro)	53177	CCC 49.2
Q (Gln)	57414	CAA 15.3
R (Arg)	52224	CAA 61.6
S (Ser)	60056	CGA 10.3
T (Thr)	56880	UCA 6.8
V (Val)	69190	ACA 12.0
W (Trp)	16046	GUU 15.7
Y (Tyr)	30103	CGU 24.5
Start	3166	UCC 27.7
Stop	3166	ACC 48.4
		GUU 16.8
		GUG 42.8
		UGG 100.0
		AGG 9.4
		UAU 58.5
		AGU 62.2
		GUU 64.2
		CCG 16.3
		CAG 38.4
		CGG 26.9
		UCG 6.8
		UCU 14.9
		ACU 25.1
		GUU 24.7
		AGA 8.7
		AGC 17.8
		ACU 25.1
		GUU 24.7
		AGG 9.4
		AGU 25.9
		UUA 22.9
		UUG 26.0
		AAU 62.5
		CCU 19.1
		CUU 8.6
		UUA 22.9
		UUG 26.0

present conveys a sucrose sensitivity to many prokaryotes including *Synechocystis* and several other cyanobacteria (reviewed by Thiel, 1994). If cyanobacteria carrying an antibiotic-resistance cassette coupled to *sacB* are transformed with a plasmid construct matching the flanking regions (but lacking the two cassettes), transformants that result will lack the antibiotic resistance and *sacB* cassettes in one of their genome copies, provided that the cyanobacterium displays homologous double recombination. Many cyanobacteria including *Synechocystis* sp. PCC 6803 contain multiple genome copies per cell. For this reason, transformed and non-transformed genome copies will need to be segregated into different cells before a mutant with a pure genotype (all genome copies in the cell being mutant) can be obtained. After several generations, in at least some cells segregation is likely to have occurred, and sucrose is provided to the medium. Cells that have lost all *sacB* copies (and therefore also all copies of the antibiotic-resistance cassette coupled to *sacB*) will survive, and a marker-less mutant is obtained (Lagarde *et al.*, 2000).

28.6 Directions of biotechnological importance

Whereas the scientific groundwork has been laid for effective cyanobacterial production systems, few applied biotechnology examples involving cyanobacteria have emerged over the past years. The reasons for this lack of industrial enthusiasm for cyanobacterial systems may include (1) solid footing of other model systems (*Escherichia coli*, yeast, etc.) in industrial applications, (2) cyanobacteria remain a relatively unknown entity among most researchers in the applied life sciences and (3) cyanobacteria are often viewed as pond scum or as toxin producers, even though the model systems that are focused on in this chapter do not have negative properties that we are aware of. The most promising cyanobacterial applications at this moment may involve compounds that are not easily produced by other methods. A large number of pharmaceutically or otherwise useful compounds are produced naturally by specific cyanobacteria, usually in small quantities (reviewed by Burja *et al.*, 2001). Using molecular genetic methodology as described in this review, appropriate genes could be put under a strong promoter and overproduced in the natural organism or introduced into a model cyanobacterium.

Another important application of biotechnology involving cyanobacteria is the production of valuable pigments, such as carotenoids, that serve both as coloring agents and as antioxidants. Cyanobacteria generally produce four main carotenoids, including β -carotene (the precursor to hydroxylated and ketocarotenoids), zeaxanthin (which is β -carotene with a OH-group on each of the two rings), echinenone (a ketocarotenoid) and an unusual carotenoid named myxoxanthophyll. Myxoxanthophyll is a glycosylated carotenoid, and it appears to be a common carotenoid component in essentially all cyanobacteria (10–40% of total carotenoids). However, myxoxanthophyll does not have a specific function assigned yet. The biosynthetic pathway of myxoxanthophyll is still largely unknown; the only known step in this pathway is a ring hydroxylation step that involves the same enzyme as the one

that is responsible for zeaxanthin formation (Lagarde & Vermaas, 1999). As myxoxanthophyll is among the most hydrophilic or amphiphilic carotenoids known in nature, it could function as an effective antioxidant and triplet quencher in locations that are not effectively served by other compounds of similar function.

Using simple knock-out mutagenesis one can create a mutant lacking several of the standard carotenoids such as echinenone and zeaxanthin. The only carotenoids in the resulting mutant are β -carotene and deoxymyxoxanthophyll (Lagarde *et al.*, 2000). The resulting mutant is apparently normal, indicating that the function of zeaxanthin and echinenone can be fulfilled by the remaining carotenoids. Depending on the biosynthetic pathway of myxoxanthophyll (either γ -carotene or β -carotene may be the last common precursor between the main carotenoid biosynthesis pathway and myxoxanthophyll), it may be feasible to further reduce or eliminate the β -carotene content of the cell and boost the deoxymyxoxanthophyll content. In this way, a cyanobacterium that produces large amounts of novel carotenoids may be obtained.

Another approach is to provide the cyanobacterium with an enzyme from another source that will lead to a pigment that is not usually found in cyanobacteria. A proof of concept has been provided by the introduction of a chlorophyll *a* oxygenase gene from algae or plants (Satoh *et al.*, 2001; Xu *et al.*, 2001). In the presence of just the *cao* gene, very small amounts of chlorophyll *b* (a pigment naturally absent from cyanobacteria) are produced in the cyanobacterium (Satoh *et al.*, 2001), whereas in the presence of both *cao* and *lhcb*, a gene for LHC II, a chlorophyll *a + b* binding protein from plants, chlorophyll *b* functionally replaced more than half of the chlorophyll *a*. Interestingly, LHC II did not accumulate in this mutant and chlorophyll *b* was incorporated in the PS II complex (Xu *et al.*, 2001). This approach, in combination with combinatorial mutagenesis by which the substrate specificity of existing enzymes may be modified, can easily lead to the production of novel compounds.

In conclusion, the methodology for genetic modification of cyanobacteria such as *Synechocystis* sp. PCC 6803 has been worked out very well, and cyanobacteria represent a phylum with much genetic variability and a proven record of production of valuable compounds. As cyanobacteria grow autotrophically, the risk of contamination of large cultures is less than that for heterotrophic bacteria. Altogether, cyanobacteria have outstanding potential for biotechnological applications, and the coming decade may bring just as many breakthroughs in the applied arena as the past decade has brought in basic science on the molecular genetics of cyanobacteria.

Note: This chapter reflects the status of the field by the end of 2001, doesn't include many interesting works published later.

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29 Microalgae as Platforms for Recombinant Proteins

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29.1 Introduction

Microalgae have not been extensively used for the production of industrial or pharmaceutical proteins. Nevertheless, they offer potential advantages over current systems. Microalgae are capable of high-level protein expression in low-cost growth media, and are easy to culture under various growth conditions, using relatively little energy. Moreover, they possess some unique characteristics that may be very important for the global economy and environment. For example, plant expression systems, the existing competent systems for protein production, are very sensitive to the absorption of sodium from salty soil or water, resulting in stress and significant biochemical changes (Serrano & Rodriguez-Navarro, 2001). In contrast, many microalgal species tolerate high salinity, growing well in salty water. In several regions around the world, irrigated land is not suitable for growing crops due to salty ground water, providing opportunity for molecular farming of microalgae of industrial value. Other important factors include microalgae's minimal negative environmental influence, the ease with which drug markers required for transformation can be removed (Cai & Wolk, 1990; Fischer *et al.*, 1996), and the relative ease with which products can be purified to meet consumer safety and effectiveness regulations.

As microplants, algae may be the best systems for the production of industrial proteins native to plants. Horseradish peroxidase, used in clinical biochemistry, cannot be produced in *E. coli* or other prokaryotic systems owing to its toxicity, the lack of glycosylation and the inability of bacterial hosts to incorporate heme and Ca^{2+} required for proper folding and activity of the protein (Smith *et al.*, 1990). Current production methods use suspension-cultured horseradish cells because they produce more enzyme than the mother plant (Japanese patent JP 2276574, 1990; Aspegren *et al.*, 1996). Using algae we may be able to achieve high level expression and at the same time avoid a lengthy process of establishing cell suspension.

29.2 Transformation of algae

In recent years, the development of gene transfer methods for an expanding number of microalgal species has laid a foundation for the introduction of

single genes encoding valuable heterologous proteins into algae. To date, about ten important microalgal species have been successfully transformed. These include green algae (e.g. *Chlamydomonas reinhardtii*, *Chlorella*, *Volvocarteri* and *Dunaliella*), diatoms (e.g. *Phaeodactylum tricornutum*) and others (e.g. *Kappaphycus alvarezii*).

29.2.1 The glass bead transformation procedure

This procedure was originally developed to introduce DNA into yeast (Costanzo & Fox, 1988), and was successfully applied to the green algae *Chlamydomonas* by Kindle (1990). The process involves agitating cells in the presence of exogenous DNA, acid washed glass beads and polyethylene glycol. Although it is convenient, it generally results in relatively low transformation efficiencies (<1000 transformants μg^{-1} DNA) and requires cell wall-less strain as hosts.

29.2.2 Electroporation

Electroporation has been widely used for the transformation of animal cells (Heiser, 2000), plant cells (Bates, 1995) and bacteria (Miller *et al.*, 1988). The application of electrical pulses opens pores in the cell membrane through which DNA can pass down a concentration gradient into the interior of the cell (Potter, 1993; Gietz & Woods, 2001; Nishikawa & Huang, 2001). Electrophoretic and electro-osmotic transport under the influence of an electric field may also facilitate DNA across the membrane (Johnson *et al.*, 1998). Transformation of algae by electroporation was first reported by Brown *et al.* (1991), and was optimized by other laboratories (Tang *et al.*, 1995; Shimogawara *et al.*, 1998). The electroporation protocol optimized for *C. reinhardtii* by Shimogawara *et al.* (1998) gave a transformation efficiency up to 1.9×10^5 transformants μg^{-1} DNA. Electroporation protocols require the delivery of an electrical pulse to cells. The specifics of cell preparation and the parameters of the pulse (field strength in kV cm^{-1} , capacitance in μF and resistance in Ω) vary between protocols. For any given strain, it may be necessary to investigate the parameters of electroporation and the treatment of cells in order to obtain sufficient numbers of transformants. The limitation of electroporation is that it works only on cell or protoplast suspensions, which is, in many cases, difficult to obtain.

29.2.3 Biostatic transformation

DNA can also be introduced into cells by *gene gun*, which propels metal particles coated with DNA at high velocities (typically 500 m s^{-1}) into cells under partial vacuum. Recent biostatic apparatus uses pressurized air (Oard *et al.*, 1990) or helium (Sanford *et al.*, 1991, Finer *et al.*, 1992, Takeuchi *et al.*, 1992) to accelerate either tungsten or gold spheres of $1\text{--}4 \mu\text{m}$ in diameter. The DNA-coated microprojectiles are suspended in solution, and placed or dried onto a larger macrocarrier. The loaded macrocarrier is launched and then suddenly stopped by a stopping plate, and the microprojectiles continue

on at high velocity toward the target cells, penetrating them and depositing the DNA. This method of transformation is discussed in detail by Sanford *et al.* (1993), and in an excellent recent article on the development of the biolistic process (Sanford, 2000). The first success in stable transformation of algae using this technology was reported by Newman *et al.* (1990). Biolistic technology is now receiving ever greater attention from algal geneticists for its reliability and the possibility to transform multicellular or macroalgae.

29.2.4 Selectable markers

As with other organisms, success in the introduction of foreign gene into algae requires a vector to harbor the genes and provide a selectable marker to separate transformed from non-transformed host cells. Foreign genes, flanked by an active promoter (usually native algal promoters) and a downstream untranslated region, are transformed into algal cells together with the selectable marker. There are two types of selectable markers used by algal geneticists: homologous selectable markers (endogenous genes) and portable dominant markers. Most homologous markers must be introduced into an appropriate host strain other than wild type. The commonly used homologous marker in *C. reinhardtii* is *ARG7* (encoding the argininosuccinate lyase), which requires an *arg⁻* auxotroph host strain. Other homologous markers carry mutations that confer resistance to antibiotics or herbicides (Boynton & Gillham, 1993), or restore photosynthetic growth to mutants defective in the photosynthetic genes that are homologous to the markers (Erickson, 1996). Portable dominant markers (heterologous genes from bacteria) are likely to be more useful in algal genetics and biotechnology since they confer host resistance to antibiotics and often can be used in a wide variety of organisms. The following bacterial genes were successfully modified and applied to algae: *aadA* gene, which confers spectinomycin resistance (Cerutti *et al.*, 1997); *APH* (aminoglycoside 3'-phosphotransferase) or *NPTII* (neomycin phosphotransferase), conferring resistance to Kanamycin or G418 (Hasnain *et al.*, 1985; Blowers *et al.*, 1989; Hawkins & Nakamura, 1999); *ble* gene, conferring resistance to the antibiotic zeocin (Apt *et al.*, 1996; Stevens *et al.*, 1996; Hallmann & Rappel, 1999; Zaslavskaja *et al.*, 2000).

29.3 Trophic type engineering

To date, only a limited number of microalgae have been successfully cultivated on a large scale. Most of these were cultivated either in large outdoor ponds, or in light reactors (Lee, 1997; Apt & Behrens, 1999; Richmond, 2000). The productivity of these cultivation methods is strongly limited by seasonal and diurnal variations in temperature and light, and by self-shading. An alternative is to use fermentation, but this is feasible only for heterotrophic algae such as *Cryptocodinium*. This mode of growth uses the same culture medium as that for light growth, except that fixed carbon compounds, such as glucose, are provided. Fermentative growth is more commercially competitive since it can be carefully optimized for maximal productivity through continuous monitoring of the levels of various nutrients, including nitrogen and phosphorus.

The recent success in trophic conversion of the microalga *Phaeodactylum tricornutum* is an exciting breakthrough in algal biotechnology. For the first time, a photosynthetic organism that strictly depends on light for energy and life was genetically converted to one that can thrive on exogenous glucose in the absence of light (Zaslavskaya *et al.*, 2001). This fundamental change in the metabolism of an organism was accomplished through the introduction of a single gene encoding a glucose transporter (*glut1* from human erythrocytes [Mueckler *et al.*, 1997], or *hup1* from *Chlorella kessleri* [Sauer & Tanner, 1989]). These foreign genes, under the control of the promoter for the *Phaeodactylum Fcp* gene encoding the fucoxanthin chlorophyll binding protein, were introduced into *P. tricornutum* by electroporation. Several zeocin-resistant transformants obtained by the authors can grow heterotrophically to a cell density that is five times higher than that of untransformed cells grown photoautotrophically (over a five day growth period). This demonstrates the power of metabolic engineering in future microalgal biotechnology.

Through this sort of trophic type conversion, high-cost, light-based algal production methods can be replaced by low-cost fermentation technology, opening the opportunity for commercial production of valuable proteins and compounds through the development of vast numbers of microalgae (see Chapter 31).

29.4 Optimization of expression levels

Heterologous genes introduced into eukaryotic systems including microalgae are often poorly expressed owing to epigenetic suppression, inefficient transcription and poor translation. In addition, many proteins are complex, containing multiple subunits, cofactors/prosthetic groups, disulfide bonds, and post-translational modifications that are essential for function. The high-level expression of active/functional proteins in any system is dependent upon a variety of factors, including promoters, codon preference, subcellular localization, mRNA stability and cofactor binding. Information about the optimization of expression levels in algal systems is scarce, but much can be learned from other systems.

29.4.1 Promoters

An ideal promoter for the expression of recombinant proteins will direct efficient transcription to allow high-level protein production, and will be tightly regulated to minimize metabolic burdens and toxic effects (Weickert *et al.*, 1996). Promoters strongly induced by either a low-cost chemical, or by shifting the growth conditions are excellent choices for either research or commercial purposes. The arylsulfatase gene either from *Volvox* or from *Chlamydomonas* is strictly regulated, and is transcribed only under conditions of sulfur starvation (de Hostos *et al.*, 1989; Hallmann & Sumper, 1994). This promoter may be utilized to drive the synthesis of foreign proteins that might be even toxic to algae. More inducible promoters suitable for biotechnological applications can be trapped and identified using promoterless reporter genes (Haring & Beck, 1997).

Promoters may behave very differently under fermentative conditions as compared to laboratory growth conditions. If the cells are to be grown in fermentors, it may be necessary to apply two-dimensional SDS/PAGE to fractionate proteins that are highly expressed under fermentation conditions. These highly expressed proteins can be recovered and identified through MS-fingerprinting, or by N-terminal sequencing, and one can then go back to isolate promoters that can be used to drive high-level gene expression.

29.4.2 Transgene silencing

RNA silencing is a remarkable type of gene regulation based on sequence-specific targeting and degradation of RNA. Transgene silencing frequently hampers heterologous gene expression in plants. There are several cases of gene silencing reported in algae (Blankenship & Kindle, 1992; Stevens *et al.*, 1996; Cerutti *et al.*, 1997). The silencing of a foreign arylsulfatase gene in *V. carteri* was attributed to high-level methylation of this gene (Babinger *et al.*, 2001). Transgene silencing can be minimized by placing matrix attachment regions (MARs) at the side of transgene (Allen *et al.*, 2000). Thorough analysis of transformants at the molecular level, and selection of transformants carrying single un-rearranged transgenes are also very important for avoiding gene silencing (de Wilde *et al.*, 2000). Because, a highly expressed gene may be silenced several years later (Babinger *et al.*, 2001), it is advisable to keep multiple highly expressed transformants as backup.

29.4.3 Introns

Introns may affect the expression of a transgene transcriptionally or post-transcriptionally. Some introns contain enhancer or repressor elements that boost or weaken the transcription of the mother genes (Goto *et al.*, 1996; Taylor, 1997). Other introns may affect transgenes through post-transcriptional events such as nuclear export or transcript stability. The expression of a transgene often benefits from the proper use of introns (Koziel *et al.*, 1996; Xu *et al.*, 2001). One example well-known among algal geneticist is the development of an efficient *ble* marker: the expression of a bacterial *ble* was greatly increased by insertion of a *RBCS2* intron into the coding region of the *ble* gene, making the marker the very best one so far for nuclear transformation of *Chlamydomonas*.

29.4.4 Codon bias

Nearly every protein-coding sequence analyzed to date displays a bias in synonymous codon usage. In general, algae have much stronger codon bias than higher plants. Highly expressed algal genes (especially plastid genes) are usually strongly biased toward a set of major codons, apparently to increase translation efficiency (Morton, 1998, 1999; Morton & So, 2000). In non-algal systems, countless examples have shown that optimization of codon usage is critical for the successful overexpression of heterologous proteins. It is likely that by taking into account the codon usage frequently found in

highly expressed algal genes, one can increase the translatability of synthetic genes, permitting high levels of expression. There are a number of ways to change the codons of a gene. One effective method is to use a set of mutually priming, overlapping oligonucleotides and to perform two runs of polymerase chain reaction (Massaer *et al.*, 2001).

29.4.5 Subcellular localization

Protein accumulation and stability are strongly influenced by subcellular localization (Moloney & Holbrook, 1997; Conrad & Fiedler, 1998). In plant systems, consistently higher accumulation levels and enhanced protein stability were observed when the proteins were retained within the lumen of the ER by the addition of a C-terminal KDEL sequence (Stoger *et al.*, 2000; Peeters *et al.*, 2001). The presence of a subset of molecular chaperones and protein-folding enzymes in this compartment may help to fold and stabilize heterologous proteins (Crofts & Denecke, 1998). The oxidizing environment of the ER may also promote disulfide bond formation (Hwang *et al.*, 1992).

29.4.6 Cofactors

Many proteins contain cofactors (e.g. Ca^{2+} , heme, flavins) that are essential for function and stability. The soluble expression yields of these proteins can often be increased by enhancing the endogenous production of cofactors, or by supplying them exogenously. Expression of functional human liver inducible nitric oxide synthase requires coexpression with calmodulin, and the inclusion of tetrahydrobiopterin in the purification buffers (Gerber *et al.*, 1997). The production of active heme-binding human cystathione-synthase in *E. coli* is increased eightfold by the addition of aminolevulinic acid (a heme precursor) to the culture (Kery *et al.*, 1995). Interestingly, the attachment of lipoic acid (cofactor) to pea H-protein was found to be greatly improved by adding chloramphenicol to inhibit protein synthesis prior to incubation with the cofactor. This approach might be useful for increasing the efficiency of other post-translational modifications (Macherel *et al.*, 1996).

29.4.7 Chaperones and artificial chaperones

Protein folding is a crucial step in the expression of heterologous proteins. Over-expression often results in the formation of inclusion bodies that require strong denaturants to solubilize. Co-overproduction with chaperones has been used in prokaryotic systems to prevent the formation of inclusion bodies with some degree of success. The accumulation of soluble, active neuronal nitric oxide synthase is greatly increased by coexpression with GroESL (Roman *et al.*, 1995), whereas the solubility of only one of two tyrosine kinases expressed in *E. coli* is improved by the same approach (Yasukawa *et al.*, 1995). The effectiveness of this approach can be complemented by *in vitro* refolding. Recently, a versatile strategy was developed that promotes the folding of chemically denatured proteins via the sequential

addition of low molecular weight artificial chaperones (polyelectrolytes, β -cyclodextrin, cycloamylose). These molecules mimic the action of chaperones in living cells. This method was shown to be effective with several model proteins under conditions that provide little or no refolding, via simple denaturant dilution (Daugherty *et al.*, 1998; Machida *et al.*, 2000; Muronetz *et al.*, 2000), and could be productive with other urea- or guanidine hydrochloride-denatured proteins that resist conventional folding/refolding strategies. Other small molecules, e.g. proline, glycerol and heparin sodium may also act as folding aids (Meng *et al.*, 2001).

29.5 Downstream processing

The isolation and purification of protein products into a useable form, popularly termed *downstream processing*, constitutes an expensive and technically challenging aspect of protein production. The intensity of downstream processing increases with the degree of product purity required and with the complexity of the raw material. Generally, diagnostic or therapeutic proteins must be highly purified for reliability, or to minimize side effects. On the other hand, industrial bulk enzymes usually do not require significant processing.

If cells were to be grown in light reactors or fermentors, it may be advantageous to fuse heterologous genes of interest to signal peptides that will direct secretion of the proteins into the growth medium (Kermode, 1996). Because fresh microalgae growth media are relatively simple, protein-free solutions, cell material can be separated from proteins of interest by vacuum filtration and subsequent clarification of the filtrate. The success of this strategy depends largely upon the stability of the proteins in the extracellular environment, which may contain secreted protease (Terashima *et al.*, 1999). Use of protein stabilizing agents such as polyvinylpyrrolidone (PVP) may improve foreign protein titers in the culture medium (Lacount *et al.*, 1997; Wongsamuth & Doran, 1997), suggesting that the medium is the site of protein degradation, at least in some plant cell culture systems (Doran, 2000). However, Sharp & Doran (1999) reported that application of bacitracin, a broad-spectrum protease inhibitor, did not prevent protein loss. Other factors also need to be taken into account. For example, excess added carbon sources (e.g. glucose) may cause acidification of the growth medium, which may block secretion of some polypeptides (Kim *et al.*, 1998). To obtain correctly folded foreign proteins after secretion is another challenge. In this regard, it is encouraging to note that functional trimeric tumor necrosis factor α was obtained from spent growth medium of *Streptomyces lividans* (Pozidis *et al.*, 2001).

Recovery and purification of proteins from biomass or cell material may require multiple separation steps such as cell disruption, protein precipitation, adsorption, chromatography and diafiltration. It is very important to develop a cost-effective means of production. For example, in an economic evaluation of β -glucuronidase production from transgenic corn seed containing 0.015% β -glucuronidase, downstream processing (including milling, protein extraction and purification) accounted for 48% of the operating cost; the cost of the transgenic corn was only 6% (Evangelista *et al.*, 1998).

Compared with other microbial expression systems, the major difference in purification of recombinant proteins from algal cells arises in the step of cell disruption. Algae usually have a thick cell wall, making mechanical disruption devices like Bead Beaters the most efficient means of cell disruption. In general, Bead Beaters (using glass or silica beads) work better with high-density cell suspensions ($3-5 \times 10^9$ cells ml $^{-1}$ for cyanobacteria). However, this method can give rise to problems due to heat generation. Beating bursts longer than 2 min cause cell suspensions to overheat. Cycles of 1 min blending followed by 2 min of cooling work best for *Synechocystis* (He *et al.*, 1999). Protocols for large-scale preparation of cell extracts using Bead Beaters can be requested from the device's manufacturer (Biospec Products Inc, Bartlesville, Oklahoma). Post-treatment protocols developed for plant systems are a good place to begin in developing algal methods. The bulk of the nucleic acids, and much of the phospholipids in the extract are removed by precipitation with polyethyleneimine (Gegenheimer, 1990). After removing inhibitory compounds by passage over a column (e.g. BioGel P-6DG or Sephadex G-50), eluted crude protein is ready for further purification using immunological, eletrophoretic, or chromatographic methods. Strategies and considerations for protein purification have been reviewed by Linn (1990).

In general, tagging proteins simplifies purification processes. One commonly used tag is the poly-His tag, which permits two-step purification of proteins tagged by polyhistidine using metal-ion affinity chromatography (see Porath, 1992 for a review). This approach allowed purification of small polypeptides that are present in trace amounts (not even detectable by western blots) in *Synechocystis* PCC 6803 (He *et al.*, 2001). Another tag is the poly(GVGVP) tag (more precisely, a protein-based polymer encoded by synthetic DNA sequences). This tag facilitate non-chromatographic, single-step purification by shifting temperature (McPherson *et al.*, 1996; Daniell *et al.*, 1997). At a temperature above transition point, linear GVGVP folds into β -spirals that further aggregate to form twisted filaments through hydrophobic interaction. This approach, involving reversible folding of proteins, is suitable for grams to kilograms scale purification (Daniell *et al.*, 2001). Other tags such as HA tag, used for immuno-localization studies in algae (Wykoff *et al.*, 1999), can also be utilized for protein purification.

29.6 Concluding remarks

The use of microalgae for the production of recombinant proteins is in its infancy. As an expression system, microalgae hold some promise as low-cost, environmentally friendly eukaryotic organisms capable of heterologous protein synthesis. Preliminary evidence of the feasibility of this approach has emerged. In an effort to express human growth hormone (hGH) in the eukaryotic alga, *Chlorella*, a level of about 200–600 ng hGH ml $^{-1}$ culture was achieved (Hawkins & Nakamura, 1999), which is comparable with the optimized production of hGH of 0.5–2.4 μ g ml $^{-1}$ culture in *E. coli* (Goeddel *et al.*, 1979) or *Bacillus brevis* (Kajino *et al.*, 1997). In the past several years, protein production technology has moved at a great pace, and protein-based therapeutics are now being tested in an increasingly diverse range of

modalities in various competing production systems (Humphreys & Glover, 2001). It is unlikely that one production method/process will dominate since different systems have different abilities to produce the very different molecules that have been engineered to confer the desired disease treating properties. The ability of algal culture to become highly competent systems for commercial protein production depends on the ability of the systems to express proteins to high levels and on the ease of downstream processing. In the future, powerful algal production systems that produce therapeutic or industrial proteins with high quality and yield whilst reducing development lag time, capital expenditure and labor costs, can be developed by combining molecular biological approaches with process engineering. Since microalgae are relatively easy to culture and transform as compared with plant cell suspensions, one potential niche for reactor-scale algae farming is the rapid development and production of low to medium volume therapeutic proteins to meet the demand of ever-changing market of protein pharmaceuticals.

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30 Bioactive Chemicals in Microalgae

Olav M. Skulberg

30.1 Introduction

A pet child has many names. The expression *bioactive chemicals* conceals another confusion, including a multitude of different *children* under the same designation. Certain cell metabolites – generally produced during the stationary growth phase of a microbial culture – are called secondary metabolites (Schlegel, 1985). They constitute some of the most important biotechnological products for mankind. A multitude of substances are involved, including organic acids, carbohydrates, amino acids and peptides, vitamins, growth substances, antibiotics, enzymes and toxic compounds. Bioactive chemicals of this nature – being of extensive industrial interest for almost a century – have drawn the attention of microbiologists and organic chemists to carry out research on the relevant molecules, their structures, *in vitro* synthesis and modes of action. The term *bioactive chemicals* was opportune to collectively cover the family of their favorite molecules.

Microalgae are generally held as the principal primary producers of aquatic ecosystems (Hoek *et al.*, 1995). As a group, they can cope with high and low temperatures, suboptimal and supraoptimal light intensities, low availability of essential nutrients and other resources. Possessing a multitude of physiological, biochemical and molecular strategies provides microalgae with biosynthetic pathways resulting in a richness of complex organic molecules belonging to the category of bioactive chemicals.

Microalgae have, moreover, an advantage over many other organisms in that they can be expediently cultured for the production and processing of desirable compounds. The search for bioactive chemicals from microalgae is becoming steadily more promising, affected by the increased number of species being brought into culture. The controlled maintenance of microalgae is a key factor in exploiting them as a source of economically important products.

30.2 Retrospective view and research continuity

Extracellular products of microalgae have long been an important objective of phycological research (Pringsheim, 1949; Schussnig, 1953). Among the first observations concerning microalgal bioactive chemicals are those of

Harder (1917), who showed in studies on the blue-green *Nostoc punctiforme* that autotoxic substances were liberated from the organism.

Extracellular products are compounds produced by healthy, actively growing microalgae which pass from the cell into the medium. Substances of this nature become the subject of much investigation, carried out both in the field and the laboratory (for a review, see Hutchinson, 1967). Important information was published, ranging from indications on the presence of bioactive substances to substantial evidence based on quantitative growth data and chemical identification. The specific agents involved could be either inhibiting or enhancing the development of autotrophic and heterotrophic organisms. References for details from this period are given by Fogg (1971). Only a few examples will be mentioned here for the purpose of coherence.

Extensive experiments with cultures of *Chlorella vulgaris* (Pratt & Fong, 1940) demonstrated production of secondary metabolites which behave as antibiotics toward Gram-positive as well as Gram-negative bacteria. Concerning vitamins and other growth stimulating substances synthesized by microalgae, the investigations carried out with cultures of fungi dependent on the aneurincomplex – vitamin B, thiamine – are illustrative (Gäumann & Jaag, 1950). Using 18 different species of soil algae belonging to the orders Volvocales, Chlorococcales, Ulotrichales and Chaetophorales, it was concluded that each of them was able to supply the fungi with the necessary requirement of vitamins. Studies on strains of *Chlamydomonas* (Nakamura & Gowans, 1964) demonstrated nicotinic acid was produced in laboratory cultures. The yield in certain mutants could be increased about eightfold – compared with the wild type (Harris, 1989), due to overexcretion controlled by a single gene.

Antagonistic reactions and antibiosis due to phycotoxins have been an intensive field of research throughout the twentieth century. The common characteristic of phycotoxins is that they exert a pronounced effect on the metabolism and biological functions of affected organisms with just a minute quantity. This is a property which may also provide a lead for the development of useful drugs.

The relationship between the occurrences of phytoflagellates and toxic shellfish was first reported in connection with red tides (Sommer & Meyer, 1937). The dinoflagellate *Gonyaulax catenella* was identified as the causative organism. The chemistry of the toxin involved – saxitoxin – was extensively studied, and the molecular structure was unequivocally established (Schantz *et al.*, 1975). Considerable progress has so far been made in the chemistry and toxicology of marine phycotoxins. Chemically the relevant bioactive substances become separated into three major types, alkaloids, polyethers and cyclic peptides (Smayda & Shimizu, 1993).

Health hazards from blue-green algae – cyanobacteria – were suspected from the beginning of the last century. Canadian scientists were the first to isolate the strains involved (Huges *et al.*, 1958). The chemistry and physiology of cyanotoxins have gradually been characterized based on the purified metabolites (for a review, see Gorham & Carmichael, 1979). In the past several decades, new sources of cyanotoxins have been identified, and many novel toxins have been isolated and their structures established (Carmichael, 1997; Chorus & Bartram, 1999; Wu *et al.*, 2000).

The recent progress of molecular biology (Lodish *et al.*, 1995) has led to an advanced understanding of bioactive molecules in general, and their biosynthesis and physiological functions in particular. The development of genetic tools – notably gene transfer systems and transposable genetic elements – may be considered as a prelude to relevant use in the genetic manipulation of microalgae. Projects on genomics are completed or underway on species in some genera, e.g. *Alexandrium*, *Chlamydomonas*, *Nostoc* and *Synechococcus*. Together with the discovery of the considerable range of novel bioactive chemicals in microalgae, this opens for an economic application in the overall scheme of modern innovation (Richmond, 1992; Borowitzka, 1999; Shimizu, 2001).

30.3 Organisms and availability

The microalgae are photosynthetic microorganisms of prokaryotic (cyanobacteria) and eukaryotic nature. With respect to diversity they include 11 divisions and 28 classes (Hoek *et al.*, 1995). The numbers of approximately 30 000 species so far described are certain to increase substantially with new knowledge (Norton *et al.*, 1996). Microalgae are characterized for their cosmopolitanism at the morphological species level, and rather low endemity is manifested. However, based on intraspecific level studies of different microalgae – using a range of molecular techniques – it has been identified genetic diversity ranging from regional and global scales to between and within populations at small scales (Gallagher, 1980; Medlin *et al.*, 1996; Phillips & Fawley, 2000).

Of particular interest in connection with microalgae for industrial purposes, it is good reason to mention that currently more than 11 000 strains – including the number of 3000 species – are maintained in culture collections (Andersen, 1996; Day, 1996). This represents the highest percentage of species of any group of microorganisms that are preserved *in vitro*.

Selected organisms from almost all the algal classes have been used for screening purposes aiming at bioactive substances or other high-value compounds. At present the cyanobacteria feature most prominently as sources of bioactive agents. Phytoflagellates are in the same way providing many promising secondary metabolites. The relevant algal divisions Heterokontophyta, Haptophyta, Cryptophyta, Dinophyta, Euglenophyta and Chlorophyta open for a vast expanse in research on the diversity of unusual chemical substances and the opportunities they offer for the future. Recent research has gained insight into how microalgae also represent the biosynthetic origin of many bioactive molecules isolated from marine invertebrates. The animals may acquire their content of the relevant substances via diet or symbiosis.

An example of dietary derived metabolites comes to light in studies on the marine gastropod *Dolabella auricularia* – a sea hare. This gastropod is a generalist herbivore, and many metabolites first isolated from the animal are of dietary origin (Faulkner, 2000). The effective antineoplastic substances named dolastatins – potent anticancer agents – have been obtained by means of large-scale collections of sea hares from marine localities. The role of cyanobacteria as biosynthetic progenitors of the relevant metabolites is now

discovered. The dolastatins are synthesized by, e.g. *Lyngbya majuscula*, *Schizothrix calcicola* and *Symploca hydnoides* (Harrigan *et al.*, 2000).

The marine sponge *Dysidea* can illustrate the presence of metabolites of symbiotic cyanobacterial origin. Species of *Dysidea* have blue-green symbionts, and bioactive compounds first isolated from the sponge have proven to be of cyanobacterial origin (Unson & Faulkner, 1993). The *Dysidea*-derived substance dysidenin, a marine toxin inhibiting, e.g. iodide transport in cells, has now been traced in populations of *L. majuscula* (Jiménez & Scheuer, 2001).

Lichens are an intricate association between a fungus and one or more green algae or cyanobacteria. They establish one of the most successful and best investigated examples of symbiosis (Rai, 1990; Masuch, 1993). More than 700 secondary lichen substances have been described. Many of these metabolites, which belong to chemically diverse classes of compounds – including aromatic substances such as depsides, depsidones, and carotenoids – are unique for the lichens concerned. This wide range of secondary products is found in intact lichens. Experimental studies with the mycobionts have, however, shown that the presence of early biosynthetic products (precursors) from the phycobiont plays a physiological role in determining which chemical products are generated (Ahmadjian, 1992; Kranner *et al.*, 2001).

Symbiotic cyanophytes are recognized as an exciting source of novel pharmaceuticals. Many of the interesting bioactive metabolites derived from macroorganisms are in short supply for further research due to rare occurrence of relevant natural populations. The isolation and culture of the cyanophyte partner involved in the biosynthesis of the bioactive substances are a challenge for new advancements in the field of natural drug discovery.

30.4 Secondary metabolic processes

The study of secondary metabolism in microalgae has undergone a progress from the isolation and recognition of bioactive chemicals, via the research on their biosynthesis and toward the study of the physiological control mechanisms involved.

Secondary metabolism is a part of normal microalgal cell growth, related to the interactions with the environment. Both primary and secondary metabolic processes involve the pyridine nucleotide/flavoprotein/cytochrome chain of electron transfer processes, and the anhydride reactivity of the thioesters and phosphoric anhydrides respectively. Figure 30.1 shows the main flow of carbon with *highways and byways of metabolism* in the synthesis of primary and secondary metabolites. The compounds formed in the production stage of secondary metabolism (idiophase) are generally built up of separate intermediate components that accumulate – either in the cells of the microalgae or in the culture medium – during the growth period (trophophase). The biosynthesis in the two phases of development are linked by the involvement of enzymes in common. There will also be important linkages through the joint use of such reagents as ADP, NADPH₂, etc., and consequently, a degree of control and interaction like that which these reagents supply within the system of primary metabolism (Richter, 1978).

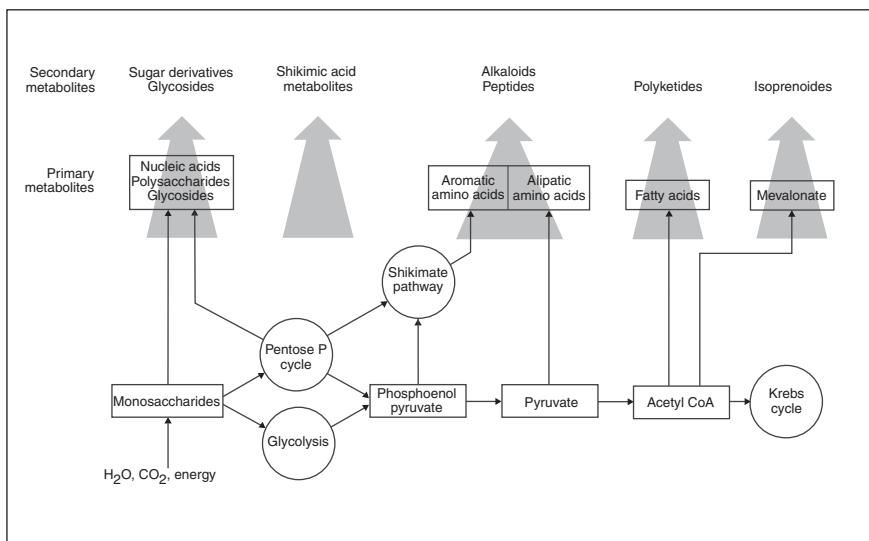


Fig. 30.1. The main flow of carbon metabolism, the products of primary synthesis and the secondary metabolites.

The identification of precursors of secondary metabolites is of particular practical importance. These precursors are intermediates in the primary metabolism, being the peculiar substrates for the special secondary biosynthesis. Once the relevant precursors are identified, a number of regulatory phenomena may be recognizable. In some cases, specific inducers of secondary metabolite production have been identified.

Genetic and physiological research on microalgae has explained that the formation of secondary metabolites is an inherent feature of the overall development of the producing culture, and is initiated or proceeds most efficiently at particular development stages. Several observations attest to the integration of secondary metabolism into the life cycle of the producer organism (Luckner & Nover, 1977; Skulberg, 1988; Rai & Gaur, 2001).

The synthesis of secondary metabolites is usually favored by suboptimal growth conditions (Rai & Gaur, 2001). It is often suppressed by inorganic phosphate and by carbon and nitrogen sources, tending to support biomass development rather than cell differentiation. Mutants of microalgae altered in their cytodifferentiation and physiological behavior can display changes in secondary metabolism. They may also lose the ability to form a separate secondary metabolite, but apparently grow and develop normally. However, the evidence is mounting that at least some of the relevant metabolites affect their producers as autoregulators of development, by triggering particular biochemical or morphological events – e.g. sporogenesis, antibiotogenesis – during cytodifferentiation (Black & Wolk, 1994).

A major goal for future phycological research includes getting knowledge of the biochemical and ecological roles of autoregulatory metabolites, to provide a clearer understanding of the biological functions of secondary metabolism in the microalgae and its effects on the ecosystem.

A critical requirement for a commercially useful culture of microalgae must be stability linked to high productivity. Genetic and biochemical instability may limit the usefulness of microalgal cultures for the production of secondary metabolites. Variation in biosynthetic activity is influenced by the origin and type of clones from which the culture is initiated as well as the culture conditions. Genomic instability exerts influence on the results. Epigenetic changes – reversible variations in gene expression – are also of importance, being closely associated with the control of the biosynthetic activity in the culture. The level of production of certain bioactive substances will be influenced by the availability of precursors together with the activity of specific pathway enzymes. The activity of these enzymes will be controlled by gene or multi-gene expression. The switching *on* and *off* of these pathway-related genes will have profound effects on the metabolism of secondary products. Research onward should investigate the biosynthetic process at the level of gene expression, so a better control of variation can be made possible.

30.5 Microalgae from the biotope through the stage of screening

The culturing of microalgae, and their maintenance in culture collections as a stable renewable resource (Hunter-Cevera & Belt, 1996), is a huge advantage in the development of their value to biotechnology. However, algal culture technology is a difficult matter concerning the scale-up and practical biomass production (Richmond, 1999). Only robust microalgae have so far been successfully cultivated for economic purpose (Vonshak & Richmond, 1985; Cohen, 1999). Among prominent examples are species of the genera *Arthrosphaera* (*Spirulina*), *Chlorella*, *Dunaliella*, *Haematococcus*, *Porphyridium* and *Scenedesmus*.

Exploring microalgae as sources for bioactive chemicals includes the effective isolation of organisms into unicellular culture, growing them for biomass, making extracts and carrying out bioassays and chemical analyses/structure determination for the characterization of the potential for delivering interesting materials (Fig. 30.2). A considerable literature describing the field and laboratory procedures for the biological and chemical work to be undertaken is at hand. A few selected references to laboratory manuals and handbooks can be given as examples (Stein, 1973; Hellebust & Craigie, 1978; Gantt, 1980; Littler & Littler, 1985; Richmond, 1986; Penn, 1991; Vogel & Vogel, 1997; Kessler, 2000).

The use of bioassays to screen microalgae for bioactive chemicals is commonplace. However, the reasoning behind screening techniques to detect and isolate compounds for drug discovery will be mentioned. The first milestone is the finding of lead compounds which may serve as molecular templates for further development. Compounds of this nature are typically active in the sub-micromolar concentration range. They are selective for a particular target interaction (e.g. ion channel or receptor, enzyme), exhibit functionality in relevant biotests, and should preferably be liable to chemical derivatization or synthesis. Solubility, bioavailability, *in vitro* and *in vivo* efficacy are properties usually optimized by the drug companies (Mulzer & Bohlmann, 2000).

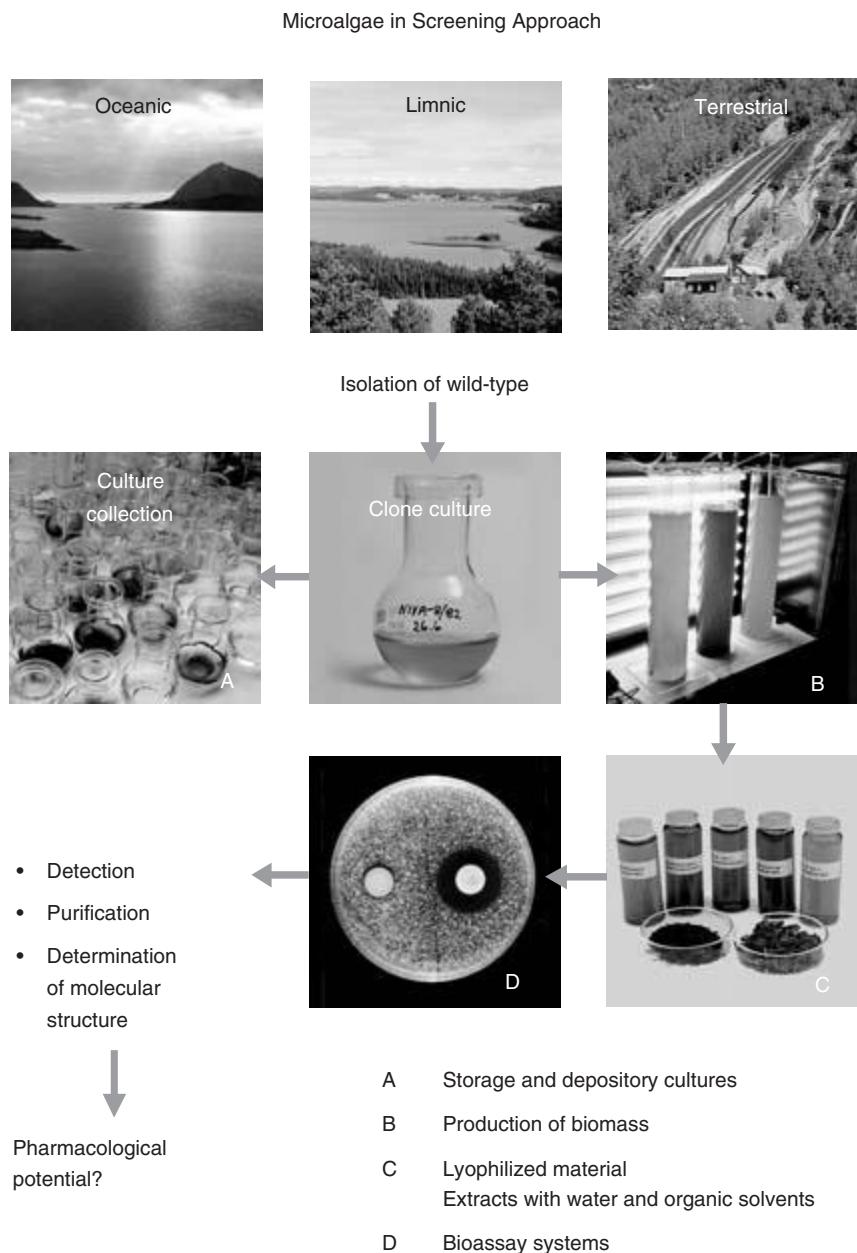


Fig. 30.2. The main steps in bringing the microalga from nature to the stage of testing for bioactivity.

Relevant assays must fulfill several criteria. A prerequisite for natural product screening is that the assay must be designed to function in the presence of samples with a broad range of physico-chemical properties. Furthermore, assays must be both sensitive (in the concentration range $<200\text{ nM}$) and highly reproducible. New technologies in drug screening – challenging present discovery programs – have evolved (Vogel & Vogel,

1997; Hildebrandt & Igarashi, 1999). The most significant of these is genomic science, which is identifying the genes, their sequences and associated proteins involved in pathophysiological processes. Screening procedure improvements are within reach, such as miniaturization of assay format, and highly sensitive, new detection methods (Osada, 2000). The drug-design process from the discovery of the bioactive substance to clinical trials and drug application may in this way be more time- and cost-effective.

30.6 Bioactive compounds in cultures of microalgae

General surveys on bioactive compounds produced by microalgae have previously been published. Several primary references are appropriate to mention (Zajic, 1970; Glombitska & Koch, 1989; Kerby & Rowell, 1992; Becker, 1994; Namikoshi & Rinehart, 1996; Goyal & Goyal, 1998; Borowitzka, 1999). These publications not only give an overview of the large spectrum of substances from microalgae with varying bioactivities, they illustrate in addition the contemporary expanding research efforts and the progress in the generation of novel bioactive chemicals (Skulberg, 2000).

In the following, a brief framework of knowledge will be provided for a selection among the main categories of bioactive compounds from microalgae. The different economic and practical interests in the use of bioactive compounds as fine chemicals and for nutritional purposes are extensively dealt with in other chapters of this handbook. However, some particular information about applications in the boundary of pharmacology and medical care can be useful to mention briefly.

30.6.1 Secondary metabolites for fine chemicals

The scientific names of the divisions and classes of algae often contain a reference to the color of the organisms they include. Three main groups of pigments – chlorophylls, biliproteins and carotenoids – are associated with absorption of radiation and photosynthesis (Richter, 1978; Richmond, 1986). Industrial cultivation of microalgae for the manufacturing of curative commodities is based on selected biliproteins and carotenoids.

Biliproteins are the red and blue bile pigment-protein conjugates of the blue-greens, red algae and cryptomonads, causing the characteristic colors of these systematic groups of organisms. The phycoerythrins (red) and phycocyanins (blue) contain the linearly arranged tetrapyrrolic bile pigments, phycoerythrobilin and phycocyanobilin, which are strongly covalently bonded to a protein element. They exist in a number of forms, differing in the arrangement of their polypeptide components, and they can be distinguished by spectral characteristics. Biliproteins usually represent 1–10% of the dry weight of the relevant microalgal biomass, although under certain circumstances it may be as much as 24% (Chapman, 1973).

The commercial utilization of phycobiliproteins is as natural dyes for the replacement of synthetic pigments (e.g. phycocyanin), as highly sensitive fluorescent reagents (e.g. phycoerythrin) in diagnostic tests, and for labeling

antibodies used in multicolor immunofluorescence analysis (Glazer, 1999). The biliprotein phycocyanin is also extracted from the biomass of blue-greens for the health-food and cosmetic market (Richmond, 1988; Becker, 1994). An inhibitory effect of phycocyanin from *Spirulina platensis* on the growth of human leukemia cells is recently reported (Liu *et al.*, 2000). These findings suggest that phycocyanin can be used as a potential antitumor substance. Unicellular red algae – i.e. *Porphyridium*, *Rhodella*, *Rhodosorus* – have been investigated as sources of phycoerythrin for production of fluorescent markers for diagnostics and food dyes (Arad, 1988) (see Chapter 15).

Carotenoids are yellow to red isoprenoid polyene pigments, widely distributed in nature serving particular biological functions. They can be synthesized *de novo* only by photosynthetic organisms, and microalgae constitute an important global source. More than one hundred different carotenoids encountered in microalgae are structurally identified (Liaaen-Jensen, 1998). They consist of the hydrocarbon carotenes and their dihydroxy derivatives (xanthophylls).

Under natural, favorable growth conditions primary carotenoids are synthesized. When nutrient starvation or other stressed conditions exert influence, cultures of microalgae are producing secondary carotenoids (Liu & Lee, 2000). These pigments are deposited in the cells in lipid globules outside the plastids. Examples of secondary carotenoids are β_1 , β -carotene and keto-type carotenoids including astaxanthin (Renstrøm *et al.*, 1981; Grung *et al.*, 1992). This last mentioned carotenoid is an extremely powerful antioxidant with several mechanisms of action making it valuable in human health care, e.g. cancer prevention, and immune system boosting (Palozza & Krinsky, 1992; Oshima *et al.*, 1993; Jyonouchi *et al.*, 1995; Tanaka *et al.*, 1995).

Relevant microalgae for production of secondary carotenoids include strains of several genera, e.g. *Chlorella*, *Dunaliella*, *Haematococcus* and *Spirulina* (*Arthrospira*). The green alga *Haematococcus* can be mentioned as a commercial producer organism of astaxanthin (Lee & Zhang, 1999). Under laboratory controlled conditions this alga accumulate up to 6–8% dry matter of astaxanthin in the aplanospores, however, due to several limitations a yield of about 2.5% of astaxanthin in biomass is recognized obtainable in industrial operations.

Polyunsaturated fatty acids (PUFAs) also need attention for their special suitable bioactivity. Extensive data are now available on the fatty acids related to all of the major classes of microalgae. Useful characteristic features have been described related to the abundance and production of particular PUFAs (Metz *et al.*, 2001). However, despite several decades of research, it is apparent that some of the biosynthetic steps, leading to the formation of relevant unsaturated fatty acids in microalgae, are still not known with certainty.

Polyunsaturated fatty acids are critical components of membrane lipids in most eukaryotes (Lauritzen *et al.*, 2001), and are the precursors of certain hormones and signaling molecules (Creelman & Mullet, 1997). Known metabolic pathways of PUFA synthesis involve the processing of the saturated 16:0 or 18:0 products of fatty acid synthase (FAS) by elongation and aerobic

desaturation reactions. Species of some microalgae used for commercial production (Cohen, 1999) are especially rich in essential fatty acids, e.g. C₁₈ linoleic (18:2ω6) and γ-linolenic (18:3ω3) acids, and their C₂₀ derivatives eicosapentaenoic acid (20:5ω3) and arachidonic acid (20:4ω6). These fatty acids are essential ingredients of the health dietary foods, and have a central position in medical biochemistry.

30.6.2 Phycotoxins (see Chapter 28)

Among naturally occurring biotoxins (lethal substances acting on whole organisms), the toxins produced by algae are designated phycotoxins. They are synthesized by certain species and adversely affect other organisms when present in extremely minute quantities. Phycotoxins are characterized by their potency (μg toxin kg^{-1} body mass), specificity of action (e.g. ionic channels in membranes) and in some cases reversibility. Physiologists and physicians carrying out research in the field of medicines and other health care substances have an eye continually on the possible importance of phycotoxins for development of new pharmacological tools and medicaments.

So far, relatively few microalgal species have drawn the attention of toxicologists and biochemists because of their toxicogenic properties. However, a wide range of secondary metabolites with toxic nature are present in prokaryotic and eukaryotic microalgae. And species with practical concern are distributed all over the diversity of organisms in aquatic and terrestrial environments (Premazzi & Volterra, 1993; Skulberg *et al.*, 1993). Microalgal cultures are a basic requisite for fundamental and applied research on phycotoxins. Both for experimental purposes and for the production of the relevant secondary metabolites are defined clone cultures, a *sine qua non*. Strains of the different species of toxicogenic microalgae are available from the international culture collections (Andersen *et al.*, 1995). In the following some well-defined phycotoxins and their biological activities will be mentioned.

The most familiar marine phycotoxins are paralytic shellfish poison (PSP), tetrodotoxin (TTX) and the ciguatera-related toxins (CTX). A prominent source of PSP is phytoflagellates, and notorious species belong to the genera *Alexandrium* (*Gonyaulax*), *Pyrodinium* and *Gymnodinium* (Baden & Trainer, 1993). The relevant toxin is a tetrahydropurine designated saxitoxin, exhibiting a relaxant action on smooth vascular muscles. The rate of rise and amplitude of action potential of cardiac muscles are depressed. This makes saxitoxin a useful tool in experiments analyzing the mechanism of transmitter release and the interaction of the transmitter with postsynaptic membranes (Evans, 1972). Although TTX has some structural differences to saxitoxin on the molecular level, both toxins have essentially the same physiological channel-blocking effect.

The ciguatera poisoning (CTX), resulting from ingestion of several varieties of fish from subtropical and tropical regions, is due to a lipid-soluble compound designated ciguatoxin being produced by dinoflagellates, e.g. *Gambierdiscus toxicus*. Ciguatoxin is a polyether with physiological actions involving both α-adrenergic and cholinergic nervous systems. The effects are

due to the opening of sodium channels at resting potential, and the inability of opening channels to be inactivated during subsequent depolarization (Scheuer, 1988). Maitotoxin (MTX) which coexists with ciguatoxin is among the most potent toxins produced by phytoplankton. This toxin is reported to act by changing the configuration of a membrane protein, transforming it into a pore which allows calcium ions to flow through (Murata *et al.*, 1991). Brevetoxins (BTX) are produced by the dinoflagellate *Phytopliscus brevis*, and exhibit similar neurotoxicity as ciguatoxin by generating membrane depolarization via interaction with sodium channel sites (Shimizu *et al.*, 1986).

Also diatoms are responsible for producing neurotoxins (Bates *et al.*, 1989). The pennate diatom *Nitzschia pungens* have domoic acid, a dicarboxylic amino acid, as a secondary metabolite. Domoic acid brings about a potent depolarizing and excitatory effect in the nervous system. The poisoning is termed amnesic shellfish poisoning (ASP). Domoic acid acts as a potent agonist of glutamate receptors, being structurally similar to glutamic and aspartic acid. Relevant species of *Nitzschia* can conveniently produce domoic acid in controlled cultures. The metabolite has interest connected with investigations of neurodegenerative diseases.

A second syndrome of poisoning in humans conveyed by consumption of shellfish is characterized by gastrointestinal disturbances (diarrhoeic shellfish poisoning – DSP). The causative agents are usually dinoflagellates. Species of the genera *Prorocentrum* and *Dinophysis* belong to this category. The principal compound involved is okadaic acid and its derivatives (Murakami *et al.*, 1982). They have structured molecular similarities to ionophoric polyethers which have been identified in terrestrial organisms as antibiotics. Okadaic acid is reported to be a powerful tumor promoter of non-phorbol ester type. As a specific inhibitor of phosphatase activity in *in vitro* preparations, okadaic acid is currently applied in pharmacological and physiological research.

Several taxa of phytoplankton are known for their ichthyotoxicity, responsible for massive fish kills in nature and in aquaculture (Underdal *et al.*, 1989; Aune *et al.*, 1992). The haptophyte genus *Prymnesium* can serve as an example. The toxic properties of *P. parvum* are attributed to a group of chemical compounds having similar composition, but different toxic effects. The toxic principle is designated prymnesin, containing proteolipids in the composition of fatty acids together with protein and phosphate (Skulberg *et al.*, 1993). Prymnesin has a broad spectrum of different biological activities *in vivo* and *in vitro*. The observed effects are classified as cytotoxic, hemolytic and ichthyotoxic (Shilo, 1971). Ichthyotoxicity has extensively been investigated with the use of microalgal cultures, and observed in strains of, e.g. *Ochromonas*, *Uroglena* (Kamiya *et al.*, 1979) and *Chrysotrichomulina* (Yasumoto *et al.*, 1990).

The toxins produced by cyanophytes – cyanotoxins (Carmichael, 1997) – are grouped into categories based on the bioassay methods used for their screening and the poisoning effects observed. A practical classification of toxic properties includes neurotoxic (Carmichael, 1992), hepatotoxic (Falconer, 1993) and protracted toxic (Skulberg *et al.*, 1994) symptoms linked to perceptible changes indicating organ damage or other health injury. The fast growing

knowledge about these secondary metabolites is reflected in the voluminous publications appearing (Watanabe *et al.*, 1996; Chorus & Bartram, 1999).

The neurotoxic compounds exert their effects on specific ion channels in nerve and muscle membranes (Wu *et al.*, 2000). Their use in investigating the exitable properties of nerve cells determines the current interest attached to selected substances as pharmacological tools.

Homoanatoxin-a, a neurotoxin produced by strains of some oscillatorialean cyanobacteria, can be used as an example. The toxin has been isolated, its molecular structure elucidated and its toxicity investigated (Skulberg *et al.*, 1992; Aas *et al.*, 1996; James *et al.*, 1998). Homoanatoxin-a is a low molecular weight bicyclic secondary amine. The structure of the toxin is 2-(propan-1-oxo-1-yl)-9-azabicyclo (4.2.1)non-2-ene. The molecular weight is 179 Da. It has potent cholinergic properties and a high toxicity. The LD₅₀ for the pure toxin is about 250 µg kg⁻¹ body weight (i.e. mouse). Toxicosis in the lethal dose range leads to severe body paralysis, convulsions, and death by respiratory arrest in 2–12 min (Lilleheil *et al.*, 1997).

Homoanatoxin-a allows the generation of a tritiated product (Wonnacott *et al.*, 1992). This compound has the necessary characteristics that enable it to be exploited in the investigations of high affinity nicotinic sites in the human brain. Neurotoxins from blue-greens are receiving attention in the exploration of how the human brain works in both health and disease. Some neurotoxins selectively damage the same neuronal systems that are affected by neurodegenerative disease. These compounds may therefore be suitable as experimental tools for the investigation of such diseases (Langston & Young, 1992).

The potential to generate pharmaceuticals from neurotoxins is a promising development (Namikoshi & Rinehart, 1996). Suitable species of cyanophytes are candidates for the bioprocessing of target compounds. Fortunately, they naturally have a superior ability to synthesize the stereospecific compounds with the relevant bioactivity (Haugen *et al.*, 1994). Toxins produced by cyanophytes are often chiral and can exist as two optically active forms (enantiomers). Enantiomers of a chiral compound have identical chemical and physical properties. They can only be transferred into each other by reflection. The ring structure of homoanatoxin contains two asymmetric centres which normally would result in two pairs of enantiomers. However, due to steric constraints of bond angles, homoanatoxin exists in only two enantiomeric forms. The chemical synthesis of such substances gives a racemate (a precise 1:1 mixture of both enantiomers), while biosynthesis is normally enantioselective producing only one enantiomer. Furthermore, in many cases one enantiomer has toxic properties, while the other one is inactive or might even have antagonistic effects.

Hepatotoxic cyclic peptides include microcystins and nodularins. Microcystins have been characterized from cultures of species of the genera *Anabaena*, *Anabaenopsis*, *Hapalosiphon*, *Microcystis*, *Nostoc*, *Oscillatoria* and *Planktothrix*. The brackish water blue-green alga *Nodularia spumigena* is the source of nodularin. Compared with many other cell oligopeptides these cyclic peptides are rather small, with molecular weight in the range of 800–1100 Da. They contain either five (nodularins) or seven (microcystins) amino acids, with the two terminal acids of linear peptide being joined to form a cyclic compound

(Sivonen *et al.*, 1989; Harada, 1996). More than 60 structural variants of microcystins have been characterized from isolated strains in cultures.

The pharmacological actions of cyanotoxins with hepatotoxic effects are connected with uptake into hepatocytes via bile acid carrier salt transport. Microcystins induce changes in the actin microfilaments of the liver cells cytoskeleton, leading to dense aggregation of the microfilaments. Destruction of the sinusoid endothelial cells results in lethal intrahepatic hemorrhage (Falconer, 1993). The hepatotoxins are strong inhibitors of protein phosphatases, enzymes which are vital to various cellular processes such as cell growth and tumor suppression. This inhibitor effect is of great benefit to drug development (see later sections).

30.6.3 Bioactive metabolites as drug leads

According to definition, the biological effects exhibited by phycotoxins are harmful to the target organism. On the other hand, compounds of this nature may have potential to induce favorable activities on non-target cells or creatures. This seeming contradiction occurs because a molecule that is detrimental at one concentration level may well be beneficial when received at a lower and more controlled dose, or delivered to a different receptor site. Although secondary metabolites, e.g. microalgal cytotoxins may be directly useful as drugs, it is more usual that the relevant metabolite merely provides a design model for the construction of an analog molecule. Moreover, many factors other than just potent bioactivity are crucial in the development of drugs for pharmaceutical use. Among important qualifications are bioavailability, lack of negative side effects and chemical stability during storage. Properties of this kind are unlikely to be found in a secondary metabolite which is synthesized *in vivo* by the microalgae for a direct purpose (Grabley & Thiericke, 1999).

Once an interesting activity has been discovered and identified with a discrete secondary metabolite, the next step is the chemical determination of the molecular structure of the relevant component (Fig. 30.2). The laboratory methods used require microgram quantities of material. This is usually obtained from extracts of biomass from clone cultures of the producer microalgae. However, for drug-design purposes it is necessary to determine the three-dimensional molecular structure. The procedure involves use of either nuclear magnetic resonance (NMR) spectroscopy or X-ray crystallography. Both techniques have a need for milligram quantities of material, sometimes up to a hundred milligram if extensive crystallization trials are required. Mass production of the microalgae can be done for the extraction of the necessary quantities of the compound, or the chemical synthesis of the compound has to be challenged. And this is only the preliminary work before clinical trials and the drug-design process are carried out based on medical and pharmaceutical competence (Shu, 1998; Jaspers & Lawton, 1998).

30.6.4 Cytotoxins and similar metabolites

A lot of secondary chemicals produced by microalgae in cultures are described under the category cytotoxins (Glombitzka & Koch, 1989; Patterson

et al., 1994; Borowitzka, 1995; Luesch *et al.*, 2000). Cytotoxins are studied using cultured cell lines (*in vitro* biotests, using virus, fungi, bacteria or cell cultures, e.g. plant cells, protoplasts, cancer cells). The substances considered are generally not highly toxic to plants and animals, but are distinctive with their selective bioactivity influencing intracellular metabolism. The cytotoxic substances themselves are not likely to be useful as pharmaceuticals, however, they can serve as models for the rational design of useful compounds. It is important to underline that *in vitro* assays alone are inadequate for rational evaluation of relevant bioactivity, and only in combination with *in vivo* investigations fruitful results can be obtained (Borowitzka, 1999). Screening for cytotoxins has first of all been carried out on cyanobacteria, and more than 200 such compounds from blue-greens have been characterized (Jaspars & Lawton, 1998).

Microalgae constitute an exciting botanical garden for investigations on secondary metabolites with ecological importance and potentials for drug discovery. A rapid increase in research on bioactive molecules has taken place highlighting compounds from natural sources (Mulzer & Bohlmann, 2000).

Screening of microalgae for antibiotics and other pharmaceuticals is receiving ever increasing interest. A range of relevant biological effects such as antibacterial, anticoagulating, antifungal, antihelminthic, antimitotic, anti-neoplastic, antiviral, etc. is in the research light. Natural products derived from microalgae are also investigated for therapeutic applications in the treatment of cancer and other malignants. The antimicrobial substances explored may target various kinds of microorganisms, prokaryotes as well as eukaryotes. The potential to produce the microalgal raw material in controlled cultures enables the manufacture of structurally complex molecules which are difficult or not convenient to synthesize chemically (Borowitzka, 1995).

In this section on metabolites with pharmaceutical potential, many relevant microalgal constituents are excluded, and a selection of compounds and organisms is made in an attempt to give a brief digest of the field. The examples chosen are intentionally restricted to publications in which the bioactivities reported are attributed to defined substances. Bioactive compounds from blue-greens are particularly dealt with, due to the prominent position of this phylum as a source of bioactive molecules.

30.6.4.1 Eukaryotic microalgae

The unicellular rhodophyte *Porphyridium* is regarded as a reliable producer of valuable biochemicals (Cohen, 1999). Among the useful substances obtained from the biomass are sulfated polysaccharides. They are agents known to possess antiviral activity against a variety of animal viruses. Recent investigations have demonstrated antiviral effect from *P. aerugineum* due to highly sulfated polysaccharides on *Herpes simplex* and *Varicella zoster* (Huleihel *et al.*, 2001). The results indicated that the relevant polysaccharides are able to inhibit viral infection by preventing adsorption of virus into the host cells and/or inhibiting the production of new viral particles inside the host cells.

Bioactive metabolites produced by chrysophytes are already mentioned in connection with the ichthyotoxicity of *Prymnesium parvum*. Acrylic acid was the first antibiotically active compound from microalgae to be unambiguously identified and correlated with a biological effect. The marine bloom forming flagellate *Phaeocystis pouchetii* is the origin of the substance (Sieburth, 1960).

The blooms of nannoplanktic chrysophytes experienced in recent years in oceanic and coastal waters of the Norwegian Sea – connected with extensive intoxication of fish, invertebrates and even to seaweeds (Underdal *et al.*, 1989) – indicate the presence of organisms with a large potential for new drug discovery. So far chrysophytes have been examined to a minor extent with respect to their content of secondary metabolites with bioactivity. Among promising results it can be referred to research with screening for potential anticancer activity. In extracts of cultured microalgae the compound malhamensilipin from *Poteriochromonas malhamensis* has been identified with protein tyrosine kinase activity (Gerwick *et al.*, 1994).

Antibiotically fatty acid derivatives from diatoms have gained research attention (Findlay & Patil, 1984). Both limnic and marine cultured species have been investigated and established as sources of lipophilic substances with antibiotic activity. Relevant compounds have been isolated from, e.g. *Asterionella japonica*, *Chaetoceros lauderii* and *Navicula dalgomei* (Borowitzka, 1999). A large-scale screening program for antifungal activity in marine microalgae resulted in detection of several species of diatoms as potentially good sources for antifungal agents (Kellam *et al.*, 1988).

The secondary metabolites of dinoflagellates have long possessed a prominent place in research on antibiosis and related effects (Metting & Pyne, 1986). A wide range of species have been investigated for antibacterial and antifungal properties (Murakami & Yamaguchi, 1989; Pesando, 1990). Macrolides with antitumor effects have been isolated from dinoflagellates. The compound amphidinolide-A is toxic to two kinds of leukaemia cells – L 1210 and L 5178 Y. A species of the genus *Amphidinium* is the producer organism (Kobayashi *et al.*, 1986). Related amphidinolides have also been characterized and found to have markedly different activities with better effects. The dinoflagellates as an entity is regarded as a very rewarding source for new drugs. They include, however, organisms which in many cases are experienced to be rather difficult to grow in mass culture.

The chlorophytes constitute an extremely diverse phylum of microalgae, having a proportional high abundance of species being investigated for various bioactive metabolites. The genera *Chlamydomonas* and *Chlorella* are generally regarded as the best studied microalgae of all (Richter, 1978; Harris, 1989). The early relevant research works on *C. vulgaris* (Pratt & Fong, 1940) became rapidly classics, and stimulated the progress of studies on growth promoting and inhibiting properties of secondary metabolites in green algae from aquatic and terrestrial habitats (Harder & Opperman, 1953). The bioactive substances from chlorophytes include among others nitrogen-containing compounds and carboxylic acids. Several interesting leads to pharmacologically active compounds are present (Yamaguchi *et al.*,

1989; Merchant *et al.*, 1990). Several relevant properties worthy of notice are asserted in cultures of organisms in the genera *Chlorella*, *Dunaliella* and *Tetraselmis*. The drug criteria reported include a multitude of qualities, e.g. analgesic-, antihypertensive-, antioedema-, antiserotonin-, bronchodilator-, muscle relaxant- and polysynaptic-block effects (Borowitzka, 1999).

30.6.4.2 Prokaryotic microalgae

The cyanophytes are the most advanced in position among microalgae with respect to extensive research, and as a major source of bioactive natural products. The rapid growing knowledge about the new compounds have fostered several review articles covering organisms and the discovered structures of agents (Patterson *et al.*, 1994; Falch *et al.*, 1995; Namikoshi & Rinehart, 1996; Jaspars & Lawton, 1998; Dow & Swoboda, 2000; Skulberg, 2000). These reviews illustrate the broad diversity of isolated compounds and the different bioactivities discovered.

The intensity and advancement of the research work carried out are evident from the extent of efforts invested. Several laboratories have performed methodical isolation of strains being maintained in culture collections and used for testing of bioactivity. Approximately a thousand extracts from cultured blue-green algae were tested for cytotoxicity utilizing an assay with human epidermoid carcinoma cells. Antineoplastic activity was also investigated by screening of effects on the differentiation of human leukaemia cells. A hit rate of ca. 7% was reported (Patterson *et al.*, 1991). Testing for inhibition of reverse transcriptase from avian myoblastosis virus and HIV-1 by lipophilic and hydrophilic extracts of 900 cultures of blue-greens, resulted in a hit rate of about 2% (Lau *et al.*, 1993). Extracts from 600 cultures of blue-greens were tested – with a hit rate of 10% – for inhibition of cellular infection of human pathogenic viruses HIV-1 and HSV-2 (herpes), and antirespiratory syncytia virus (Patterson *et al.*, 1993). Inhibition of tyrosinase related to melanin production in skin diseases was tested using 154 extracts of 127 marine blue-greens and other microalgae, ten of the organisms were effective inhibitors (Wachi *et al.*, 1995). Based on more than a hundred strains of cyanophytes exocellular polysaccharides containing non-saccharidic components – such as peptidic moieties, acetyl, pyruvyl and sulfate groups – released into the culture medium, have been investigated in the course of the last decades (De Philippis *et al.*, 2001).

Cyanophytes are recognized as a rich source of compounds with pharmacologically as well as structurally interesting secondary metabolites. A selection of characteristic blue-greens and their respective bioactive substances will be referred to.

Antibiotic agents

An investigation based on 20 strains of cultured limnic and terrestrial blue-greens – using hydrophilic extracts – demonstrated extensive presence of antimicrobial activity (Falch *et al.*, 1995). Out of 54 extracts tested, 78% showed antibacterial and 45% antifungal activities. Three compounds

exhibiting antibacterial, antifungal, cytotoxic, molluscicidal, anti-inflammatory, and antiviral activities (*loc. cit.*), were obtained via bioassay-guided fractionation of the lipophilic extracts of *Fischerella ambigua* – isolated from soil. Another antibiotic producing species is *Scytonema hofmanni* – a terrestrial species. This slow-growing organism excrete a hydrophobic metabolite which is toxic to most blue-greens and also to some green algae (Gleason & Wood, 1987). The compound is a chlorine containing diaryl-lactone designated cyanobacterin. Also *Hapalosiphon fontinalis* – a limnic species – is producing lipophilic compounds with antialgal and antifungal activity (Moore *et al.*, 1987). An indole alkaloid – hapalindole A – and its oxidation product anhydrohaplooxindole have been isolated from strains in culture. Marine blue-greens contain similar secondary metabolites. The euryhaline species *Nodularia harveyana* possess lipophilic bioactive substances with effects against cyanobacteria, eubacteria and some invertebrates (Pushparaj *et al.*, 1999). Also *Hormothamnion enteromorphoides* – a tropical marine species – an organism producing hormothamnin A can be mentioned (Gerwick *et al.*, 1992). This cyclic undeca peptide has both antibiotic and cytotoxic effects.

Antiviral agents

Antiviral compounds from blue-greens include the chemical categories peptides and glycolipids. The peptide cyanovirin-N has been isolated from a culture of *Nostoc ellipsosporum*, a terrestrial species. This agent is active against highly drug-resistant primary isolates of HIV-1, but is inactive against herpesvirus type 1 (Boyd *et al.*, 1997). The peptide binds irreversibly to the HIV viral surface envelope glycoprotein, and aborts cell to cell fusion and transmission of HIV-1 infection. The laboratory cultured *Lyngbya lagerheimii* – a limnic species – belongs to the glycolipid producing type. The active sulfolipids made up almost 10% of the organic extract from the producer organism. They caused different degrees of protection to cells infected with HIV-1. The field of antiviral agents synthesized by blue-greens will have a promising future (Jaspars & Lawton, 1998).

Anticancer agents

Anticancer activity is the so far most frequent reported property of bioactive molecules from cyanophytes. An extensive discovery program carried out by the National Cancer Institute in USA has contributed substantially to this advance (Jaspars & Lawton, 1998; Harrigan *et al.*, 2000). Scytophyccins are polyketide-derived macrolides isolated from *Scytonema pseudohofmanni* (Ishibashi *et al.*, 1986). They inhibit the proliferation of a variety of mammalian cells, including the human epidermoid carcinoma (KB) cell line. In addition, they are active against intraperitoneally implanted lymphocytic leukemia and lung carcinoma (Furusawa *et al.*, 1994). The depsipeptide cryptophycin is a prominent anticancer compound. This metabolite was first isolated from a cultured strain of *Nostoc* sp. – ATCC 53789 (Schwartz *et al.*, 1990). The total structures of the cryptophycins are cleared up and reported (Trimurtulu *et al.*, 1994), and they may probably be approved as cancer chemotherapeutics by

virtue of their greater activity than existing agents (Barrow *et al.*, 1995). Another very promising anticancer agent is curacin A isolated from *L. majuscula*, a tropical marine species (Gerwick *et al.*, 1994). This is an antimitotic agent with potent brine-shrimp (*Artemia salina*) toxicity. Curacin A binds to the cholchicine binding site on tubulin, and potently inhibits the growth of tumor cells (Blokhin *et al.*, 1995). *L. majuscula* was initially harvested via field collections for the research purpose. Sustainable supplies of relevant material were later successfully obtained by mass culturing of the organism (Rossi *et al.*, 1997).

Enzyme inhibitory agents

Many biological processes are controlled by proteolytic reactions, and the related enzyme systems are major gateways to drugs for health care and cure of sickness. Cyanophytes are becoming famous for the various peptide protease inhibitors, which have been isolated and identified in cultures during the last decade (Borowitzka, 1999).

Protease inhibitors have many applications in medicine for treatment of, e.g. strokes, coronary artery occlusions and pulmonary emphysema, and they are also being used in the therapy of HIV infections. Aeruginosins are linear peptides isolated from *Microcystis aeruginosa* with inhibitor activity on thrombin, plasmin and trypsin respectively (Murakami *et al.*, 1995). Also the cyclic peptides of cyanophytes – microcystins and nodularins – are well known for their proteinase inhibitor effects. Several unusual depsipeptides such as micropeptin, microcystilide, cyanopeptolin, oscillapeptin and nostocyclin, can be mentioned in this connection (Weckesser *et al.*, 1996). These compounds have the common feature of being – in various ways – inhibitors of the enzymes trypsin, plasmin, thrombin and chymotrypsin (Patterson, 1996).

Regulation of the eukaryotic mitotic cycle includes reversible protein phosphorylation, accordingly different phosphatases are also considered as important components in the control of plant growth and development (Smith & Walker, 1996). The growth suppressing ability of extracts of *Microcystis aeruginosa* observed on vascular plants (Kós *et al.*, 1995) is interpreted as caused by the peptide protease inhibitory effects of microcystins.

30.6.5 Allelochemicals and akin substances

Allelopathic interactions among organisms are mediated by bioactive chemicals regarded as secondary metabolites (Whittaker & Feeney, 1971; Rice, 1984; Keating, 1999). The chemical nature of relevant microalgal metabolites is reported as mainly organic acids or phenolic compounds (Ahluwalia, 1998). Phenols and brominated phenolic agents produced by some algae are water soluble and acting as potent allelochemicals. The growth inhibition caused by *Chlamydomonas reinhardtii* on other microalgae is due to a long chain fatty acid (McCraken *et al.*, 1980). Acrylic acid is identified as the antibacterial and antifungal agent of the chrysophyte flagellate *Phaeocystis poucheti* (Sieburth, 1960). Aquatic macrophytes can be impaired by growth inhibiting substances from *Pandorina morum* and *Scenedesmus quadricauda* (Sharma,

1985). A substance synthesized by *P. morum* inhibited the electron flow with methyl viologen as electron acceptor (Patterson *et al.*, 1979). Compounds with bioactivity against photosynthesis in prokaryotic and eukaryotic organisms belong to the category of allelochemicals (Smith & Doan, 1999).

Related phenomena reported from investigations based on microalgal extracts include various regulating effects on cell growth and development. A glycoprotein-rich extract of *C. vulgaris* may activate mature leukocytes, and stimulates certain stem cells in the bone marrow of rodents when administered subcutaneously or orally (Konishi *et al.*, 1996). An active principle in extracts from selected strains of *Chlorella*, *Euglena* and *Spirulina* has been found to be the plant hormone jasmonic acid (Ueda *et al.*, 1991). In cultures of several species of blue-greens and green algae the presence of cytokinin and gibberellin is demonstrated (Burkiewicz, 1987; Ördög & Pulz, 1996; Stirk *et al.*, 1999). Many other active principles in these extracts have so far not been characterized.

Some chemical substances play an important role in interactions among organisms by eliciting a physiological or behavioral response. Information chemicals may be divided into two major groups: Pheromones – intraspecific infochemicals, and allelochemicals – interspecific infochemicals. Secondary metabolites of microalgae encompass both types (Lürling, 1999). In freshwater systems consumer-induced defenses may be found in planktic microalgae. A grazing-mediated chemical defense in the marine flagellate *Emiliania huxleyi* has been reported (Wolfe *et al.*, 1997). The smell and taste of water may also depend on organic chemicals with special bioactivity related to allelopathy and intraspecies regulation. Metabolites of prokaryotic and eukaryotic microalgae with olfactory effects are characterized as volatile organic biogenic substances (VOBS – Jüttner, 1987).

30.7 Perspective

Applied phycology is on the threshold of a very exciting time, being part of the scientific drug discovery and development community. From chemistry to disease mechanisms, from molecules to ecosystems, from target discovery to pharmacoconomics, there are more breakthroughs for bioactive chemicals from microalgae than ever before. The renewed interest experienced in the use of natural sources in drug discovery fosters fresh advances and collaboration in multidisciplinary teams of biologists, research chemists and physicians. Combinatorial chemistry has made the path from the hit to the development of a drug candidate much more effective than with conventional methods. And algal culture technology will offer several logistical advantages in exploiting the potential of microalgae for the manufacturing of economically important secondary metabolites. It is opportune *to make hay while the sun shines*.

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31 Heterotrophic Production of Marine Algae for Aquaculture

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31.1 Introduction

Extensive research has been devoted to developing microorganisms such as yeast, molds, bacteria, fungi, and microalgae as additives to aquaculture feeds. A major advantage in the use of unicellular organisms is that the technology exists to produce industrial quantities under controlled and environmentally safe conditions. Several studies have demonstrated that the partial replacement of fish-based ingredients with yeast and bacteria does not compromise fish survival, growth, or disease resistance (Anon, 1977; Dabrowski, 1982; Dabrowski *et al.*, 1985; Murray & Marchant, 1986). Moreover, the composition of many microorganisms can be manipulated through media enrichment supplements to produce high levels of protein and lipid (Kangas *et al.*, 1982; Tan & Johns, 1991; Sanchez *et al.*, 1995; Day & Tsavalos, 1996).

Microalgae are an integral component in the aquaculture food chain, and inclusion of photosynthetic microalgae in aquaculture feeds has been practiced for many years (Day *et al.*, 1990; Zhou *et al.*, 1991; Laing & Millican, 1992; Day & Tsavalos, 1996; Langden & Onal, 1999). This approach, however, has not resulted in a large commercial application to replace fish-based ingredients, mostly due to culture inefficiency and high production costs (Borowitzka, 1992; Chaumont, 1993; Wilkinson, 1998). Photosynthetic production of algae either in out-door or indoor photobioreactor systems is costly, since cultures must be maintained at low densities ($1\text{--}2\text{ g l}^{-1}$). Consequently, large volumes of media must be processed to recover small quantities of algae, and since most algal cells are very small, expensive harvesting processes must be employed.

An alternative source for algal substitutes may be biomass production using heterotrophic fermentation. Heterotrophic growth has several distinct advantages over phototrophic growth that make it a preferred mode of growth for rapid and high-density accumulation of algal biomass. When grown heterotrophically, algae utilize an organic carbon source (e.g. glucose or acetate) as both a carbon source and energy source for growth. A major

advantage of heterotrophic algal growth is a substantial reduction in the cost of biomass production. Other advantages include, far higher cell densities ($>75\text{ g l}^{-1}$ dry weight), which reduce harvesting effort, the readily available large-scale growth fermentation vessels (up to 500 000 l volume), and the use of existing and standardized fermentation technology. Altogether these factors result in a lower cost and reliable production method for algal biomass.

31.2 Strain selection and improvement

By using established fermentation techniques for culturing strains of chrysophytes, cryptophytes, dinoflagellates, thraustochytrids (marine micro-heterotroph) or fungi, essential fatty acid mixtures can be obtained with high levels of eicosahexaenoic acid (20:5 $n - 3$, EPA), docosahexaenoic acid (22:6 $n - 3$, DHA) and arachidonic acid (20:4 $n - 6$, ArA) (Cohen *et al.*, 1995; Behrens and Kyle, 1996; Apt and Behrens, 1999). Examples are the species, *Schizochytrium* sp. (thraustochytrid) and *Cryptothecodium cohnii* (dinoflagellate) which can produce high levels (up to 50%) of DHA (Barclay & Zeller, 1996; Behrens & Kyle, 1996). The dried biomass from *Nitzschia* sp. (Boswell *et al.*, 1992) and *Navicula* sp. (Tan & Johns, 1996) (both diatoms) can offer a rich source of EPA (Kyle *et al.*, 1989; Apt & Behrens, 1999). Moreover, extracted oils from the heterotrophic fungi, *Mortierella* sp., which contain up to 54% ArA can be readily produced and combined with other heterotrophic products. Additional waste stream co-products of the fermentation process are phospholipids rich in DHA and ArA. Recent studies have shown that these essential lipids are effectively absorbed during enrichment process of larval prey (Harel & Place, 1999; Place *et al.*, 1999). Feeding trials with striped bass (*Morone saxatilis*), sea bream (*Sparus aurata*) and halibut larvae resulted in a significant improvement in survival, growth and pigmentation rates. For example, halibut larvae fed on *Artemia* enriched with DHA rich phospholipid grow over 50% faster and were fully pigmented (100%) as compared with larvae fed on other commercially available fish oil products.

Marine oils rich in DHA isolated from specific fish tissues (cod liver oil, tuna orbital oil), or through special extraction procedures (silage, cold acetone) have been recommended as supplements to broodstock diets and larval rearing enrichments (McEvoy & Sargent, 1999; Sargent *et al.*, 1999). However, the availability of these DHA containing oils is limited and often prohibitively expensive to produce. Fish oil is used in fish feed mainly because it offers a range of fatty acid classes, including the long chain $n - 3$ Poly unsaturated fatty acid (PUFA), that contribute to the energy, growth and reproductive demands of the fish. In addition, the $n - 3$ fatty acids in fish oil impact natural immunity of the farmed fish thereby reducing or eliminating the need for medications.

Recent research in the UK and Italy (McKenzie *et al.*, 1997) has demonstrated that a reduction in rearing tank oxygen with growing sturgeon can be ameliorated with fish fed $n - 3$ fatty acids. These same experimental fish fed with saturated fatty acids showed a sharp reduction in activity levels, rates of oxygen consumption, and cardiac performance. Even herbivorous fish appear

to have a more robust immune response when ω -3 fats are included in their diet (Pilarczyk, 1995). These data suggest that, among many well-known benefits, fish fed with $n - 3$ rich fatty acids may survive and resist the stresses of intensive aquaculture better than fish fed on other types of fats. However, the level of $n - 3$ PUFA in fish oil varies depending on fish species, extraction procedures and storage conditions. In fact, standard available fish oils (menhaden, anchovy, herring, etc.) do not offer sufficient levels of DHA or DHA/EPA ratios to satisfy the nutritional demands for reproduction and larval growth (Harel *et al.*, 1994; Sargent *et al.*, 1997; Harel *et al.*, 2000). In addition, diets formulated with standard available fish oils are usually low in ArA. This is relevant in light of the increasing body of evidence attesting to the species-specific requirement of this essential fatty acid (Castell *et al.*, 1994; Bessonart *et al.*, 1999; Harel *et al.*, 2000; Koven *et al.*, 2001).

In addition to fatty acids, productions of natural astaxanthin from heterotrophic strain of algae (e.g. *Haematococcus* sp.), or natural L-ascorbic acid (vitamin C) from *Chlorella pyrenoidosa* (Running *et al.*, 1994) are additional examples of the potential of algal fermentation biotechnology. Astaxanthin is the primary source of pigmentation in salmonids, crustaceans, and ornamental fish. Normally carotenoids are obtained via the natural diet of organisms in the wild; however, in aquaculture, carotenoids have to be specifically added to the artificial diet. Recent studies have demonstrated the key role of dietary carotenoids in the health status of animals, including fish. Astaxanthin acts as a natural antioxidant extending the shelf life and improving the quality of the final fish product. Until recently, the aquaculture industry has depended on synthetic astaxanthin for the production of pigmented fish products (accounting for 20–30% of total feed costs). However, the market share of algal astaxanthin is increasing rapidly as it represents an effective and more natural alternative source of carotenoids in aquaculture. The advantages being: (1) Microalgal astaxanthin has characteristics similar to the natural dietary sources of carotenoid in wild salmon and trout because it is present as bioactive esters and is the (3S, 3'S) stereoisomer, (2) Production of microalgal astaxanthin is an effective use of clean technology, driven by fermentation process (Zhang & Lee, 2001), (3) It is essentially waste-free as the algal biomass can be fully integrated into feeds. Extruded feeds containing pre-mixed synthetic astaxanthin have a 30% loss, because it more easily leaches into the water. While micro-encapsulation can reduce such a loss, uptake efficiency is reduced. Microalgae rich in astaxanthin can be incorporated in fish oil and sprayed on extruded feeds after extrusion. This improves incorporation efficiencies and precludes the need for extraction. Preliminary estimates show that once the fermentation process is optimized overall production costs may be 20–30% lower than the synthetic and yeast-based alternatives.

Another potential use of heterotrophic algal products is to partially substitute or supplement a live algae diet of the larva of marine finfish, shrimp, and bivalves. Although a complete replacement of living algal supplements has not been successful, the partial replacement of up to 40% using spray-dried heterotrophically grown algal biomass have been reported for a number of marine organisms, such as mussels, *Mytilus galloprovincialis*

(Davis & Campbell, 1998; Langden & Onal, 1999), juvenile bivalve *molluscs* (Laing & Millican, 1992), and juvenile clams, *Tapes semidecussata* (Boieng, 1997). The value of live-algae supplement to the culture medium of many marine larvae is well established (Reitan *et al.*, 1993; Harel *et al.*, 1998; Harel & Place, 1999). However, live photosynthetic algae production is costly and inconsistent and can represent up to 30% of the cost of larval production (Coutteau & Sorgeloos, 1992). Consequently, many hatcheries, mostly in Japan and more recently in Europe, are replacing their on-site algal production with commercially available concentrates of fresh water *Chlorella* sp. This alga can be easily grown in standard fermentors to a high biomass, without losing its chlorophyll pigments, and even be strain-selected to produce specific essential nutrients (Running *et al.*, 1994). Using a semi-balanced medium containing glucose in an inorganic salt solution and several high and low oxygen tension regimen cycles, Nishimura *et al.* (1988) have demonstrated that even photoautotrophically grown marine *Chlorella* species are able to complete cell division under anaerobic condition in the dark, and to achieve a division index of practically 100%.

31.3 Media design strategies for fermentation

Algal strains which have improved characteristics over wild-type strains have been selected. Such developments have been made by traditional techniques of screening and mutation followed by further selection. Recently, even recombinant techniques have been widely suggested for algae. However, for a variety of reasons, recombinant transformation techniques have not been successfully developed for wide-scale production of selected algal strains.

The biochemical composition of microalgae can be modified through environmental manipulations, including nutrient availability, light intensity, pH, temperature, and salinity. For example, harvesting cells in the exponential phase of growth can produce algae containing high levels of protein and $n - 3$ PUFAs. The culture can be further manipulated to become nutrient limited, or nitrogen limited for a suitable time, if higher lipid content and $n - 3$ PUFAs are desired. The initial nitrogen content of the growth medium can be provided such that nitrogen becomes depleted late in the exponential phase. Nitrogen limitation stimulates total lipid production while maintaining high levels of $n - 3$ PUFAs as long as the induction period is kept short, usually 6–24 h (Barclay, 1997). Raising or lowering temperature, depending on the strain employed, can manipulate length of the induction period. Alternatively, the algae can be cultured on a continuous basis in a medium with a high carbon–nitrogen ratio, enabling continuous production of high lipid content (and high $n - 3$ content). In the absence of nitrogen, algae continue to fix carbon but cannot synthesize proteins, and as a result carbon is utilized mainly for the production of nitrogen poor compounds such as lipids and carbohydrates. PUFAs in microbial products, when exposed to oxidizing conditions can be converted to less desirable unsaturated fatty acids or to saturated fatty acids. However, saturation of $n - 3$ highly unsaturated fatty acids (HUFAs) can be reduced or prevented by the introduction of synthetic antioxidants or naturally occurring antioxidants, such as β -carotene,

vitamin E and vitamin C, into the microbial products. The amount of anti-oxidants incorporated in this manner depends, for example, on subsequent use requirements, such as product formulation, packaging methods, and desired shelf life.

Some algal strains, although capable of growing rapidly in the dark, may lose their nutritional value and physiological characteristics. The marine Prasinophyte *Tetraselmis*, a wildly used algal species in aquaculture, probably presents one of the most extreme examples in its response to environmental changes. Several strains of this green alga can be equally cultured under both mixotrophic and heterotrophic conditions, but the pigment levels, and lipid content, and composition are radically affected by heterotrophic conditions (Day *et al.*, 1991; Day & Tsavalos, 1996). These changes can result in an unattractive color and nutritionally deficient feed when compared to the photosynthetic product. Similarly, growth conditions that support maximal biomass production may be different from the conditions that would yield maximum content of the required nutrients (Sukenik, 1991). However, such nutritional differences can be reduced through process development and classical strain improvement. Running *et al.* (1994) exemplified the efficacy of such a program in which extensive screening and process optimization resulted in a greater than 70-fold increase in intracellular ascorbic acid expression compared to the parent strain of *Chlorella pyrenoidosa*.

Marine microalgae generally exhibit a sodium dependent uptake mechanism of nitrate and urea (Rees *et al.*, 1980), and without added NaCl grow slowly and accumulate more saturated fatty acids and storage lipids. However, adding salt in excess to the media can significantly increase the cost factor, and cause corrosive damage to stainless steel fermentor vessels. Similarly, removing silica from diatom growth media also results in an enhanced lipid production (Roessler, 1988). All of these operational parameters may be exploited to maximize the production of *n* – 3 rich algal biomass, as was demonstrated with the heterotrophic dinoflagellate, *Cryptocodinium cohnii*, in which nearly 30–50% of its constituent fatty acids is DHA, further increases with the increasing concentration of NaCl in the medium (Jiang & Chen, 1999), as well as elevating the cell lipids content by depriving inorganic nitrogen from the media before harvesting.

31.4 Scale-up from laboratory to industrial fermentors

The ability to grow rapidly in the dark at high culture densities and on inexpensive and easily sterilized media is a prerequisite for selecting heterotrophic algae strains for scale-up production. The selected algal strain should be able to grow in low salinities to reduce media cost and corrosion of the fermentation system. Gladue and Maxey, screened 121 algal strains for heterotrophic growth. Fifty-seven strains were tested positive with a capacity to grow on glucose as the carbon source and in the presence of complex nutrients (yeast extract and tryptone). However, only five algal strains, including *Chlorella vulgaris*, three strains of *Tetraselmis* and the two diatoms *Cyclotella cryptica* and *Nitzschia alba* exhibited the required minimum rate (generation time less than 12 h) of growth for scale-up (Fig. 31.1).

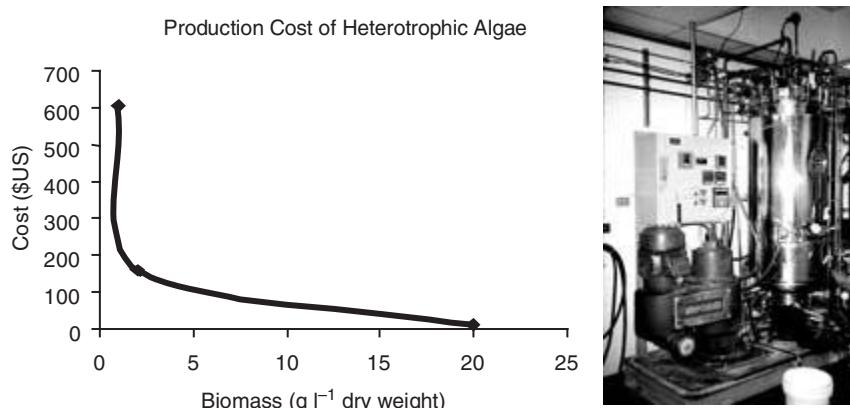


Fig. 31.1. Relationship between culture density and production cost of heterotrophic algae in a standard industrial fermenter (courtesy Dr P. Beherns, Market BioSci. Corp, Columbia, MD).

Some commercially important diatoms are able to grow heterotrophically (a list of 35 such diatoms and references to experimental work is provided by Tuchman, 1996). It is believed that many diatom species out-compete other phytoplankton in the nature by having mechanisms to supplement photosynthesis with heterotrophic uptake, and to utilize two different pathways for assimilation of organic nitrogen under limited-light conditions such as highly turbid waters. Relative to other microalgae, many diatom species can be successfully cultured by fermentation, because they are evolutionarily suited to directly absorb organic nutrients, and their high resistance to shear forces. In fact, many diatoms can grow heterotrophically as rapidly as autotrophically, and some species grow even faster. A few examples are the marine pennate diatom, *N. alba*, and the centric diatom, *Cyclotella meneghiniana*, which grows heterotrophically in the dark within a range of glucose concentrations between 5 mg L⁻¹ and 10 g L⁻¹ (Hellebust, 1971; Droop, 1974; Hellebust & Lewin, 1977).

In a joint research project between the University of Maryland Biotechnology Institute's Center of Marine Biotechnology (COMB, Baltimore, MD) and the National Center for Mariculture (NCM, Eilat, Israel), we have screened for heterotrophic growth of several diatom strains that are used extensively in abalone culture. We found that *Amphora* T3, *Navicula tennerima* and three strains of *Cylindrotheca fusiformis* (2083, 2086 and 2087 UTEX cultures strains, Austin TX) retained the best potential for heterotrophic culture. *Amphora* T3 has been described as an excellent feed for post larvae abalone, while a feed mix containing photosynthetically grown *Amphora* T3 and *Nitzschia laevis* or *Navicula lenzi* permit the highest growth and survival rates (Dr Muki Shpigel, NCM, personal communications). Both *Nitzschia laevis* and *Navicula lenzi* failed to grow in a highly enriched media with organic carbon and complex nutrients. Of interest was our difficulty to obtain sterile cultures of *N. lenzi*. This diatom grew poorly in the presence of antibiotics. It is possible that *N. lenzi* can grow only in the presence of symbiotic bacteria. Studies have shown that healthy microflora can actively

release dissolved organic carbon or essential nutrients to the culture medium, which is then utilized by the algae (Jones & Cannon, 1986). For example, vitamin B₁₂ deficient marine diatoms can have their requirement satisfied through association with B₁₂-producing bacteria (Haines & Guillard, 1974). Evidence also exists for other filamentous *Navicula* species (*Navicula confervacea*) that bacteria adhere to diatom specific mucilage-free sites near areas of active nutrient uptake and release (Rosowski, 1992).

As a result of repeated cycles of selection and propagation of diatom colonies that grow in the dark, significantly faster growing strains were obtained. Addition of organic acids (a mixture of acetate + lactate + glutamate) as the carbon source resulted in poor culture growth and extensive bacterial contamination. Most of the strains that tested positive for heterotrophy were capable of growth on glucose. Mutations were induced after about ten cycles of repeated strain selection through a treatment with N-Nitrosoguanidine (NTG). *Amphora* T3 responded poorly to this treatment and no further growth improvement was achieved. On the other hand, *Cylindrotheca fusiformis*, UTEX strains 2083 and 2086 and *Navicula tenerima* were very responsive, producing healthy and dense blooms (at concentration of ca. 10⁴ cells ml⁻¹) after 14 days in the dark. Although current doubling times in the dark are low (in the range of 20–40 h per generation), repeated cycles of selection and mutation over a longer time period, should result in much faster growth rates, especially for *Amphora* T3 and *C. fusiformis*, UTEX strain 2083, as they demonstrated a significant growth improvement. *Navicula tenerima* seems to respond less than the other two diatoms to this selection procedure, however, several colonies that grow in the dark were obtained. Although our attempts to culture the diatom *Nitzschia laevis* in heterotrophic system was not successful, other studies have indicated that some strains of this diatom can be grown in the dark and to produce large quantities of EPA as well (Fig. 31.2).

31.5 Commercial considerations and production costs

Autotrophic production of microalgae is expensive and inconsistent (\$50–400 per kg dry weight, Coutteau & Sorgeloos, 1992). On the other hand, high-density heterotrophic production technology can inexpensively produce selected algal species of high economic value. For example, maximum production efficiency of phototrophs is around 0.4 g l⁻¹ d⁻¹ whereas heterotrophic production can be over ten times higher (over 5.8 g l⁻¹ d⁻¹, and even as high as 75 g l⁻¹ d⁻¹). In terms of energy investment, a production of 1 kg phototrophs may cost over \$11.22 in electricity (mostly for light production) while in heterotrophic culture conditions, the cost of energy is only \$2.01 (cost of glucose). This comparative information was obtained from Dr Paul Behrens at Martek BioSci Corp, Columbia, MD. Generally, the required doubling time is less than 12 h. Several algal strains can grow even faster; for example, the doubling time in a fermentor of a selected strain of *N. alba* is 6.5 h, very similar to that of *C. vulgaris*. Such doubling rates are translated to a production cost of ca. \$12 kg⁻¹ dry weight product, whereas production

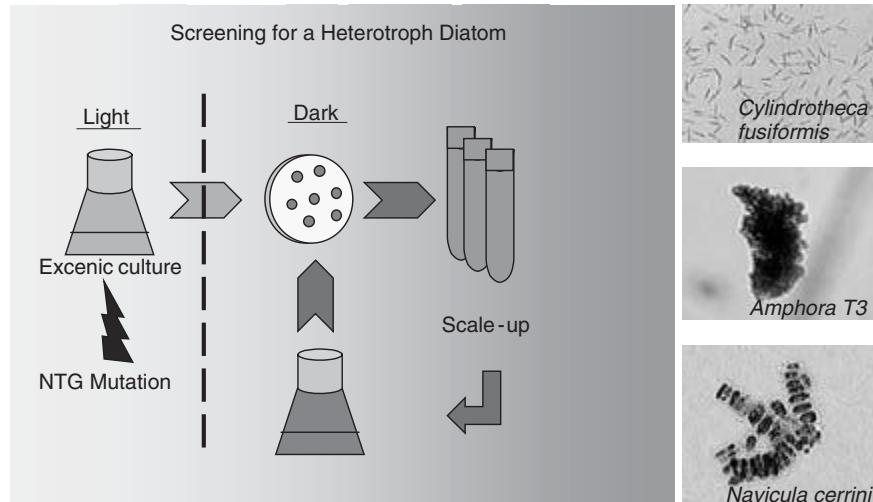


Fig. 31.2. Selection and mutagen treatment of *Cylindrotheca fusiformis*, *Amphora T3* and *Navicula cerrini*. Diatoms were initially cultured in the dark on agar plates. Several healthy and isolated colonies were selected from the agar plates and placed in vials containing sterile F/2 and glucose media + 40 µg ml⁻¹ N-Nitrosoguanidine (NTG) for 20 min. Diatom mutants transferred to culture flasks and allowed to bloom in the dark. Pictures show a sample of average size colony ($\times 6$ ocular magnification).

cost of ca. 20 h doubling time algae would range from \$160 to 600 kg⁻¹ dry weight (Kalk & Langlykke, 1986).

Even though *Tetraselmis* sp. and *N. alba* can produce high levels of EPA, the abundant and cheap alternative source of EPA from fish oils makes the algal product economically uncompetitive. On the other hand, the dinoflagellate, *Cryptocodinium* sp. can produce 15–20% of its dry weight as DHA with only a negligible amount of EPA. Current production cost for DHA rich algal dry biomass (*Cryptocodinium* sp. or *Schizochytrium* sp.) is about US\$25 kg⁻¹, whereas menhaden oil costs about US\$2.50 kg⁻¹. Yet, a true comparison should include the efficiency of the material to deliver this essential fatty acid to the fish. The level of DHA in *Cryptocodinium* sp. meal is an order of magnitude higher than menhaden oil, therefore, on a weight basis, the amount of algal meal required to deliver sufficient levels of DHA would be much less than that of fish oil. Similarly, the natural form of astaxanthin from *Haematococcus pluvialis*, which is only 1.5–4% of dry weight, still values this alga at about US\$45 kg⁻¹ in spite of the alternative availability of a synthetic product. Moreover, the algae as a whole can have additional intrinsic value as a high quality feed and protein source for a range of aquaculture species and larvae.

The significant advantage of heterotrophic cultivation of microalgae, i.e. higher yields, allows for a more efficient and economical harvest and downstream processing, including spray drying and extraction. Based on several processing models, the final cost of the algal biomass is more sensitive to algal productivity. A 50% improvement in growth rate will result in about 20% cost decrease (Borowitzka, 1992). Therefore, any factor that can increase

productivity (product concentration in the algae) and growth rate through repeated cycles of strain and genetic selections or recombinant techniques is of fundamental importance. Harvesting also represents a significant cost factor, since 20% improvement in harvest efficiency will equally decrease the biomass cost, while the cost of drying and packaging can add ~10% to the biomass cost. The viable option of adding raw algal biomass directly to the fish feed, without extraction, purification or spray-drying can save another major cost component of the overall extraction process. For example, the extraction of astaxanthin from *H. pluvialis* is a costly process; however, the use of this alga in fish feed as a pigmenter requires no further extraction following cell breakage.

31.6 Conclusion

The heterotrophic, large-scale production of algal biomass represents a potential high quality substitute for fish-based ingredients in aquaculture feeds. Specifically, whole cell preparations and use of algal and fungal oil extracts can be superior alternative sources for DHA, ArA, astaxanthin, and ascorbic acid enrichment products in a wide variety of fish and shellfish feeds, including both larval and broodstock feeds. The current production levels of heterotrophic algae are insufficient to replace the worldwide use of fish by-products in aquaculture. To date, experience shows that fish meal and fish oil have played a decisive role in the achievement of improved growth and feed efficiency of marine species. This is not surprising given the balance of nutrients and their natural role. On the other hand, the natural fishery is a dwindling resource that can vary in both quantity and quality, and where supplies and processing costs are likely to rise. These are compelling reasons to more closely examine heterotrophic production as a cost effective and stable source of marine type lipids and other essential nutrient sources in fish feeds and for the enrichment of the live feed. In addition the consumer's perception on what is safe, natural, and environmentally friendly will increasingly dominate future formulation decisions.

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32 N₂-fixing Cyanobacteria as a Gene Delivery System for Expressing Mosquitocidal Toxins of *Bacillus thuringiensis* subsp. *israelensis*

Sammy Boussiba and Arieh Zaritsky

32.1 Introduction

Extensive screening of novel strains of cyanobacteria with high biotechnological value has been conducted over the decades worldwide with very limited economic gain. Attempts to improve desired characteristics of existing strains by chemical mutagenesis were hindered due to the poor plasticity of genetic traits and reduced stability of selected mutations in microalgae. Various aspects of strain improvement for commercial purposes have been achieved in cyanobacteria with recombinant DNA techniques (Golden *et al.*, 1987; Elhai & Wolk, 1990; Haselkorn, 1992; Elhai, 1994; Houmar, 1994; Vermaas, 1996; Apt & Behrens, 1999). The most remarkable achievement involves conferring cyanobacteria with herbicide resistance (Golden & Haselkorn, 1985; Brusslan & Haselkorn, 1988; Windhoevel *et al.*, 1997), to prevent annihilation of indigenous cyanobacteria species by heavy application of herbicides in rice fields.

Also promising is the use of genetically modified cyanobacteria for degradation of residual pesticides in water bodies, a potential serious hazard to public health (Kuritz & Wolk, 1995).

We aimed to engineer cyanobacteria for mosquito control (Boussiba & Zaritsky, 1992; Boussiba & Wu, 1995; Boussiba *et al.*, 1997). Mosquitoes and blackflies are vectors of tropical disease such as malaria, filariasis, encephalitis, dengue fever, yellow fever, hence constituting a threat to public health (de Barjac & Sutherland, 1990; Porter *et al.*, 1993; Margalit

& Ben-Dov, 2000). Effective control and prevention of such epidemic diseases, a persistent major public concern, is achieved by reducing population sizes of the transmitting vectors. Their impact on civil life has been partially alleviated by the heavy use of pesticides. Chemical insecticides used to control mosquitoes cause ecological problems and enhance resistance development of the pests. Environmentally friendly alternatives are, thus, of high priority; biological control being considered permanent, inexpensive and free of environmental hazards. Subsp. *israelensis* of the bacterium *Bacillus thuringiensis* (Bti) is a safe and efficient agent to control mosquito larvae. One approach to overcome the low efficacy and short half-life nature of current formulations of Bti is the use of genetically engineered cyanobacteria as a gene delivery system for heterogeneously expressing Bti toxins for the control of mosquito borne diseases.

32.2 *Bacillus thuringiensis* subsp. *israelensis* (Bti) as a biopesticide against mosquitoes

Bti, the most valuable agent for controlling mosquito and blackfly larvae (Mulligan *et al.*, 1980; Lacey & Undeen, 1986; Chungjatupornchai *et al.*, 1988), was discovered in the Negev region of Israel over two decades ago by Margalit (1990). It is highly specific against water-dwelling dipteran species while causing no harm to other organisms including predators of the vectors (de Barjac & Sutherland, 1990; Entwistle *et al.*, 1993). Bti's capacity to synthesize insecticidal crystal proteins (ICPs) has spurred intensified research which led to a comprehensive understanding of their structure and function, the encoding genes and their expression (Hofte & Whiteley, 1989; Margalit & Ben-Dov, 2000). This knowledge, consequently, improved the efficiency of biocontrol formulations, the construction of effective toxins and delivery systems.

The use of Bti is limited by low persistence under field conditions, where it does not reproduce (de Barjac & Sutherland, 1990; Entwistle *et al.*, 1993). The major reasons for low efficacies of current Bti preparations include: sinking to the bottom of the water body, absorption to silt particles and organic matters, inactivation of its toxins by sunlight and consumption by other organisms (Margalit & Ben-Dov, 2000).

32.3 Advantage of cyanobacteria as a BTI toxin delivery system

Much effort has been expended so far around the world to solve these problems. Improving the formulations of Bti to enhance its persistence in natural habitats of dipteran larvae and isolating new mosquitocidal strains or species are such examples. As a high-tech approach, a number of studies have described cloning the genes responsible for toxicity for expression in other organisms that are ingested by the targets (Chang *et al.*, 1992; Porter *et al.*, 1993; Liu *et al.*, 1996; Khampang *et al.*, 1999; Servant *et al.*, 1999). Among these are cyanobacteria, which are abundant in nature (Thiery *et al.*, 1991). Introducing an engineered indigenous strain of cyanobacteria should present

minimal side effects and avoid the problem of disturbing the fine ecological balance. Their ability to float in the upper layer of water bodies and to resist different environmental conditions are unique features (Thanabalu *et al.*, 1992; Boussiba & Wu, 1995). Shuttle vectors harboring cyanobacterial replicons have been constructed and used in a number of strains (Porter, 1987; Elhai & Wolk, 1988).

The last decade saw some progress in expressing mosquitocidal toxin genes of *Bacillus* sp. in cyanobacteria. In a pioneering work, the binary toxin genes from *B. sphaericus* 1593M were expressed in *E. coli*, *B. subtilis* and the cyanobacterium *Anacystis nidulans* R2 (Tandeau de Marsac *et al.*, 1987). All the three transgenic organisms showed reasonable levels of larvicidal activity against *Culex pipiens* larvae despite expression from its original promoter (*E. coli*, *B. subtilis* and unicellular cyanobacterium *A. nidulans* R2). Soon afterwards, the larvicidal toxin gene *cry4Ba* from *Bti* was expressed in *Agmenellum quadruplicatum* PR-6 through the shuttle vector pAQE19LPC Δ Sal under the regulation of the cyano-phycocyanin (CPC) promoter (Angsuthanasombat & Panyim, 1989). The larvicidal activity of the transgenic cells against *Aedes aegypti* was very low in this attempt due to degradation of the product, *cry4Ba*. The same gene was later placed under the control of *PpsbA*, which originated from *psbA*, a chloroplast gene of *Nicotiana tabacum* (Chungjatupornchai, 1990).

Cyanobacteria, which proliferate near the water surface, may thus provide an alternative for prolonged delivery of *Bti* δ -endotoxin to mosquito larvae that breed in the same habitat. Toxicities of the transgenic unicellular cyanobacteria were however very low, even under the regulation of strong promoters, too low to be effective *in vivo*. Certain improvements have been reported by Murphy & Stevens (1992), and Stevens *et al.* (1994). The gene coding for *cry11Aa* was fused in frame with the first six codons of *cpcB* and cloned into shuttle vector pAQE19 Δ Sal for expression in *A. quadruplicatum* PR6 under *P_{cpcB}* promoter. The fused protein was stable in *A. quadruplicatum* PR6 cells, which was readily ingested by the freshly hatched *C. pipiens* larvae, but toxicity was also low. It was the first genetically engineered cyanobacterium that killed mosquito larvae by feeding living cells.

In parallel to the use of unicellular cyanobacteria, the filamentous nitrogen fixing cyanobacterium *Anabaena* PCC 7120 was used for expressing the *B. sphaericus* binary toxin genes (Xudong *et al.*, 1993). Transformed cells carrying the larvicidal toxin genes (*p51* and *p42*) from *B. sphaericus* 2297 were bioactive upon feeding mosquito larvae, with higher toxicity against *C. pipiens* than against *Anopheles sinensis*. The much higher toxicity in the latter study than in Tandeau de Marsac *et al.* (1987) can be attributed to the multicellular character of *Anabaena* compared to the unicellular *A. nidulans* originally used.

32.4 Expression of *Bti* cry genes in *Anabaena* PCC 7120

The broader spectrum of toxicity against mosquito larvae of *Bti* than *B. sphaericus* (Skovmand & Sanogo, 1999) makes the former more useful.

Efficacy of engineered strains expressing *cry* genes in multicellular nitrogen fixing cyanobacteria should be much higher than existing commercial formulations of Bti due to the low cost of mass production and longer persistence in the field (Boussiba, 1991, 1993; Boussiba & Zaritsky, 1992; Zaritsky, 1995).

Three of the Bti toxin genes (*cry4Aa*, *cry11Aa* and *p20*) had been isolated and cloned in all seven possible combinations for expression in two vectors of *E. coli* (Ben-Dov *et al.*, 1995). The two most toxic clones (pHE4-ADR, with all three genes, and pHE4-4AD, without *p20*) were subcloned into *E. coli-Anabaena* shuttle vector for expression under the control of the strong constitutive promoter *P_{psbA}* (Elhai, 1993) or tandem promoters *P_{psbA}-P_{A1}* (Wu *et al.*, 1997). The final constructs, under control of the tandem promoters, were designated pSBJ2 and pSBW2 (Wu *et al.*, 1997; Lluisma *et al.*, 2001; Manasherob *et al.*, 2002). They were introduced into *Anabaena* PCC 7120 by tri-parental conjugal mating (Elhai & Wolk, 1988). Two clones with pSBJ2 and four with pSBW2 were found toxic to larvae of *A. aegypti*, and then studied as described (Wu *et al.*, 1997). Our study was the first to coexpress several Bti *cry* proteins in this cyanobacterial species, and resulted in clones with the highest toxicities reached so far (LC₅₀ of about 0.3 µg total soluble protein per ml). The high toxicities remained surprisingly stable during three years of constant cultivation, whether under drug selection or not.

The lack of necessity for antibiotic resistant genes is crucial for field applicability of these transgenic strains. Furthermore, we have also developed a sensitive method for tracing the transgenic cyanobacteria after release by gene amplification with several pairs of primers targeted uniquely to each of the Bti toxin genes (Ben-Dov *et al.*, 1997).

The expressed proteins in the transgenic organisms were analyzed by SDS-PAGE and Western hybridization with antisera directed against each of them or against Bti whole crystals. *Cry11Aa* was detected in both strains, but in different quantities, as in *E. coli*. In *Anabaena*, large amounts of *Cry11Aa* were found in both the pellet and supernatant of cell crude extracts, but only trace *Cry4Aa* was detected in the pellet. The abundance of *Cry11Aa* and synergism with trace amount of *Cry4Aa* seem to account for the high toxicity. *p20*, That was readily detected in the three *E. coli* strains carrying *p20*, pHE4-ADR, pHE4-AR and pHE4-DR, in mass ratios of 1:2:10, respectively, was not found in the transgenic *Anabaena*.

Large differences in toxicities exist among the clones expressing mosquitoicidal toxin genes in cyanobacteria (Soltes-Rak *et al.*, 1995; Sangthongpi-tang *et al.*, 1997), from non-toxic to very toxic, by feeding with intact cells with LC₅₀ as high as 10⁵ cells ml⁻¹. The bioassay conditions were varied over a wide range from feeding newly hatched larvae during six days to the third instar larvae for 24 h (as in standard assays). These marked differences in bioactivity and stability among the reported clones can be explained by various reasons as follows. Previous work expressed a single or binary toxin, mostly in unicellular cyanobacteria. The very high bioactivity achieved in our work can thus be due to coexpressing in a filamentous strain two toxin genes that synergize each other (Porter *et al.*, 1993; Poncet *et al.*, 1995). Moreover, the codon usage of Bti's *cry* genes fortuitously resembles that of *Anabaena*.

PCC7120, rather than all those unicellular strains (Wada *et al.*, 1992; Soltes-Rak *et al.*, 1995). The poor expression of Bti *cry* genes in higher plants has indeed greatly been improved by modifying them to match the codon usage of the transgenic plants (Perlak *et al.*, 1991, 1993; van der Salm *et al.*, 1994; van Aarssen *et al.*, 1995). Such codon usage bias may well account for the poor expression of Bti *cry* gene in unicellular cyanobacteria.

32.5 Transformation of indigenous filamentous cyanobacteria and release of genetically modified microorganisms

Laboratory strains such as *Anabaena* sp. PCC 7120, not adapted to natural environments, may be inferior in nature because they are selected against (by competition with indigenous species) too quickly to yield the desired control efficacy. Reintroducing recombinant indigenous species that express cloned *cry* genes into their natural habitat would provide a solution to this problem. As a model organism, we picked the rice field nitrogen fixing isolate *Anabaena siamensis*; a fast growing strain proliferating under adverse conditions prevailing in rice field environments (Boussiba, 1993).

Two major problems are often encountered in establishing a transformation system for a newly isolated strain, restriction endonuclease activities and host range of shuttle plasmids (Boyer, 1971; Moser *et al.*, 1993; Elhai, 1994; Soper & Reddy, 1994). Strong restriction activities in the host often chop foreign incoming DNA before it is established. Endogenous plasmids, found in many strains of cyanobacteria (Houmar & Tandeau de Marsac, 1988), are often not suitable for replication in several strains of high interest, such as *Spirulina* sp. (Vachhani & Vonshak, 1997). New vectors should therefore be constructed for transforming indigenous strains.

The major risks associated with the release of recombinant microorganisms into the environment include unintentional spread of transgenes into other organisms (i.e. through horizontal gene transfer) and adverse ecological impacts of the introduced strains. In the case of insecticidal organisms, the development of resistance among target species is to be seriously considered, but for a number of reasons, these risks are minimal concerning the application in nature of recombinant cyanobacterial strains carrying the Bti's *cry* toxin genes. Firstly, a dried formulation with cells killed during the process can be used. It can be argued that the transgenic organisms carry unaltered Bti toxins, already shown to be highly specific against larvae of mosquito and black flies, and harmless to all non-target organisms tested so far, including other insects, fish, frogs, crustaceans and mammals, not to mention humans (Margalit & Ben-Dov, 2000). Moreover, Bti, hence its *cry* toxins and their encoding genes occur naturally in the environment. These facts, coupled with the observations that no resistance was developed in field populations of mosquitoes and no adverse effects on human or animal health have been detected after more than 15 years of large-scale, worldwide, and extensive field use of Bti (Margalit & Ben-Dov, 2000), further argue for the safety of using recombinant cyanobacterial strains as biocontrol agents.

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33 The Enhancement of Marine Productivity for Climate Stabilization and Food Security

Ian S.F. Jones

33.1 Food from the ocean

The sea is 70% of the surface of the globe but provides only 6% of human protein needs. Can the ocean play a bigger role in providing food security for low income food deficient countries? By combining foreign income from carbon sink credits and fish protein for human consumption, enhancement of the productivity of the oceans may be attractive in the near future. It is now possible to estimate the increase of sustainable fish catch as a result of providing extra nutrients to the open ocean. Such ocean nourishment in selected areas will lead to an increase in new primary production. The implications of increasing by 50% the protein available from the ocean can be examined.

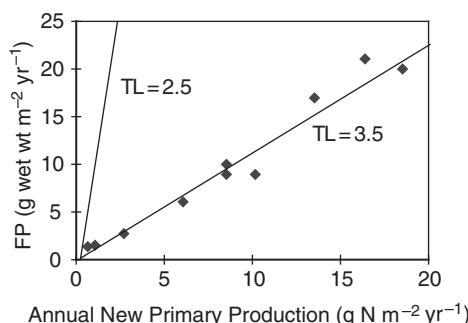
Over the last 25 years the green revolution has increased the global supply of food allowing 2 billion additional people to be fed. Uneven distribution of food, however, means that over 800 million people are hungry each day (World food summit, 1996) and 40% of people suffer from protein deficiencies (FAO, 1992). While this needs to be addressed, an even greater challenge is to provide the food and particularly the protein for the anticipated further 2 billion (Marshall, 1996) that will cover the earth during the next quarter of a century.

Despite a wish for a decreasing world population, the neglect of educational opportunities, particularly for women, means that many of those able to reproduce will not be persuaded to limit reproduction. The more fortunate of us have an ethical duty to contribute to feeding these additional people. The extra food production will inevitably require further change of the environment. Providing the nutrients that are in short supply in the sea, as has been done on the land in the green revolution, may be the alternative to dramatically increasing agricultural production of food.

33.2 The technology

Primary production, on a global average, has a Redfield ratio of C:N:P of 106:16:1 by molecules. If more phytoplankton could be grown by adding 1 t of nitrogen, 5.7 t of phytoplankton carbon would be produced. Some of this nitrogen would be exported from the ocean photic zone and some would be used to generate a secondary crop of phytoplankton. In steady state, this export of carbon is equal to new primary production. The ratio of new primary production to primary production is designated f , and Aufdenkampe *et al.* (2001) found for the tropical Pacific an average f of about 16%. This suggests that globally 1 t of new primary production supports $1 + (1 - f) + (1 - f)^2 + \dots = 1/f = 6.25$ t of primary production per year. Thus, our 1 t of nitrogen would lead to ~ 36 t of primary production. Pauly & Christensen (1995) suggest exchanges between trophic levels are 10% efficient. This is 10% of the available carbon in each trophic level (TL), not of the carbon ingested. Thus TL 2, that is zooplankton, would have 3.6 t of organic carbon as a result of our nitrogen introduction. Clupeids are TL 2.5 as they feed on both phytoplankton and zooplankton. Carnivorous fish are TL 3.5. Our 1 t of nitrogen would give 0.11 t of TL 3.5 fish carbon. As fish are about 11% carbon, Strathmann (1967), we see that our 1 t of reactive nitrogen would support $0.1 \times \sqrt{0.1} \times 6.25 \times 5.7/0.11 = \sim 10$ t wet weight of clupeids or 1 t wet weight of carnivorous fish.

To calibrate these crude calculations, we have taken the observations of carnivorous fish and squid biomass production reported by Iverson (1990) together with the relationship between new primary production and total phytoplankton production. This net production after predators have taken their share is shown in Fig. 33.1. In addition, we have plotted the expected production based on the TL transfer efficiencies discussed above. There is good agreement for the carnivorous fish production and this gives some confidence for the clupeid calculation.



Green2a

Fig. 33.1. Hypothetical fish production at different TLs. (♦) Data from Iverson (1990) for different geographical areas excluding coastal upwelling regions where clupeids are prevalent. New primary production is the flux of nitrogen into the photic zone. Reprinted with permission from Elsevier Science Ltd (Green house Gas Mitigation).

The calculation suggests that the addition to the photic zone of 1 t of reactive nitrogen is associated with 100 t of cupeids and 1 t of TL 3.5 of new fish production. If we were to provide the same amount of nitrogen (100 Mt) to the ocean as we do at present to the 10% of the earth's area used for agriculture, carnivorous fish production could be expected to increase by 100 Mt year⁻¹. If the nourishment was in areas adjacent to present regions of dense clupeid production, the existing stocks might easily colonize the new areas and provide a greater source of marine protein. While only about 25% of the standing stock can be caught for a sustainable fishery, all the additional fish of one age class can eventually be captured (less those taken by predators and those that die) in an ongoing fishery. In the absence of any experience we will take 50% as the capture efficiency. The above would suggest that 100 Mt of reactive nitrogen might increase the carnivorous fish catch by 50%.

Throughout most of the coastal and open ocean, primary production is often limited either by nitrogen or iron (Howarth, 1988). Consumed with 100 Mt of N is about 14 Mt of phosphorous – less than Tiessen (1995) estimates – is introduced annually to the ocean by anthropogenic activities. Although repeated nourishment with only iron and/or nitrogen would eventually deplete the ocean of any excess supply of trace nutrients, it is difficult at present to estimate how long this would take. With increased new primary production comes additional oxygen consumption (Matear & Elliott, 2001) in the deeper ocean. This may place a limit on the desirable increase in productivity. The thermocline water is refreshed by contact with the atmosphere only after some time. Species change might be expected if phytoplankton have reduced phosphate or silicate availability. However, it is unlikely to affect the net export of carbon since in steady state, export equals the new primary production. The connectivity of the ocean ecosystems requires that the introduction of ocean nourishment be combined with careful monitoring. Satellites will increasingly provide a cost effective way of doing this.

Such eco-engineering naturally sparks ethical debates. It involves intervening in the natural order of life. These debates, however, should be a balancing of the rights of extant human beings, to basic needs, with the rights of future generations who may have to cope with a changed environment. Will the risks involved in increasing the sustainable yield of protein from the sea be lower than making the same increase on the land? This issue is developed below.

33.3 Climate stabilization

The UNFCCC is an agreement amongst most of the sovereign nations of the world to slow the rate of climate change by controlling the concentrations of Greenhouse gases in the atmosphere. Strategies to moderate the build up of these gases can be approached either by reducing emissions or increasing the sinks of carbon. Sinks can either permanently sequester the carbon (for thousands of years) or store the carbon for hundreds of years until the supply of fossil fuels are exhausted and alternative energy sources are developed. CO₂ is the most important Greenhouse gas.

The most attractive use of CO₂ in flue gases is for conversion by photosynthesis to food. Much current thinking is concentrated on capture at the

source, transport and sequestration. If storage in the ocean is achieved by increasing productivity there is no need to capture the CO₂ in the flue stack. The atmosphere is a good transporter of CO₂ and despite a geographic clustering of anthropogenic CO₂ sources, the atmospheric concentration of CO₂ varies by only about 5% over the whole of the earth surface. The carbon recycled from the ocean as fish catch is a small fraction of the carbon sequestered. From sequestering can come revenue, since Jones & Caldeira (2003) predicted from modelling that macro nutrient nourishment leads to quasi-permanent sequestration.

Thus, with the concept of ocean nourishment we can address two important issues of our times – food security for the rising population and climate stabilization despite the increasing burning of fossil fuels.

33.4 The ocean nourishment process

33.4.1 Shore-based system

Figure 33.2 shows a conceptual land-based factory fixing nitrogen from the atmosphere and enhancing photosynthesis at the edge of the continental shelf. The alternative of floating ocean nourishment plants has some advantages discussed in Jones & Cappelen-Smith (1999). At the core of the system is the production of the macronutrient, reactive nitrogen. This can be in the form of ammonia (NH₃), synthesized from methane (CH₄). Such a process produces carbon dioxide and this needs to be subtracted from the amount of atmospheric carbon sequestered by the introduction of nitrogen to the upper ocean.

Greenhouse Gas Mitigation Process

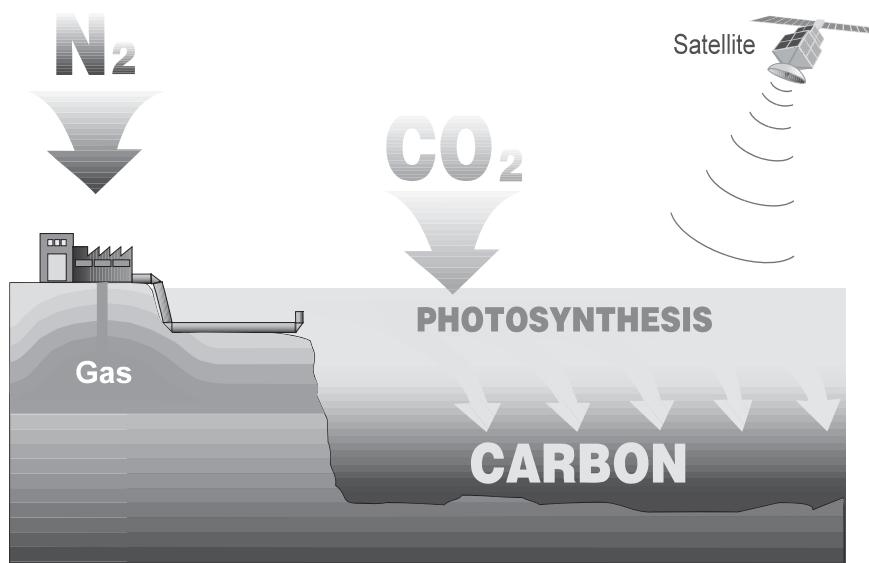


Fig. 33.2. A schematic view of the ocean nourishment scheme.

33.4.2 Costs of fixing and delivering nitrogen

Some properties of a modern nitrogen fixing plant are listed below:

Ammonia production	2000 t d^{-1}
Natural gas consumption	$30 \text{ GJ t}^{-1} \text{NH}_3$
Freshwater consumption	$1000 \text{ Kg t}^{-1} \text{NH}_3$
Electricity consumption	$20 \text{ kWh t}^{-1} \text{NH}_3$
Labor for operation	6 persons shift $^{-1} \times 4$ shifts
Labor for maintenance	6 persons shift $^{-1} \times 4$ shifts
Operating days per year	330
Yearly NH_3 production	660 000 t

The costs in US dollars we have assumed for the construction of a $2000 \text{ t d}^{-1} \text{NH}_3$ plant at a greenfields location are:

Capital cost	\$290 M
Natural gas costs	variable
Other costs per tonne NH_3	\$11.3

There are more details in Shoji & Jones (2001). In addition, the nourishment delivery system has been estimated to cost \$50 M and it can be operated by the same staff who would be involved in the production of NH_3 . Thus we have a total capital cost of \$340 M. It is considered that \$300 M of this could be in the form of non-recourse debt funding and three interest rates are examined, 4%, 6% and 8%. The remaining \$40 M is equity funding which needs a much higher rate of return on a new technology such as ocean nourishment. On a yearly basis the expenses are for 19 800 000 GJ of natural gas, interest on \$300 M debt, and (other) operating costs of \$7.46 M.

The costs incurred in nourishing the ocean could be recovered according to Shoji & Jones (2001) by selling carbon credit sinks produced by the additional new primary production. Firstly, let us assume, the value of the extra fish produced is not captured by the operators of the nourishment plant but provided as a collateral benefit. Using the figure from Jones & Otaegui (1997) of 12 t of CO_2 sequestered per tonne of NH_3 provided, a yearly supply of 7.9 Mt of CO_2 as carbon credits would be provided to the market. The ratio of 12:1 of CO_2 to ammonia assumes a Redfield ratio for phytoplankton and allows for the carbon dioxide produced in the ammonia synthesis process. Jones & Otaegui (1997) used an uptake efficiency of 70% which gives scope for a greater number of credits if operating experience shows a higher efficiency. If this rose to 80% the most favorable example of credits would be about $6.75 \text{ t}^{-1} \text{CO}_2$ avoided. This is a yearly income of \$53.5 M.

It seems highly possible that extra fishing rights could be auctioned as a number of countries have such auction concepts in place. At 70% uptake efficiency, the plant above would produce a sustainable clupeid catch of 3 Mt wet weight year $^{-1}$. If the fish harvested had to sustain all the expense of ocean nourishment, the cost would be in the order of $\text{US\$20 t}^{-1}$ of fish landed. There might be increases in the carnivorous fish and a reduction in fishing costs as well. These plants could be located adjacent to regions of low income

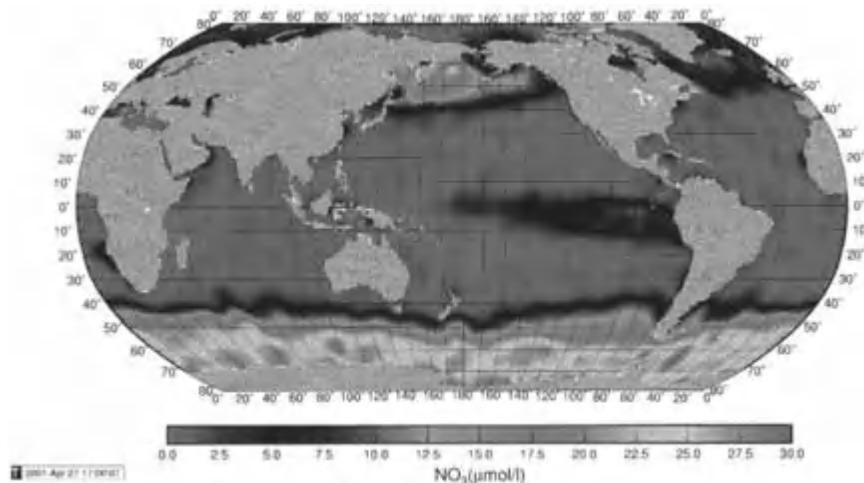


Fig. 33.3. The surface distribution of nitrogen (nitrate) using the World Ocean Data 98. Areas of HNLC can be seen.

food deficient coastal communities. This makes ocean nourishment attractive to the poor (Fig. 33.3).

33.4.3 Injection

The design of the nutrient injection system needs to address the issues of: the density of the injected fluid; the concentration of nutrients; the depth of injection and the volume from any one injection point. Let us assume for the purpose of illustration that the nutrient properties are those of ammonia.

Ammonia in solution (ammonium hydroxide) is less dense than water and so forms a buoyant plume that rises from the injection point. The more injection points used, the more rapid the initial dilution and the decrease in buoyancy. Thus an injection depth should be chosen so that the plume does not break the ocean surface while still concentrated. Otherwise there is the danger of ammonia escaping to the atmosphere. As phytoplankton generally grows fastest in the moderate light levels typical of the middle of the photic zone, this would seem to be a good depth for release. One wishes the concentration of nitrogen in the plume to stay above 1μM for a couple of days so that significant phytoplankton growth can be achieved before dilution and consumption lowers the concentration of nutrients to levels where new primary production is negligible. Ammonia concentration lethal for continuously exposed marine life can be expected in a region near the injection point.

33.5 Phytoplankton growth rates (see Chapter 8)

Large area, i.e. patch fertilization, has been demonstrated in the IRONEX (Coale *et al.*, 1996) experiments. It is possible to model the response of the

phytoplankton to additional nutrients. If the nutrients are introduced over a large area, patch fertilization, the phytoplankton increase is modified by reducing nutrient concentration and predation by zooplankton and other predators. When the nutrients are introduced into a current from a fixed injection point, the dilution of the nutrients by diffusion must also be modelled.

However, modelling higher TLs of the ocean ecosystem such as zooplankton or clupeids is more challenging because our understanding of the processes is still limited. The hypothesis used here is that while the average biomass of clupeids varies from year to year due to unknown causes, the fattening of the fishery is limited by the availability of food. Thus providing additional phytoplankton outside the existing upwelling times or in areas adjacent to upwelling regions will increase the available fish stock.

In some areas of the ocean, the addition of the micronutrient iron will increase phytoplankton concentrations. Practical use of the high nutrient low chlorophyll (HNLC) area have been proposed by Makels & Barber (2001) and while this paper concentrates on carbon sequestration, other discussions in the press and in patent applications talk about the enhancement of the marine fisheries.

Jones (2001a,b) looks at some of the impacts of widespread use of ocean nourishment and the requirements for energy and area. Proposed experiments in the Sulu Sea to monitor the impacts of macronutrient additions are described by Young & Gunuratnam (1996) whereas the technical issues are addressed in Jones & Otaegui (1997). The roles ocean nourishment might have in providing both income and food to developing countries were investigated. Matear & Elliott (2001) model the efficiency with which the addition of macronutrients sequester carbon from the atmosphere and found them in the range estimated by Shoji & Jones (2001).

33.6 Carbon sequestration

Our hypothetical ocean nourishment plant will sequester 8 Mt of CO₂ per year⁻¹. In this calculation we have assumed that carbon chemistry of the water does not change in the process of adding nutrients and the generation of organic carbon by phytoplankton. The partial pressure of carbon dioxide in seawater depends upon the pH and concentration of dissolved organic carbon (DIC). Alternatively it can be expressed in terms of DIC and alkalinity. The additional nutrients can change the pH and this needs to be considered when choosing the form of reactive nitrogen provided.

There are a number of organisms that incorporate calcium carbonate in their bodies. The most important may be coccolithophorids which form intensive blooms extending over large parts of the subpolar ocean. They utilize the reaction



This process involves the uptake of 2 moles of alkalinity (2HCO₃⁻) and one mole of DIC and so cause a shift in the carbonate equilibrium in the upper ocean, such that the partial pressure of carbon dioxide initially rises. Some of the implications are reviewed in Zondervan *et al.* (2001) but for our purposes

the effect is a reduction in the apparent efficiency of carbon dioxide sequestration as a result of ocean nourishment. For the present ocean in equilibrium with the atmosphere, 1 mole of C incorporated into CaCO_3 releases approximately 0.7 moles of CO_2 due to changes in total alkalinity. For *E. huxleyi* while it is incorporating one mole of carbon into its carbonate shell, it is converting about 1.1 moles of inorganic carbon into organic carbon (POC). Overall the efficiency of sequestration for *E. huxleyi* is 1.1 mole of organic carbon plus 1 mole of CaCO_3 less 0.7 mole of CO_2 released. This is a net uptake of 1.4 moles of carbon. The sequestration efficiency is $(2.1 - 0.7)/2.1 = 0.66$. Restating, if ocean nourishment induced 1 gm of C as *E. huxleyi*, it would only be equivalent to 0.66 gm of carbon not being released into the atmosphere. It is obvious that one would try and avoid areas or conditions that encourage organisms that use calcium carbonate in their structures.

33.7 Legal and societal issues

33.7.1 Legal constraints

If the operations are confined to the exclusive economic zone (EEZ) of a sovereign state, the Law of the Sea would apply to ocean nourishment activities. The legal framework in which carbon credits can be traded and thus provide the resources to fund ocean nourishment would seem to be covered by the UNFCCC. The credit trading option however has its critics, including the Group of 77 & China alliance.

If the new fisheries created extend into the ocean commons, we have little international law regulating such activities. The London convention, signed in 1972, endeavors to manage the dumping of pollutants on the high seas. The purposeful introduction of nutrients would not seem to fall under the definition of dumping. Dumping as defined under the Convention does not include the placement of matter in the sea for purposes other than mere disposal.

33.7.2 Societal issues

The widespread introduction of ocean nourishment would impact on society and would need to take into account societal values. Historically, there has been resistance to innovation and so it can be expected that there will be opposition to a new method of food production.

Genetically modified food is now in the firing line. Often campaigns will be mounted against even the testing of new technologies on the argument that such demonstrations encourage their premature implementation. Such attitudes impede progress.

The malnutrition of a large number of people could be addressed by those in the developed world foregoing high-level animal protein from their diet, such as pork and beef. A vegetarian diet could feed the world population and not make such ecological demands on the land. While resources are unevenly distributed throughout the world, however, affluent countries will use these resources for the products that best suit their taste and cultures.

While ocean nourishment will undoubtedly improve the common good by providing more economical protein, it may impact on particular groups who are relying on some niche in the ecosystem. For example, a particular species of fish, the target of one group of fishermen may be unable to compete with the increased clupeids that may prosper under an ocean nourishment regime. Compensation may be necessary.

At a more general level there are those who proclaim that it is presumptuous to attempt to manage nature by processes such as ocean nourishment. Others say we have despoiled the land and shown that we do not have the skills to manage the environment and therefore should not start interfering with the ocean – the last pristine wilderness. Of course the ocean is not the last pristine wilderness, as unmanaged fishing and whaling have already had a significant impact on the ecology of the ocean. Carbon dioxide, as well, is invading the upper ocean because of fossil fuel burning and changing its acidity.

Mankind's interventions in nature have involved many mistakes in the past due to lack of knowledge but slowly many of these mistakes are being corrected. Initially, when farmers first ploughed the land in straight lines they made a fivefold gain in produce until erosion washed away the soil. Now contour ploughing is used to halt the erosion. It is hard to avoid unforeseen consequences in manipulations of nature but an active management of nature demands that we stand ready to adjust our activity in the light of experience. To provide the protein for the extra people, the ocean, being 70% of the surface of the globe, can be used more prudently rather than more intensively using the land.

33.7.3 Public outreach

Liberal democracies require that the public be informed of the issues involved in experimenting on the increase of ocean productivity. The public has the right and the ability to influence policy that constrains technical solutions. Projects like ocean nourishment need to address these areas and it may be as important as solving the technical problems.

Public policy is influenced by small groups in society and some of these are hostile to the exploitation of the ocean. While their concerns may be insensitive to the needs of the poor illiterate farmers and fishermen of developing countries, they have considerable influence in making policy. The malnutrition of millions is not given the priority it deserves.

33.8 Conclusions

The idea of free-range enhanced fish stocks adjacent to areas of rapidly rising populations is attractive. It differs from that of current aquaculture that must concentrate on the high-value species for the affluent of the world because of the intensity of capital and labour required. Ocean nourishment is a concept to both sequester carbon and in the same process produce extra fish resources at low cost for the rapidly rising population. Policy makers have the challenge ahead of them to help eliminate poverty and hunger amongst all people.

Public perception of new technologies can influence their adoption. Geo-engineering projects on the scale of climate management involve unfamiliar and untested concepts. The ocean *commons* hold a special place in the minds of some people and they are likely to oppose new uses of the ocean such as an enhanced carbon sink. A holistic view of new technologies tries to balance the risks and the benefits because new ideas must be introduced for mankind to advance. These societal issues may be as important as the technical constraints in creating a major sink of carbon dioxide in the ocean.

The major determinates of the costs of ocean nourishment are the interest that must be paid on the capital the cost of the feedstock, natural gas. We have used for discussion purposes interest between 4 and 8% per annum and natural gas costs of US\$0.5 to \$2 GJ⁻¹. If we assume no income from fisheries enhancement, concessional loans for a green project in a developing country and natural gas prices of \$1.20 GJ⁻¹, the selling price of \$8.70 t⁻¹ of carbon dioxide (avoided) returns about 40% per annum to the private investor. If a rate of return of 30% per annum was deemed suitable, the price of carbon credits could be reduced by about \$0.50 t⁻¹.

Fishing quota licences on the other hand, in the absence of any carbon credits, could be issued for costs less than \$100 t⁻¹ of fish. This estimate is more uncertain than the carbon credit costs.

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