

Parte C: DOCUMENTO CIENTÍFICO**C.1. PROPUESTA CIENTÍFICA****State of the art**

The anthropogenic emissions of greenhouse gases are producing an accelerated global warming of the planet, posing one of the greatest scientific challenges of the last decades. According to the last IPCC assessment report (IPCC AR5, 2013) the global surface temperature rise is likely to exceed 1.5°C by 2100, and may reach 4.8°C in the worst predicted scenario. Most of the heat accumulates in the ocean, particularly in surface waters, which are predicted to undergo a 0.6 to 2°C increase by the end of this century. There is compelling evidence that the present rate of ocean warming will have far reaching ecological consequences, from individual organisms to the ecosystem level in the next decades. Changes in distribution and timing of annual events such as migration and reproduction of different species have been already observed during the last century in association with climate change (Hoegh-Guldberg & Bruno 2010, Poloczanska et al. 2013). At the biogeochemical level, a reduction of global primary production has been projected, which can be propagated to secondary producers leading to a reduced capacity of oceans to regulate climate through the biological carbon pump (Chust et al. 2014). Thus, obtaining an in-depth understanding of the effects of increasing temperature on oceanic organisms takes new urgency.

The sensitivity of marine microorganisms to global warming is in the spotlight due to their fundamental role in ecosystem functioning. Microbes mediate most of the transformation of major elements on Earth such as carbon (C), nitrogen (N), phosphorus (P) or sulphur (S) (Falkowski et al. 2008), and participate in climate regulating processes such as the dynamics of greenhouse gases and the formation of clouds (Simó & Dachs 2002). Furthermore, oceanic microbes are found in enormous abundances in seawater (up to millions of cells per mL), and their functional performance has a global impact. For example, half of Earth's photosynthesis is mediated by marine phytoplankton (Field et al. 1998), and the CO₂ fixed by this process is rapidly remineralized by a large number of heterotrophic bacterial taxa. Only a small amount of carbon escapes this degradation and is exported to the deep ocean, where it remains immobilized for hundreds to thousands of years. Understanding how temperature will affect the coupling between these two processes (primary production/microbial respiration) is therefore crucial to understand whether the ocean can act as a source or sink of CO₂, and the capability of plankton to mitigate climate change effects. Effects of temperature on phytoplankton size-distribution (Morán et al. 2010) and changes in the timing of phytoplankton blooms have been reported, but the effects on bacterioplankton, a major contributor to oceanic respiration, are poorly understood (López-Urrutia and Morán 2007, Vázquez-Domínguez et al. 2007). In general, the effects of temperature on a myriad of metabolic processes carried out by marine microorganisms have been unexplored.

The recent development of Omics approaches is providing an invaluable 'tool box' to study the physiology and metabolic capabilities of microorganisms in the environment. In particular, the fields of genomics, transcriptomics and proteomics focus on the analysis of the collection of genes, messenger RNA transcripts, and proteins found in these organisms. The application of high-throughput technologies has reduced the cost of DNA sequencing by orders of magnitude and allows the development of more ambitious projects targeting the metabolic performance of organisms or even communities at a greater scale. Such advances are rapidly changing research directions in Environmental Microbiology. Before the Omics age, it was typical to have only limited information on the activities of cultured microorganisms, such as growth curves or range of compounds utilized. With these new approaches, the molecular basis that drives the cellular functioning of these organisms can be addressed (e.g., Paloovara et al. 2014). Moreover, these approaches are setting the path to new discoveries when applied to environmental samples where microorganisms are extremely difficult to culture. The first metagenomic analysis of marine samples (within the Global Ocean Sampling Initiative) unveiled some of the most important discoveries in microbial oceanography in the recent years, such as the high abundance of proteorhodopsins in surface waters (i.e., molecules that drive energy production in non-photosynthetic bacteria, Venter et al. 2004). Novel metabolic pathways sustaining the growth of widespread marine microbes have been also unveiled by this approach (e.g., Alonso-Sáez et al., 2012). In marine systems, Omics approaches have been used in experimental

settings too in order to understand changes in gene expression of microbial isolates or communities induced by perturbations, such as the addition of particular substrates or altered CO₂ conditions (Vila-Costa et al. 2010). However, the direct effect of rising temperatures on the regulation of microbial metabolic pathways is poorly understood.

As microorganisms respond to changing environmental conditions largely through changes in gene expression, the study of transcript inventories (transcriptomics) is a very sensitive window into the environmental cues perceived by the microorganisms and their physiological adaptations. For example, significant changes in gene expression along the diel cycle have been reported for the N₂ fixing cyanobacterium *Crocospaera watsoni* (Shi et al. 2010) or marine coastal bacterial communities (Poretsky et al. 2009). In a global warming context, understanding how temperature will impact the metabolism of marine microorganisms at the molecular level receives particular attention. Effects of thermal stress on gene expression have been studied in terrestrial and laboratory model organisms using microarrays or transcriptomics (Hoffman 2005, van der Linden et al. 2010). Although some gene expression patterns are expected (e.g. the upregulation of heat shock protein genes in response to heat stress), a large number of potentially sensitive metabolic pathways can be only detected by examining whole expression patterns by transcriptomics. A recent metatranscriptomic study focusing on marine eukaryotic phytoplankton along a latitudinal gradient found a relationship between temperature and the expression of genes involved in the synthesis of phosphate-rich ribosomes. These results indicate that phytoplankton modify the allocation of cellular resources (such as N and P) under different temperature regimes, with strong biogeochemical implications (Toseland et al. 2013). In the case of marine bacterioplankton, despite their key role in different biogeochemical processes, there have not been any studies analysing the effect of temperature on their patterns of gene expression. The acquisition of this knowledge would crucially help to establish hypotheses about the different bacterial physiological aspects that will vary in response to temperature, and provide insights into the functional performance of different bacterial taxa in a warmer ocean.

Along with a better understanding of the physiological response of microorganisms to temperature, there is a basic need to put together this knowledge in a theoretical framework, which may eventually allow us to make predictions. It has been long known that temperature, together with body size, and resource availability are three primary factors controlling metabolic rates. The Metabolic Theory of Ecology (MTE) has emerged as a unifying theory that provides a quantitative framework to better understand how temperature and body size combine to affect metabolic rate, and how metabolic rate, in turn, influences the regulation of ecosystem metabolism (Brown et al. 2004). The MTE predicts that metabolic rates will be proportional to the size of the organism in $3/4$ power relationship ($M^{3/4}$, West et al. 1997) and exponentially to temperature, as characterized by the Boltzmann-Arrhenius term $e^{-E_k/T}$, where T is the temperature, E is the average activation energy (measured in eV), and k is Boltzmann's constant (Gillooly et al. 2006). Such exponential increase in growth rates only applies to a range of temperatures where each species is under normal growth conditions, as below or above this range there is a sharp reduction of metabolic activity and growth. Thus, the thermal niche of marine planktonic species is usually unimodal and negatively skewed, and the characterisation of these thermal tolerance curves is fundamental for our understanding of marine plankton distribution and the effect of temperature on community diversity patterns (Thomas et al. 2012).

In an ecological context, initial applications of the MTE model have focused on testing whether complex processes such as community metabolic rates or biodiversity patterns exhibit a temperature dependence with the same central value of activation energy (usually 0.65 eV). When the temperature response of different organisms is analysed in detail, a spread of activation energies has been observed around this central value, with systematic deviations having biologically meaningful interpretations (Dell et al. 2011, Delong et al. 2010). Yet, basic predictions of the MTE hold across a surprisingly large range of organism sizes, from the smallest microbial cells to metazoans. Overall, this theory represents a very powerful theoretical framework to predict changes in ecosystem functioning under global warming scenarios. For example, based on different activation energies of the key enzymes for photosynthesis (Rubisco) and respiration (ATP), the balance of primary production and respiration has been projected by MTE in warming conditions (Allen et al. 2005). The MTE has only recently started

to be applied in oceanic environments (Duarte 2007), mainly to derive relationships between phytoplankton cell-size, biomass and primary productivity (Li et al. 2006, Morán et al. 2010) and make predictions on the metabolic balance in a warmer ocean (López-Urrutia et al. 2006).

The MTE proposes that the Boltzmann-Arrhenius model, derived from chemical reaction kinetics, can be used to systematically predict the rise of many biological processes. So far, this theory has been applied at the organism, population and community levels, but it has been rarely applied at the molecular level. A remarkable example is the finding that rates of DNA substitutions increase with temperature following MTE predictions (Gillooly et al 2005). Whether changes in the production of gene transcripts of different pathways induced by temperature also follow the predictions of the MTE is completely unknown. Obtaining in-depth understanding of which metabolic pathways are more sensitive to warming in microbial individuals, and to what extent changes in the expression of some of these pathways quantitatively fit in the MTE model will open a new venue for integrating transcriptional data, which could be routinely obtained in marine samplings in a close future, in predictions of a warmer ocean functioning.

Previous results of the Research Group

This project builds on the previous results of the research project COMITE “Coastal Ocean Microbes and TEmpérature” (2010-2013), coordinated by X.A.G. Morán, and where several members of the Research Team were also involved (L. Alonso-Sáez, T. Huete-Stauffer, and N. Arandia). The project COMITE aimed at understanding the effect of temperature on different aspects of the metabolism of marine bacterioplankton in the framework of the MTE. The general objective was to study the relationships between the bacterial cell size, abundance, biomass, growth rates, and transformations of organic carbon modulated by temperature. The first part of the project included a retrospective analysis of a 10 years time-series of bacterial abundance and biomass in the Southern Bay of Biscay, with the aim of finding macroecological patterns of bacterial variables in relation with temperature. We found that coastal bacteria are already responding to the hypothesized temperature-driven decrease in body size, with a significant linear trend of increasing standing stocks in low-nucleic-acid content bacteria (5% year⁻¹, as estimated by flow cytometry) accompanied by decreasing cell sizes (-1% year⁻¹, Morán et al., under review in Proceedings of the Royal Society B).

The second part of the project COMITE included experimental work over two seasonal cycles where the effects of temperature on bacterial standing stocks and growth rates was tested. A marked seasonal pattern of bacterial biomass and growth rates within the environmental temperature range (12.7 to 21.2°C) was found, and the activation energy for the growth of marine bacteria was on average 0.53 ± 0.05 eV, slightly below the mean value proposed by the MTE (0.65 eV, Huete-Stauffer et al., in prep). A concurrent positive association of E with chlorophyll (as a proxy of trophic state) suggested that increases in growth rate due to increasing temperatures in the future ocean will be strongly dependent on the availability of substrates for bacterial growth and metabolism, as hinted previously (López-Urrutia and Morán 2007). A more targeted analysis on specific bacterial taxa (using Fluorescence In Situ Hybridization techniques) showed different temperature dependence in the growth of distinct bacterial phylogenetic groups. The average activation energy of one of the most abundant bacterial clades in the ocean (SAR11) was the closest to the theoretical value (0.65 eV), higher than that of other groups (Bacteroidetes or Gammaproteobacteria, Arandia et al. in prep). Finally, changes in functional diversity (defined as the number of substrates utilized by bacterial communities using BIOLOG Ecoplates) were also observed in response to increasing temperatures, indicating that new functional capabilities are expressed by marine bacteria under warming conditions (Alonso-Sáez et al., in prep).

In summary, we found evidence of ongoing long-term changes in biomass and cell-size of marine bacteria according with the temperature-size rule, average activation energies for bacterial growth in the range of predictions of the MTE, and different temperature dependencies for distinct bacterial taxa in the framework of the previous project COMITE. TECCAM will focus on the next challenging step of examining the temperature dependence of marine bacterioplankton at the molecular level, focusing on gene expression patterns by transcriptomics. With this new approach, we will test the temperature sensitivity of a myriad of metabolic functions carried out by marine microorganisms, and test whether gene expression patterns follow the predictions of the MTE. In order to achieve the objectives of the

project, we will establish a collaboration between the researchers in AZTI and IEO with the Research Professors X.A.G Morán and X. Irigoien, presently at KAUST University (South Arabia).

Hypothesis and general objectives

Temperature affects all biological processes, from enzyme kinetics to metabolic rates. However, while gene expression is the first process involved in the modulation of cells functioning, the impact of temperature on transcriptional changes of most marine microorganisms is largely unknown. An underlying assumption of TECCAM is that temperature has direct effects at the transcriptional level in bacterial cells. In some organisms, temperature appears to produce changes mainly in the rate of processes, rather than de novo synthesis of enzymes (Rabus et al. 2002). Yet, there are compelling evidences that temperature can affect the differential expression of relevant pathways across all domains of life, such as the generation of secondary metabolites in fungi (O'Brian et al. 2007), changes in membrane composition in bacteria (van de Vossenberg et al. 1999), the utilization of specific carbon compounds in marine microbial communities (Alonso-Sáez et al., in prep), or mechanisms of virulence in pathogens such as adhesion, chemotaxis, and production of toxins (Banin et al. 2000).

The **two hypotheses** that will be specifically tested in TECCAM are:

1. Marine model bacterial isolates acclimated to different temperatures will present different transcriptional metabolic maps in direct response to temperature. In other words, temperature acclimation will produce changes in the expression of selected metabolic pathways, which will be traduced in phenotypic changes of these microorganisms.
2. Some of the transcriptional changes occurring at the cellular level under warming conditions show a systematic change with temperature, and can therefore be integrated, and eventually predicted, in the framework of the MTE.

This project aims at providing answers to general questions such as:

- What types of transcriptional changes in the global network of metabolic pathways are needed to achieve thermal acclimation in different bacterial isolates?
- What metabolic pathways are up/downregulated under increasing temperatures, including conditions of thermal stress?
- Can temperature be a valid predictor of changes in the expression of selected metabolic pathways involved in the transformation of C, N, P or S in marine microorganisms?
- Can we identify which metabolic pathways explain the decrease in fitness of marine microorganisms undergoing thermal stress?
- Can we quantitatively correlate transcriptional changes with metabolic rates (e.g., growth rates) of temperature acclimated microorganisms?

The **two general objectives** of TECCAM are:

1. To compare global patterns of gene expression of autotrophic and heterotrophic marine bacteria over an increasing range of temperatures, in order to understand the molecular changes that microbial cells undergo in response to warming and thermal stress, and how increases in temperature may affect their transformations in the C, N, P and S cycles.
2. To analyse quantitative changes in the production of gene transcripts involved in different metabolic pathways in response to warming in the theoretical framework of MTE, which will allow us to make predictions of the effect of temperature in the expression of metabolic pathways in a warming scenario.

Appropriateness of the project to the Spanish Strategy Plan for Science, Technology and Innovation, Horizon 2020 or any other national or international strategy for R & D + i.

The assessment of how global warming will affect natural ecosystems requires urgent action, and is one of the most important research areas in National and International Research Strategy plans.