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EFEITOS DA RADIAÇÃO SOLAR E ULTRAVIOLETA NO BACTERIONEUSTON

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palavras-chave

Radiação solar, radiação ultravioleta (RUV), microcamada superficial (MCS), água subjacente (AS), bacterioneuston, bacterioplâncton, metabolismo heterotrófico, diversidade filogenética procariótica, matéria orgânica dissolvida (MOD)

resumo

Apesar de desempenharem um papel fundamental nos fluxos de energia e de matéria através das redes tróficas aquáticas, os microrganismos são mais susceptíveis aos efeitos da RUV devido às suas reduzidas dimensões.

Dada a sua localização, a SML pode ser considerada como um nicho ecológico modelo para o estudo dos efeitos da radiação solar, particularmente to tipo UV-B, podendo potencialmente ajudar a obter mais informações sobre os efeitos do aumento dos níveis de RUV-B à superfície da Terra nas redes tróficas aquáticas.

Foi objectivo deste trabalho inferir o impacto da radiação solar e da RUV-B nas actividades metabólicas e diversidade do bacterioneuston e bacterioplâncton, através de estudos diários *in situ* e de microcosmos em laboratório. Para tal usaram-se como descritores do metabolismo heterotrófico bacteriano as taxas de actividade ectoenzimática bacteriana (leucina-aminopeptidase, β-glucosidase, lipase, fosfatase, β-glucosidase e sulfatase) e as taxas de incorporação de monómeros (acetato, glucose, leucina). A análise da composição das comunidades bacterianas foi feita por DGGE (Denaturing Gradient Gel Electrophoresis).

Através das experiências *in situ* não foi possível detectar padrões diários consistentes de actividade e diversidade bacterianas, demonstrando a dificuldade do estudo de ciclos dia-noite em áreas costeiras, onde relações complexas entre a MOD e as bactérias, bem como de ciclos tidais, dificulta a determinação dos efeitos directos da radiação solar. Apenas um reduzido número de diferenças estatisticamente significativas entre as taxas de actividade heterotrófica no bacterioneuston e no bacterioplâncton foram observadas. Para além disso, a observação de fortes correlações entre os níveis de actividade e diversidade no bacterioneuston e bacterioplâncton permite sugerir que a comunidade microbiana da microcamada superficial tem origem nas águas subsuperficiais, acumulando-se à superfície através de processos físicos.

Experiências de microcosmos usando radiação artificial UV-B revelaram forte inibição das actividades bacterianas aquando da exposição, tanto para o bacterioneuston como para o bacterioplâncton. Em geral, as taxas de inibição observadas foram similares em ambas as comunidades. Em alguns casos foi ainda possível observar a estimulação das actividades metabólicas pela RUV-B, mais substancial no bacterioneuston, possivelmente resultante de efeitos positivos indirectos da radiação UV-B na labilidade da MOD. A análise da composição das comunidades verificar a ocorrência de diferentes respostas nas duas comunidades, sugerindo uma possível adaptação do bacterioneuston a elevadas intensidades de RUV-B.

resumo

A comparação dos efeitos da RUV nas actividades metabólicas das comunidades bacterianas estuarinas e dulçaquícolas permitiu ainda verificar um impacto negativo mais acentuado da radiação nas amostras de água doce. Isto sugere que os ecossistemas dulçaquícolas poderão ser particularmente vulneráveis ao efeito do aumento da RUV associado à deplecção da camada do ozono.

keywords

Solar radiation, ultraviolet radiation (UVR), surface microlayer (SML), underlying waters (UW), bacterioneuston, bacterioplankton, heterotrophic metabolism, phylogenetic prokaryote diversity, dissolved organic matter (DOM)

resumo

Playing a crucial role in the fluxes of energy and matter through aquatic food webs aquatic microorganisms are more susceptible to the effects of UVR as a result of their small size.

Due to its location, the SML can be considered a model ecological niche to the study of the effects of solar, in particular UV-B radiation, with the potential to help gaining insights about the effects of increased UV-B radiation reaching the Earth as a results of ozone depletion on aquatic trophic webs.

The aim of this work was to infer about the impact of solar radiation and artificial UV-B radiation on the metabolic activities and diversity of bacterioneuston and bacterioplankton from the study of *in situ* diel cycles and laboratorial microcosms. For that, we used as descriptors of bacterial heterotrophic metabolism, the rates of bacterial ectoenzyme activity (leucine-aminopeptidase, β-glucosidase, lipase, phosphatase, β-glucosidase and sulphatase) and monomer incorporation (acetate, glucose, leucine).

Consistent diel patterns of bacterial activity and diversity were not detected from *in situ* experiments, demonstrating the difficulty of assessing day-night cycles in coastal areas, where complex relations between DOM and bacteria, as well as the occurrence of tidal cycles, hamper the assessment of the direct effects of solar radiation. Only a small number of statistically significant differences were found between heterotrophic activity rates in bacterioneuston and bacterioplankton. Furthermore, the occurence of strong correlations between activity levels and diversity in bacterioneuston and bacterioplankton suggests that the microbial community at the microlayer could be originated at the underlying waters, accumulating at the surface due to physical processes.

Microcosm experiments using artificial UV-B radiation sources revealed strong and similar inhibition of bacterial activities upon exposure, for bacterioneuston and bacterioplankton. However, in some cases it was also possible to observe stimulation of bacterial activities by UV-B radiation, slightly higher for bacterioneuston, possibly as a result of indirect positive effects of UV-B on DOM lability. The variation of the bacterial diversity during the irradiation period showed differential responses of bacterioneuston and bacterioplankton, being the subsurface community much more affected, thus suggesting a possible adaptation of bacterioneuston to high intensities of UV-B radiation.

The comparison of the effects of UVR on the metabolic activities of estuarine and freshwater bacterial communities also allowed verifying a stronger negative impact of radiation in the samples from the freshwater site. This suggests that freshwater ecosystems may be particularly vulnerable to the effects of increased RUV associated with ozone depletion.

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LIST OF ACRONYMS AND ABBREVIATIONS

μL	Microliter
μm	micrometer
μM	Micromolar
ANOVA	Analysis of Variance
ATP	Adenosine triposphate
cAMP	Cyclic adenosine monophosphate
CDOM	Coloured dissolved organic matter
CFC	Chlorofluorcarbons
Chl	Chlorophyll
CO	Carbon monoxide
CO_2	Carbon dioxide
CPD	Cyclobutane pyrimidine dimer
Da	Dalton
DAPI	4', 6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
EDTA	Ethylenediaminetetraacetic acid
EF	Enrichement factor
FISH	Fluorescent in situ hybridization
H_2O_2	Hydrogen peroxide
Hm	Ectoenzyme activity rate
Hm	Maximum hydrolysis rate
L	Liter
LMW	Low molecular weight
m	Meter
MAAs	Mycosporine-like amino acids
MCA	7-amino-4-metilcoumarone
mL	Milliliter
MUF	4-methyl-umbelliferone
NaCl	Sodium chloride
NER	Nucleotide excision repair
nm	Nanometer
nM	Nanomolar
nmol	Nanomol
O_2	Molecular oxygen
O_3	Ozone

OTU	Operational taxonomical unit
PAH	Polyaromatic hydrocarbon
PAR	Photosynthetically active radiation
PBS	Phosphate buffer system
PCR	Polymerase Chain Reaction
PER	Photoenzymatic repair
POC	Partciulate organic carbon
POC	Particulate organic carbon
R	Correlation coeficient
ROS	Reactive oxygen species
SDS	Sodium Dodecyl Sulphate
SML	Surface microlayer
TBN	Total bacterial number
UV	Ultraviolet
UVR	Ultraviolet radiation
UW	Underlying water
V	Volt
V/v	Volume/volume
Vm	Monomer incorporation rate
Vm	Maximum incorporation rate
W	Watt
W/v	Weight/volume

CHAPTER 1

INTRODUCTION

Environmental exposure to solar radiation has been an integral part of the evolution of many species and communities of terrestrial and aquatic organisms. However, anthropogenic induced stratospheric ozone depletion caused by the release of large amounts of organohalogen compounds, especially chlorofluorocarbons (CFCs) and bromofluorocarbons, have resulted in an increase in the intensity of UVR reaching the surface of the Earth during the last three decades (Blumthaler and Ambach, 1990; Helbling and Zagarese, 2003).

The discovery of the ozone hole, initially over Antarctica, prompted investigations concerning the impacts of UVR on phytoplankton, owe to their importance in primary production. However, after several years of investigation, it is now clear that UV radiation also exerts a significant impact on the microbial loop, at the basis of the aquatic trophic net, and beyond on higher trophic levels (Cotner and Biddanda, 2002; Alemanni et al, 2003; Häder et al, 2007). Solar UV radiation results in reduced productivity, affects reproduction and development, and may be involved in the increase in the mutation rate of phytoplankton, macroalgae, reproductive stages of fish and other aquatic animals. This decreased productivity is expected to result in negative effects on species diversity, ecosystem stability and on the interactions between different trophic levels. Ultimately, global biogeochemical cycles can also be impacted (Häder et al, 2007). Decreased productivity can also result in the reduction of the oceanic sink capacity for atmospheric carbon dioxide, one of the main green-house gases that have been associated with global warming and climate change (Zepp et al, 2007). Accumulating evidence also implicates it in changes in zooplankton community composition (Marinone et al, 2006), reductions in amphibian population abundance and diversity (Blaustein et al, 2003), coral bleaching syndrome (Hallock et al, 2004), and increased incidence of human skin cancer and other diseases (Russel-Jones, 1992).

Thus, ultraviolet radiation represents today an important stressor that has already begun to globally influence ecological systems, and even man.

Covering over 70% of the Earth, the surface microlayer, located at the interface between the atmosphere and the hydrosphere, is naturally exposed to high levels of UV radiation (Hardy, 1982). However, evidence of increased abundance and activity rates of microorganisms at the water surface suggest that life in this compartment might have adapted to cope with UV-induced stress (Agogué et al, 2005b). Early investigations suggested that the high proportion of pigmented bacteria at the microlayer could indicate a protective role of pigmentation (Maki, 1980; Norkrans, 1980; Hermansson et al, 1987). Efficient DNA repair mechanisms in the surface communities have also been indicated as a possible explanation to the high activity rates at the microlayer (Agogué et al, 2005b). Yet, the information on the effects of solar and artificial UV radiation on the metabolic activities of bacterioneuston is still scarce, while the predominant DNA repair mechanism in these communities are virtually unknown.

ULTRAVIOLET RADIATION AND THE OZONE LAYER

The ultraviolet region of the electromagnetic spectrum can be subdivided into three ranges termed UV-A, UV-B and UV-C. These subdivisions are somehow arbitrary, and differ depending on the discipline involved. However, environmental photobiologists usually consider the wavelength regions as: UV-A, 400-320 nm; UV-B, 320-280 nm; and UV-C, 280-100 nm (Henderson, 1977).

Shorter wavelengths (in the UV-C range) are unlikely to reach the Earth's surface, except at high altitudes, being almost completely absorbed by the ozone layer and thus have little biological significance (Henderson, 1977).

Radiation in the UV-B spectra can penetrate the ozone layer in the stratosphere. Consequently, decreases in the amount of total-column ozone at the atmosphere, result in the increased incidence of solar UV-B radiation reaching the Earth's surface. UV-B radiation is the most energetic component of sunlight that arrives at the surface, showing profound effects on plants, microorganisms, materials, and air quality (Tevini, 1993). UV-B is also the main responsible for the biophysical effects on human body as a result of cumulative exposure to sunlight, with consequences including minor effects that range from suntan and sunburn, to major effects such as photoaging and skin cancer (de Gruijl and van der Leun, 2000).

The longest wavelengths of UV-A can also penetrate the ozone layer in the stratosphere as well as through window glass, though with minimum effects to the skin if the exposure is reasonable. UV-A rays can, however, result in skin erythema, and tanning. Prolonged, continuous exposure can also produce skin aging and malignant changes (Oumeish, 1998).

Ozone (O_3) is created by the dissociation of oxygen (O_2) by short wavelength UVR (λ < 242 nm) in the stratosphere at altitudes between about 25 and 100 km, into individual oxygen atoms (atomic oxygen). The resulting atomic oxygen then combines with unbroken O_2 to create ozone (O_3), an unstable molecule that upon absorption of UV radiation with a wavelength up to about 320 is split back into a molecule of O_2 and an atom of atomic oxygen. These consequential processes result in the ozone-oxygen cycle (Figure 1.1), that at 10 to 50 km above the Earth's surface creates the ozone layer in the stratosphere. It is the dissociation of O_3 by UVR that prevents ultraviolet region of the electromagnetic spectrum with a wavelengths >290 nm from reaching the Earth's surface (Chapman, 1930).

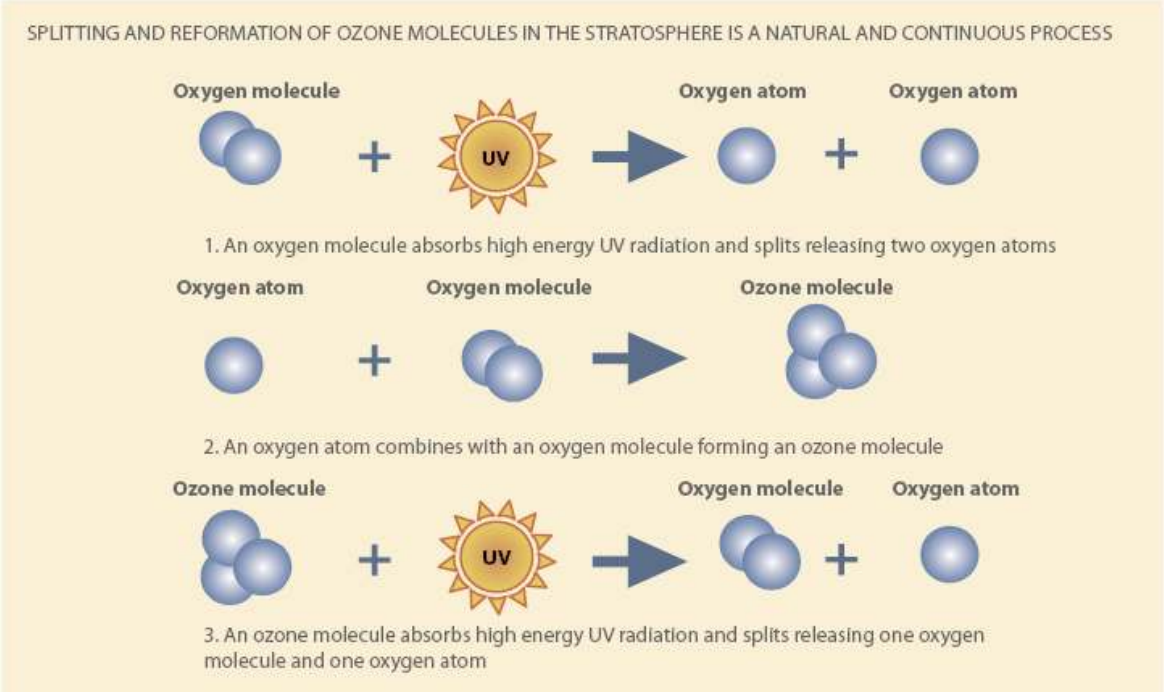

Figure 1.1. Formation and splitting of ozone molecules by UV radiation. Source: UNEP (2007).

THE OZONE LAYER DEPLETION AND THE OZONE HOLE

In the early 1970s, investigations conducted by Mollina and Rowland (1974) definitively associated CFCs with stratospheric ozone depletion. The model proposed at the time stated that CFCs diffused upward into the stratosphere, where they were broken down by UV radiation, in a process that released free chlorine which then reacted catalytically with ozone leading to its destruction (Fig. 1.2).

However, only with the discovery of significant reduction in the column ozone concentration over Antarctica during September and October in 1985, public concern over ozone destruction emerged. Two years latter, the largest seasonal reduction ever was registered: an overall decrease of 50% and up to 95% at altitudes between 15 and 20 Km (Hofman et al, 1992).

The discovery of the Antarctic ozone hole caused considerable controversy among scientists, since it had not been predicted by the models existent at the time, and several theories, based on both natural and anthropogenic sources, were initially proposed to explain the observed ozone depletion (Solomon et al, 1986). Soon, however, compelling evidence supported the early model of Mollina and Rowland and the ozone hole was definitively associated to CFCs and other anthropogenic pollutants, with long atmospheric lifetimes (75-100 years) upon release to the atmosphere, though meteorological conditions unique to the Antarctic also account for the problem (Kerr, 1986).

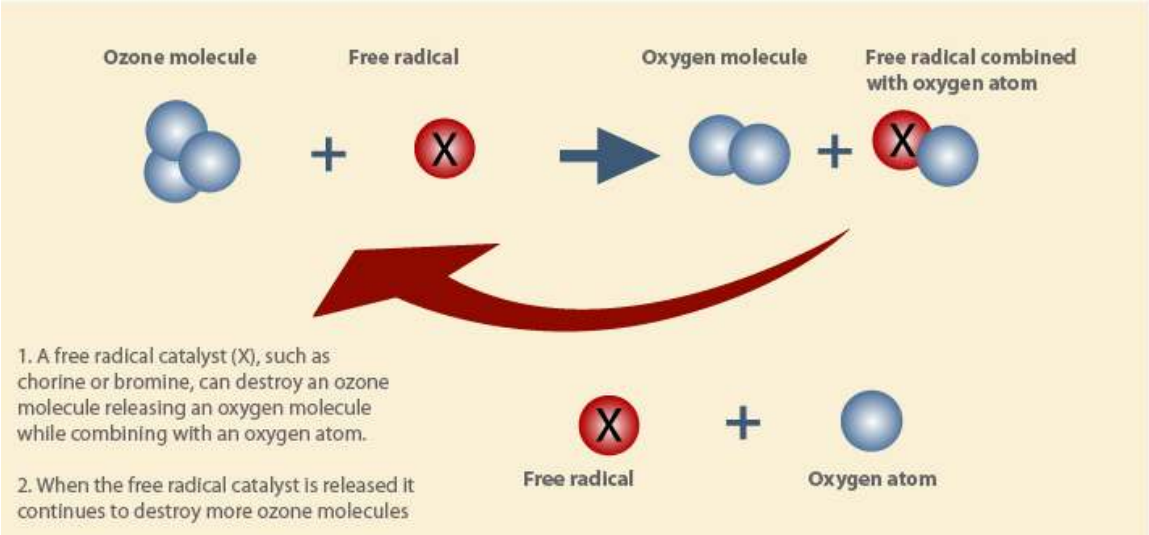

Figure 1.2. Destruction of an ozone molecule by a free radical. Extracted from UNEP (2007).

It is now recognized that ozone depletion is not confined to the Antarctic, but is a global phenomenon. Reductions in the ozone column as big as 70% over the Southern Hemisphere spring over Antarctica continue today, and in the Artic region ozone loss rates have reached values comparable to those recorded over the Antarctic in recent years (Rex et al, 1997) and are predicted to continue to decline for one or more decades (Shindell et al, 1998). In middle latitudes, declines have been about 3% below pre-1980 values for 35–60°N and about 6% for 35–60°S (UNEP, 1998).

The discovery of the ozone hole was the first definitive evidence that human activities are changing the global environment, and this totally unsuspected finding attests to the limits of our present understanding of global environmental changes (Leaf, 1993).

The “Montreal Protocol on Substances that Deplete the Ozone Layer”, which outlined specific measures and timetables for the reduction and consumption of CFCs and halons, significantly contributed to the stabilization or reduction of the most important chlorofluorocarbons involved in the ozone depletion (UNEP, 1998). However, the Antarctic ozone hole will persist in the following decades. Ozone concentrations in the lower stratosphere over Antarctica will increase by 5%–10% by 2020. Only by the year of 2070 ozone will return to pre-1980 levels, almost 20 years later than earlier determinations predicted, as a result of revised estimates of atmospheric concentrations of Ozone Depleting Substances and a larger predicted future usage in developing countries.

Ozone depletion may also be aggravated by the draw-down of nitrogen oxides from above the stratosphere due to changes in wind patterns associated with climate change (Hartmann et al, 1999). Recent projections have also indicated that the estimated recovery could actually suffer a delay of decades due to interactions with other long-term atmospheric changes, e.g. increasing concentrations of greenhouse gases. Other factors that can influence the recovery include non-ratification and/or non-compliance with the Montreal Protocol and future volcanic eruptions. Nevertheless, the recovery phase for surface UV-B irradiances will probably not be detectable until many years after the ozone minimum (UNEP, 2007).

EFFECTS OF UV-B RADIATION ON ORGANISMS

Decreases in stratospheric ozone concentration are responsible for an higher amount of ultraviolet-B (UV-B) radiation reaching the Earth's surface and the correlation between decreases in column ozone and the increase in surface UV-B radiation has further been demonstrated and quantified by ground based instruments (McKenzie et al, 2003). These measurements have shown that stratospheric ozone levels are today near their lowest value since records began, so current UV-B radiation levels are thought to be close to their maximum. Increases in surface erythemal UV radiation relative to the values in the 1970s are estimated to be about 7% at Northern Hemisphere mid-latitudes in winter/spring, 4% at Northern Hemisphere mid-latitudes in summer/fall, 6% at Southern Hemisphere mid-latitudes annually, 130% in the Antarctic in spring and about 22% in the Arctic in spring (UNEP, 2007). Long term predictions of future UV-B levels are still difficult and uncertain. Nevertheless, current projections suggest that a slow recovery to pre-ozone depletion levels may be expected during the first half of this century.

Obviously, the main public concern when considering the ozone hole is the direct effect of the increase of UV-B radiation on human health, i.e., increased damage to the eyes, the immune system and the skin. However, increased UV radiation reaching the Earth's surface can have an even more pronounced impact at the ecosystem level (Zepp et al, 2007).

MOLECULAR AND CELLULAR PHOTOBIOLOGY

Ultraviolet radiation can induce several different effects on biological species, most of them not lethal to cells, depending upon the absorption of UV photons by important molecules, most notably nucleic acids, and to a lesser extent proteins and other molecules (Harm, 1980). Radiation damage to DNA is potentially dangerous to cells since the incidence of a single photon in a single molecule can have potentially lethal effects. DNA damage can also result from free radicals and reactive oxygen species formed in various photochemical processes (Mitchell and Karentz, 1993).

Nucleic acids show maximum absorption between 260-265 nm that is due to the nucleotides bases that are the cromophores. Upon absorption of UVR by DNA, the major photoproducts that result are pyrimidine derivatives (Errera, 1952), including cyclobutyl-type dimers (also called cyclobutane pyrimidine dimers, CPDs, that are the most significant), pyrimidine adducts, pyrimidine hydrates and DNA protein crosslinks (Patrick and Rahn, 1976) (Figure 1.3). The accumulation of photoproducts can block DNA transcription and replication and, if the cell cannot repair the damage, mutagenesis or even cell death may occur (Sinha et al, 2001).

Proteins also absorb most strongly in the UV-B and UV-C regions, though their absorbance is lower than that of nucleic acids. Other molecules with the ability to absorb UV radiation include porphyrins, carotenoids, steroids and quinones (Diffey, 1991).

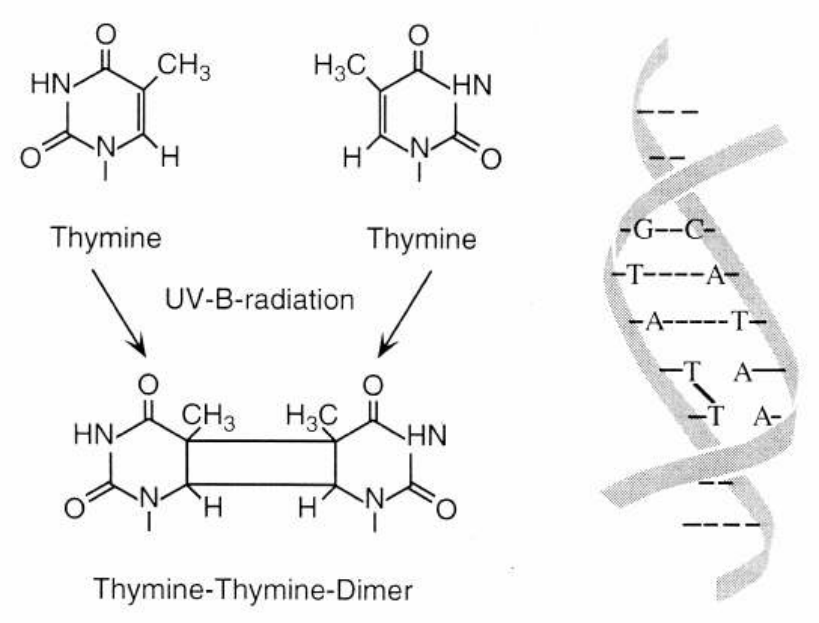

Figure 1.3. Absorption of UV-B photons may lead to disruption of the hydrogen bonds between the base pairs thymine and adenine. As a result, cyclobutane dimers of thymine groups may be formed. Extracted from Rozema et al (2002).

Effects of Ultraviolet Radiation on Aquatic Ecosystems

Aquatic ecosystems are key components of the Earth's biosphere, responsible for the production of more than 50% of the biomass on our planet and incorporating approximately the same amount of atmospheric carbon dioxide as terrestrial ecosystems (Zepp et al, 2007). The oceans also play a vital role with respect to global warming. Since marine phytoplankton represents an important sink for atmospheric CO_2, it has an important role in the development of future trends of CO_2 concentrations in the atmosphere. Hence, it is crucial to assess how enhanced exposure to solar UV-B radiation might affect the productivity of aquatic systems. Furthermore, the potential impact of ozone depletion on atmospheric carbon dioxide uptake as a result of marine primary production inhibition is uncertain and more detailed studies are urgently needed.

After the discovery of the ozone hole (Farman et al, 1985) and the concomitant increase in intensity of biologically harmful UV-B radiation reaching Antarctic waters, the majority of the studies on the effects of UV-B focused on phytoplankton, because of their role as primary producers (Lorenzen, 1979; Smith, 1989; Karentz et al, 1991; Behrenfeld et al, 1993a; Cullen and Neale, 1994; Neale et al, 1994; Prezelin et al, 1994; Vernet et al, 1994; Häder, 1995; Helbling et al, 2003). Solar short-wavelength radiation can reach ecologically significant depths in many freshwater and marine ecosystems (USEPA, 1987; Smith et al, 1992; Scully and Lean, 1994; Häder, 1995; Booth et al, 1997), so depletion of the ozone levels results in increased amounts of solar UV-B radiation penetrating within the euphotic zone, where phytoplankton productivity takes place. Clear lakes in alpine and polar regions, where UV penetrates deep into the water column, may be particularly vulnerable. In addition, ozone depletion has also been shown to alter the UV-B: UV-A: PAR (photosynthetically active radiation) ratio, potentially

impairing crucial light dependent processes in aquatic organism, that include photosynthesis, photo-orientation, photoinibition and photoprotection (Smith et al, 1992; Häder et al, 1995; Gerber et al, 1996; Jiménez et al, 1996; Häder, 1997) and resulting in a significant stress for the diverse aquatic ecosystems (IASC, 1995).

Effects of Ultraviolet Radiation on Aquatic Bacterial Communities

The pelagic plankton communities function through a web of exchanges of energy and nutrient mediated by a diverse array of producers and consumers, which ultimately depend on the energy supplied by sunlight. Bacteria are pivotal components of the microbial food web (Figure 1.4), playing a vital role in nutrient cycling and are the principal route of carbon flow in aquatic ecosystem. They contribute up to 40% of the planktonic carbon (Cho and Azam, 1990), process up to 80% of the primary production (Azam et al, 1983; Cho and Azam, 1990; Ducklow and Carlson, 1992) and show nutrient uptake potentials around 100 times faster than that of phytoplankton (Blackburn et al, 1998). Bacterioplankton also consume dissolved organic matter (DOM) to form bacterial biomass, thereby repackaging carbon and making it available to higher trophic levels (e.g. Pomeroy and Weibe, 1998). However, very little is known on the role of UV radiation on the microheterotrophic components of the aquatic food webs in spite of their importance for the carbon and energy flows through the microbial loop (Azam et al, 1983; Ducklow and Fasham, 1992).

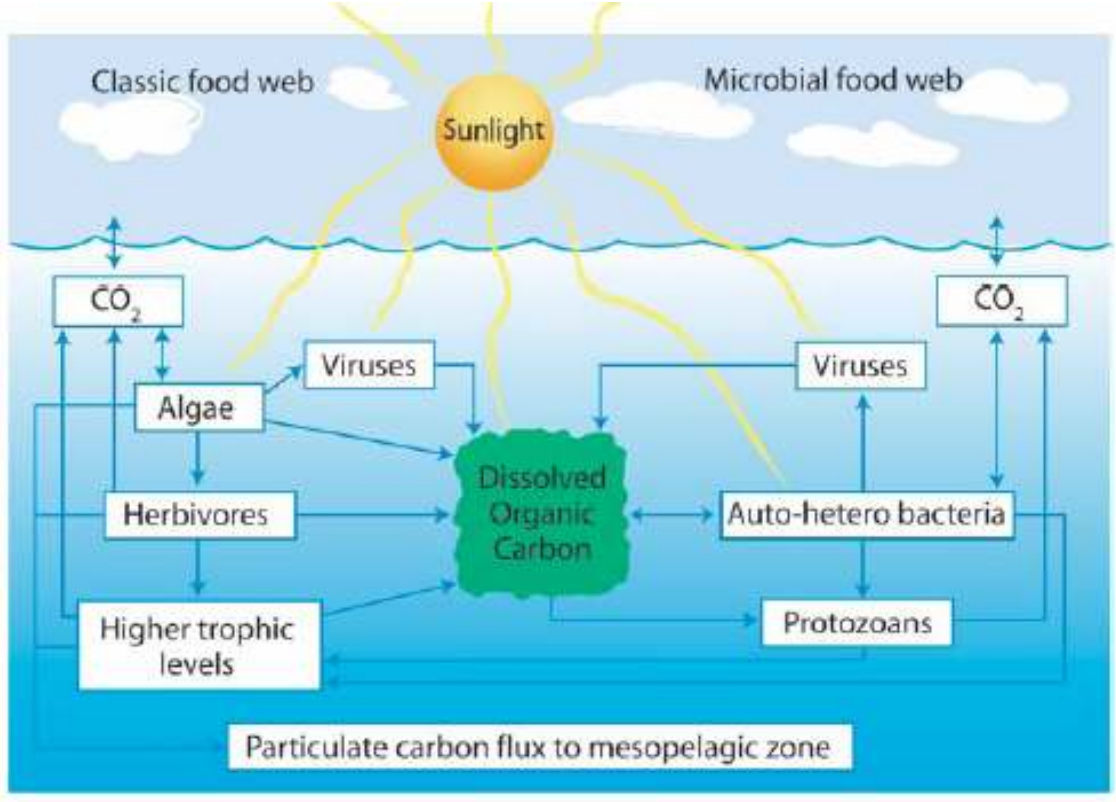

Figure 1.4. Schematic diagram of classic and microbial marine food webs illustrating the flow of carbon and energy through the systems. Extracted from Häder et al (2007).

Effects of UV Radiation on Bacterial Metabolism and Community Structure

Heterotrophic bacteria play a very important role in marine ecosystems because they are responsible for degradation and cycling of organic matter in the sea (Azam et al, 1983). It has been suggested that heterotrophic bacteria are likely to be more susceptible to UV stress than larger organisms, because they have simple haploid genomes with little or no functional redundancy, are small sized, have short generation times (Häder et al, 1995) and lack UV absorbing pigments (Karentz et al, 1994). This results in enhanced sensitivity of bacterioplankton to UV-B stress, comparatively with larger eukaryotic organisms producing, upon exposure, about the double the amount of cyclobutane dimers (Jeffrey et al, 1996a; Jeffrey et al, 1996b). Thus, the size of the organism can be assumed to be inversely correlated to the extent of UV-induced damage it receives.

UVR may have both direct and indirect effects on bacterioplankton (Figure 1.5). Direct effects arise from damage to cellular macromolecules as a result of direct absorption of UVR, especially by DNA. The wavelength and intensity of the exposure determines the type and extent of damage to DNA. UV-A generally causes indirect damage to DNA as a result of the formation of chemical intermediates, such as oxygen and hydroxyl radicals which interact with DNA to form strand breaks, alkali labile sites and DNA protein crosslinks (Peak and Peak 1989).

The most important consequence of the absorption of UV-B radiation by DNA is the formation of photoproducts such as pyrimidine (6-4) pyrimidone and cyclobutane pyrimidine dimers (CPDs) (Karentz et al, 1994). The formation of photoproducts disturbs the error-free DNA replication and RNA transcription in cells (Karentz et al, 1994) and it has been describe in bacterioplankton (Jeffrey et al, 1996a, b; Visser et al, 2002), in the marine diatom *Cyclotella* sp. (Buma et al, 1997), in phytoplankton (Karentz et al, 1991) and in phagotrophic protists (Somaruga et al, 1996). A CPD can be lethal if the lesion blocks DNA synthesis and RNA transcription or can be mutagenic if the lesion is bypassed by DNA polymerase (Sancar and Sancar, 1988).

The direct detrimental effects of UVR on bacterioplankton are manifested by reduced DNA and protein synthesis (Aas et al, 1996; Herndl et al, 1993), reduced exoenzymatic activity (Müller-Niklas et al, 1995), reduced amino acid uptake (Bailey et al, 1983), reduced oxygen consumption (Pakulski et al, 1998), and a decrease in bacterial abundance (Müller-Niklas et al, 1995; Pakulski et al, 1998).

Bacterial viability is also impacted by UV exposure, as supported by studies conducted in Antarctic waters which showed decreased viability, based on colony-forming units (CFU), of natural bacterial assemblages and 2 marine isolates (*Acinetobacter* sp. and *Bacillus* sp.) (Helbling et al, 1995). The reduction in viability by UV-A was consistently higher than that induced by UV-B, though marked differences were found in the tolerance of the natural assemblage and the 2 isolates to solar radiation, revealing heterogeneity in sensitivity among species.

Indirect effects of UVR are associated to altered bioavailability of dissolved organic matter (DOM) by UVR (Lindell et al, 1995, Herndl, 1997, Obernosterer et al, 1999) or to the

sensitivity of bacterioplankton grazers to UVR (Sommaruga et al, 1996). Photolytic cleavage of DOM by UV produces several compounds (Zafiriou et al, 1984; Kieber et al, 1990; Scully et al, 1995; Zepp et al, 1995; Kieber et al, 1996; Scully et al, 1996). Some of these products are low-molecular weight organic acids that can be taken up efficiently by the bacterioplankton, leading to enhanced bacterial activity (Lindell et al, 1995; Lindell et al, 1996; Reitner et al, 1997). Other photoproducts, like free radicals, have detrimental effects on the plankton organisms (Palenik et al, 1991; Helz et al, 1994), inhibiting bacterial activities. The net effect of UV radiation on bacterial metabolism is, thus, the result of the balance between these opposing processes.

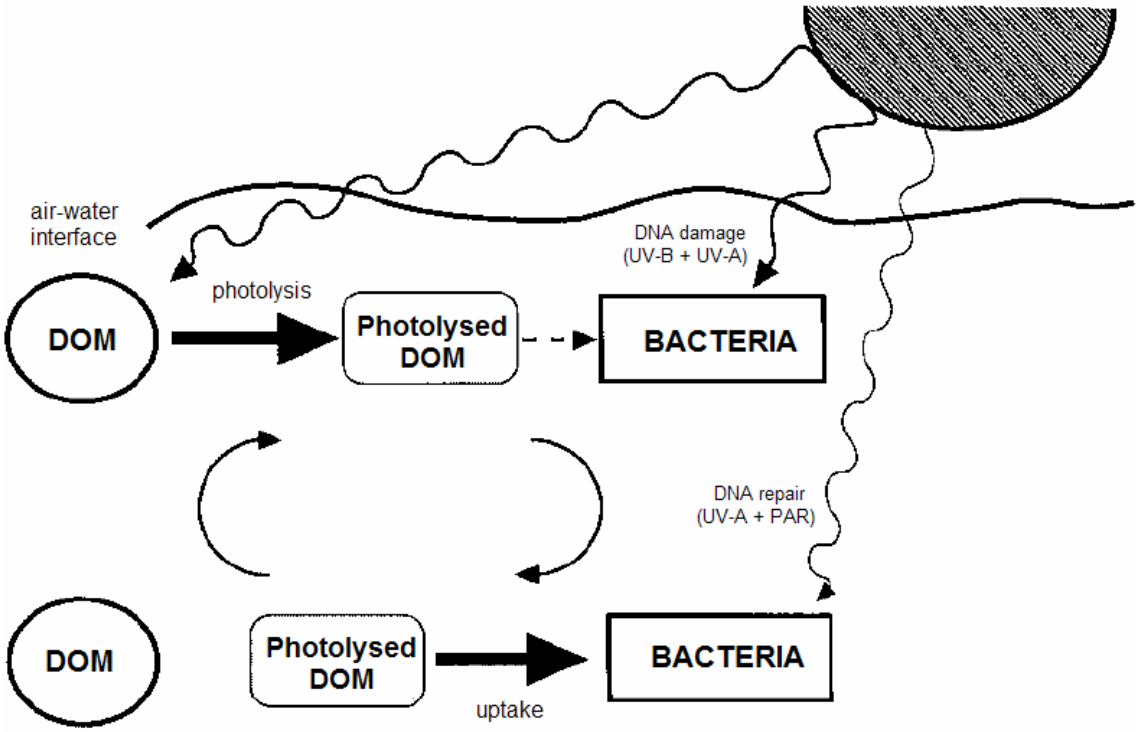

Figure 1.5. Role of solar radiation on bacterioplankton and dissolved organic matter (DOM) in the upper mixed water column. In the top surface layers of the water column DOM is photolytically cleaved and bacterioplankton activity retarded due to high levels of UV-B radiation. If mixed into deeper layers, bacterioplankton repair damage via photoenzymatic repair mechanisms induced by UV-A and the photolysed DOM is taken up. Extracted from Herndl et al (1997).

Solar UV can also have a decisive role in bacterioplankton community structure in marine surface waters (Arrieta et al, 2000). However, information on how UV radiation affects bacterial community composition is scarce (Winter et al, 2001; Norris et al, 2002; Kadivar and Stapleton, 2003; Van Mooy et al, 2004).

Solar radiation might induce changes in bacterial community composition due to a diverse array of both direct and indirect potential mechanisms: (i) direct detrimental effects of UVR on bacterial activity and survival, as a result of DNA damage (Jeffrey et al, 1996) and damage associated to the photochemical production of free radicals (Scully et al, 1996). The sensitivity of surface and deep-water communities has been described to be similar, suggesting that bacterial communities do not respond to solar exposure by increased UVR resistance

(Herndl et al, 1993). In any case, variability in the sensitivity among bacterial strains (Joux et al, 1999; Arrieta et al, 2000; Agogué et al, 2005b) can induce changes in bacterial community composition; (ii) selection of phototrophic bacteria. Besides the capacity for chemoorganotrophy, the dominating bacterial groups in the ocean possess photosynthetic capabilities (Beja et al, 2000; Kolber et al, 2000; Kolber et al, 2001; Beja et al, 2002), which may give them an advantage under sunlit conditions. Accordingly, solar exposure can favour mixotrophic members of the bacterial community; (3) indirect effects due to changes in phytoplankton composition and biomass. "Satellite bacteria" are frequently closely associated to different phytoplankton species (Schäfer et al, 2002), and systems dominated by different phytoplankton communities also differ in bacterial community (Pinhassi et al, 2004). The decay of phytoplankton after blooms can induce clear changes in bacterial community composition (van Hannen et al, 1999; Riemann et al, 2000). Thus, variations in the light regime that affect phytoplankton also influences indirectly bacterial community composition; (4) UV-radiation affects bacterivory by heterotrophic nanoflagellates (Sommaruga et al, 1996) and can trigger the lytic cycle in lysogenic bacteriophages (Maranger et al, 2002). Bacterial mortality due to bacterivory and viral lysis is selective (e.g. Jürgens and Matz, 2002; Weinbauer, 2004), so UV-induced changes in these mortality factors can also affect bacterial community composition; and (v) alteration of the bioavailability of dissolved organic carbon (DOC) to bacteria by UVR. Upon exposure of DOC to solar radiation, low-molecular (LMW) weight compounds easily degradable by bacteria (Bertilsson and Tranvik, 1998; Kieber et al, 1989; Wetzel et al, 1995), or less available to bacteria can result (Obernosterer et al, 1999; Tranvik and Bertilsson, 2001). Hence, UVR can promote bacterial community members specialized on the photochemically modified DOC compounds.

Defence Mechanisms against UV-induced Damage in Aquatic Prokaryotes

During evolution organisms have developed mechanisms of protection and mitigation of UV-B radiation damage, that include UV avoidance, radiation shielding (pigmentation), removal of toxic oxygen species and specific damage repair systems (Friedberg, 1985; Sancar and Sancar, 1988).

Motile organisms, such as motile cyanobacteria, can escape from ultraviolet radiation by migrating into habitats with reduced light exposure. Such strategies include phototactic, photokinetic and photophobic responses (Häder, 1987a, b), vertical migration (Leech and Williams, 2001; Rhode et al, 2001) or floating behaviour by a combination of gas vacuoles and ballast (Reynolds et al, 1987; Bebout and Garcia-Pichel 1995;). This allows them to change their position in the water column as environmental conditions change and thus always ensure a nearly constant external environment.

In order for UV radiation to be effective in most organisms upon exposure, it must effectively penetrate into the tissues and be absorbed. In cyanobacteria, the production of ultraviolet absorbing substances, such as mycosporine-like aminoacids (MAAs) and scytonemin confer significant shielding against high UV radiation stress (Garcia-Pichel and Castenholz,

1991; Garcia-Pichel et al, 1993; Büdel et al, 1997; Sinha et al, 1998; Dillon and Castenholz, 1999). For example, in *Nostoc commune*, MAAs play an important role in photoprotection, because they are located in the extracelular glycan. Two out of three photons are absorbed by the pigment before cell membranes or targets within the cell are reached (Böhm et al, 1995).

The accumulation of detoxifying enzymes or radical quenchers and antioxidants can also provide protection by scavenging harmful radicals or oxygen species (Mittler and Tel-Or 1991; Middleton and Teramura 1993), For example, carotenoids have a well known antioxidant activity, removing singlet oxygen and inhibiting lipid peroxidation, at least in cyanobacteria (Edge et al, 1997). UV-A and UV-B can cause oxidative stress by photodynamically generating reactive oxygen intermediates (Cunningham et al, 1985; Shibata et al, 1991). In response to UV-A and UV-B radiation, an increase in the carotenoid/chlorophyll a ratio of cyanobacteria occurs (Quesada and Vincent, 1997), supporting its role as reactive-oxygen-quenching pigments. Scavenging enzymes such as superoxide dismutase and peroxidases can also help to decrease reactive oxygen species, being its synthesis inducible by UV-B radiation in some microalgae (Rao et al, 1996). Cyanobacteria produce scavenging enzymes such as ascorbate peroxidase and catalases (Miyake et al, 1991). The production of extracelular polysaccharides can also provide protection against UV damage, and it has been reported that UV-B irradiation stimulates the extracelular glycan production of *N. commune*, probably to provide a matrix for the UV-A/B-absorbing oligosaccharides – mycosporines – located in the sheath of *N. commune* (Ehling-Schulz et al, 1997).

In response to UV damage, bacteria have also developed different repair pathways, including photoenzymatic repair (PER), nucleotide excision repair (NER) (also called dark repair), and recombinational repair (post-replication repair) (Friedberg, 1985).

PER involves the binding of a photoreactivating enzyme (photolyase) to the UV-induced pyrimidine dimers in the dark and in the presence of favourable temperature. Upon exposure to radiation between 330 and 660 nm (i.e., near-UV or visible) the enzyme is activated and separates from the dimer, repairing the DNA photoproduct in the process (Sancar, 1994).

PER has been observed in marine bacterioplankton exposed to artificial UV-B after secondary irradiation with UV-A or photosynthetically active radiation (PAR). The existence of PER in natural populations has been inferred from the results of CPD kinetic analyses performed with bacterioplankton samples obtained from the marine water column throughout the solar day (Jeffrey et al, 1996). Low temperature can slow this enzymatic repair of DNA damage (Britt, 1996; Takeuchi et al, 1996); therefore, microbes in cold environments may suffer from a less favourable balance between damage and repair than others. These environments are coincidently those exposed to the greatest ozone depletion.

In order to evaluate the potential role of the different repair mechanisms in bacterioplankton, Herndl et al (1997) transferred natural bacterial communities after exposure to UV-B or surface solar radiation levels to different radiation regimes. The authors concluded that the most efficient repair mechanisms in bacterioplankton was photoenzymatic repair and reported that only a short exposure of UV-A was sufficient to yield a significantly higher bacterial

activity than in the dark control. However, in the aquatic environment, the equilibrium between UV damage and photorepair may be influenced by the passive movements of the cells within the mixing layer, where they are alternately exposed to high levels of damaging solar UV radiation near the surface and beneficial UV-A/blue light at greater depths (Sancar, 1994).

NER, or dark repair, involves the enzymatic removal of the defective region in one of the two DNA strands, with the subsequent replacement with normal nucleotides through complementary base pairing with the intact strand (Friedberg, 1985). UV-B induced DNA damage in bacterioplankton has been show to fluctuate in a diel pattern (Jeffrey et al, 1996b), accumulating during the afternoon hours and being repaired between sunset and the following sunrise, which indicates the presence of active dark repair processes in natural bacterioplankton communities (Jeffrey et al, 1996b).

In post-replication repair, UV-damaged DNA replicates leaving gaps in the daughter strand opposing the damages sites. These gaps are subsequently filled by DNA synthesis (Sancar and Sancar, 1988).

Mutagenic or SOS repair reduces the fidelity of the DNA polymerase, allowing it to bypass damaged nucleotides. This can result in the incorporation of an inappropriate nucleotide in the daughter strand, resulting in a mutation (Freifelder, 1987).

Several genes present in microorganisms are regulated by UV-B, and changes in UV-B can alter gene expression with potentially important consequences (Strid et al, 1994; Jordan, 1996; Bender et al, 1997). Though the exact mechanisms involved in the perception of UV-B radiation by organisms and how signals are transduced are not yet well understood, active oxygen can be one of the triggers that can change gene activity (Mackerness et al, 1998).

RecA is a crucial component of the bacterial response to DNA damage, participating directly in DNA repair and regulating the expression of other genes whose functions promote increased survival following DNA damage (Walker, 1984). The expression of the *recA* gene is also regulated by UVR. For example, UV-A and UV-B have been shown to induce increased levels of RecA in *Pseudomonas aeruginosa* and in *Vibrio natriegens* (Booth et al, 2001).

Whether bacterial photoadaptation occurs in aquatic environments has not been clearly established. However, Pakulski et al (1998) observed recovery of bacterial production and respiration in subtropical coral reef bacteria incubated at a fixed depth during a second day of exposure to natural sunlight. These authors explained the findings by either photoinduced selection for light-tolerant cells or physiological adaptation to ambient light regimens that occurred during exposure. Furthermore, survival and CPD data obtained from *Vibrio natriegens* subjected to two UV-B exposure periods interrupted by a repair period (photoreactivation plus dark repair) suggested the occurrence of photoadaptation (Joux et al, 1999).

Factors that Influence the Effect of UV-R in Aquatic Bacterial Communities

Apart from depletion resulting from the anthropogenic release of halocarbons (e.g. chlorofluorocarbons), the ozone column shows natural cycles, both annual and longer term (e.g. associated with the solar cycle and El Niño oscillations), that influence the intensity of solar ultraviolet radiation inciding on organisms and ecosystems (Paul and Gwynn-Jones, 2003).
Cloud cover usually reduces UV incidence, often very substantially, resulting in unpredictable fluctuations in UVR on timescales varying between minutes and years, probably representing the major determinant of short-term variation in UV at most sites (Bjorn, 1999).

Pollution of the troposphere by particulate materials and by anthropogenically created ozone can also absorb solar UV, especially UV-B, reducing the intensity of radiation reaching the surface of the Earth (Wenny et al*,* 2001).

Dissolved and particulate organic carbon (DOC and POC) are the main attenuating substances in freshwater and coastal marine waters (Frenette et al, 2003). DOC has been found to be mainly involved in the attenuation of UV-B radiation, while POC mainly decreases the UV-A radiation in the water column (Bracchini et al, 2005). UV attenuation is widely variable in aquatic environments. Due to the runoff of silt and dissolved organic carbon (DOC) from shores, transparency is lower in coastal areas and shallow continental shelf waters.

In freshwater ecosystems, UV absorbance is usually high due to high inputs of inorganic and decaying organic material, but is also affected by eutrophication (Bracchini et al, 2004).

The inundation and water saturation of soils within watersheds will most likely be reduced by warmer, drier climates, thus diminishing the inputs of DOC to adjacent lakes and streams (Williamson and Zagarese, 2003). The combination of acidification and climate change has led to the significant increase in underwater UV penetration, in some cases (Williamson and Zagarese, 2003) and changes in DOC concentration and thus water colour and turbidity in inland aquatic ecosystems (Williamson and Zagarese, 2003). Future climate-DOM-UV radiation interactions may induce strong variations in phytoplankton abundance with unexpected effects for bacterial communities (Leavitt et al, 2003).

Zooplankton and phytoplankton have usually a small effect on UV attenuation (Vähätalo et al, 2005), but bacterioplankton plays a major role (Zepp et al, 2007). The slow photodegradation of DOC in the water column by solar radiation produces smaller fragments which are consumed by bacterioplankton (Klug, 2005). This results in the increase of UV transparency of the water column (Pérez et al, 2003) and thus deeper UV-B penetration (De Lange et al, 2003). Besides that, photobleaching increases UV transparency. Enhanced temperatures as a result of global climate change may also lead to the enhanced utilization of DOM, increasing the penetration of UV-B radiation into the water column (Molot et al, 2004).

Phytoplankton density can influence the vertical distribution of bacterioplankton. Dense diatom populations occurring during summer in the Antarctic waters result in UV attenuation in the top layers of the water column. As a result, bacterioplankton experience large UV-B induced DNA damage at the water surface, but it was protected from solar UV-B below the diatom population (Buma et al, 2001).

Spectral attenuation coefficients in the water column and the time pattern of exposure and protection for the organisms, as they passively move in the mixing layer, also determine the effect of solar UV on bacterioplankton (Herndl et al, 1997).

The formation of crusts and biofilms are additional protective measures against environmental factors including desiccation, temperature changes and solar UV (Prakash et al, 2003).

The concentration of viruses can also be important to determine the effect of UV radiation on bacterial populations, since at least in phytoplankton hosts such as *Phaeocystis*, viral infection can apparently provide some protection from solar UV radiation, though the reason for this phenomenon is not known (Jacquet and Bratbak, 2003).

Effects of UV Radiation on Microbial Food Webs

The potential of UV radiation to inhibit both the phytoplankton and bacterioplankton activities (Lorenzen, 1979; Herndl et al, 1993; Holm-Hansen et al, 1993) and their ecological potential as well as biogeochemical implications has fuelled increased interest in the effects of UVR on biological and chemical processes.

The net effect of UV-B to microbial food webs may be dependent not only on the direct deleterious effects to primary producers and bacteria, but also on the effects to higher trophic levels. However, the limited numbers of studies that have simultaneously considered several levels of aquatic trophic webs are widely contradictory, with different investigations either confirming or rejecting the influence of UV-B radiation on the relationship between predator and prey (Bothwell et al, 1994; Vinebrooke and Leavitt, 1996; Wickham and Carstens, 1998; Chatila et al, 2001).

Ultraviolet radiation has been shown to result inhibition of algal consumers (Diptera: Chironomidae) (Bothwell et al, 1994). In an oligotrophic lake, high sensitivity of a heterotrophic nanoflagellate (*Bodo saltans*) to solar ultraviolet radiation was found, resulting in reduced bacterivory (up to 70%) (Sommaruga et al, 1996). The harmful effects of ultraviolet radiation on the grazing activity of heterotrophic flagellates on autotrophic picoplankton have also been demonstrated (Ochs, 1997). In mesocosm experiments, the enhancement of UV-B radiation during the occurrence of winter/spring blooms was found to result in decreased abundance of organisms at different trophic levels. Relative to natural conditions, phytoplankton abundance and biomass tended to be reduced under UV-B stress (Mostajir et al, 1999). The same effect was detected in total copepod abundance (adults, copepodites and nauplii) (Keller et al, 97). Also in mesocosm experiments, significant reduction of phytoplankton abundance and biomass and of copepod nauplii numbers under UV-B stress was observed in a stratified coastal system. However, no significant effects of UV-B radiation on microzooplankton were detected (Mostajir et al, 1999), despite the 10-fold increase in biologically damaging UV-B applied. The lack of effects was related to the rapid extinction of UV-B in highly coloured coastal waters (Keller et al, 1997).

Effects of UV Radiation on Aquatic Biogeochemical Cycles and Interaction with Climate Change

Global biogeochemistry controls life processes, climate and their interactions, including effects on atmospheric greenhouse gas concentrations, and can be affected in several ways by changes in stratospheric ozone and, hence, solar UV-B radiation (Zepp et al, 2007).

UV-B radiation influences aquatic carbon, nitrogen, sulphur and metals cycling that affect a wide range of life processes. Modifications of carbon cycling processes by UV radiation, including both UV-B and UV-A, are mediated through changes in its capture through photosynthesis, storage in biomass and non-living organic matter, and release, mediated by respiration and photochemical decomposition (Zepp et al, 1998, 2007; Häder et al, 2007). The effects of UV radiation on carbon cycling are also associated with its effects on the cycling of metals and mineral nutrients such as nitrogen. Other components of climate change, including warming, elevated CO_2 and altered patterns of precipitation, processes that interact with UV radiation, can also strongly affect carbon and nutrient cycles (Figure 1.6) (Zepp et al, 2007).

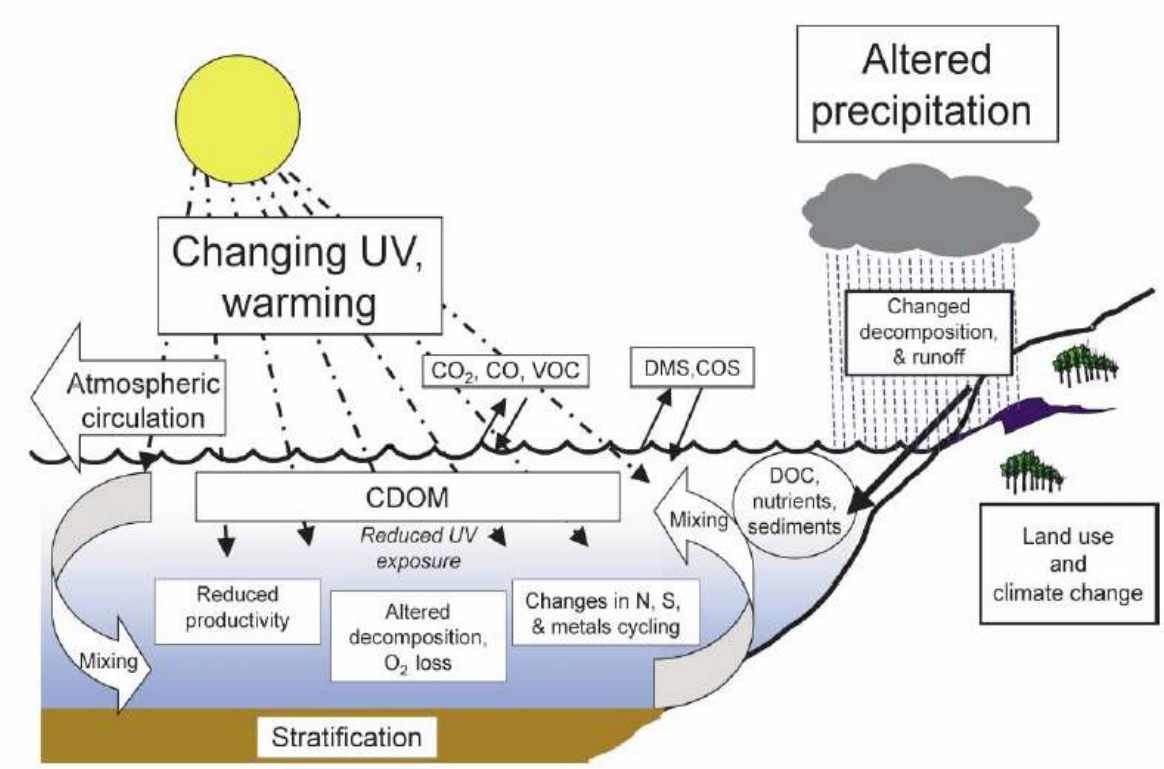

Figure 1.6. Aquatic biogeochemical cycles affected by UV radiation and their interaction with other co-occurring environmental changes such as global warming and land use change. Key: DMS, dimethyl sulphide; CDOM, coloured dissolved organic matter, the primary UV absorbing substance in aquatic environments; DOC, dissolved organic carbon. Extracted from Zepp et al (2003).

Nitrogen is a limiting nutrient in remote parts of the open ocean and thus impacts of UV radiation on important fixing bacteria could be ecologically significant (Berman-Frank et al, 2001). UV radiation affects nitrogen cycling in various ways, including effects on nitrogen-related enzymatic activity by microorganisms through photoinhibition of nitrogen fixing organisms (Häder et al, 2003) and indirectly through effects on the biological availability of essential trace elements, such as iron, that stimulates the growth of nitrogen fixers. Enhanced

decomposition of persistent dissolved organic nitrogen to biologically labile nitrogen photoproducts can also affect nitrogen cycle (Fuhrman and Capone, 2001; Zehr et al, 2001).

Phosphorus cycling can also be potentially affected by exposure to UV and recent research suggests that UV photolysis of phosphatase-humic substance complexes can enhance phosphorus cycling in aquatic environments (Boavida and Wetzel, 1998; Espeland and Wetzel, 2001).

Metals, specially iron and copper (Fig. 1.7), play an important role in the upper ocean and freshwater biogeochemistry, both by participating in UV-induced processes that produce and consume peroxides and other oxidants that participate in biogeochemical processes and also as essential trace elements for plankton (Barbeau et al, 2001). Therefore, UV radiation affects the bioavailability of and other metals in aquatic ecosystems, with the potential to affect metal toxicity and the growth of phytoplankton and other microorganisms that are involved in carbon and nitrogen cycling. In general, UV-induced photoreactions of organic complexes with metals increase their biological availability (Sunda, 1994).

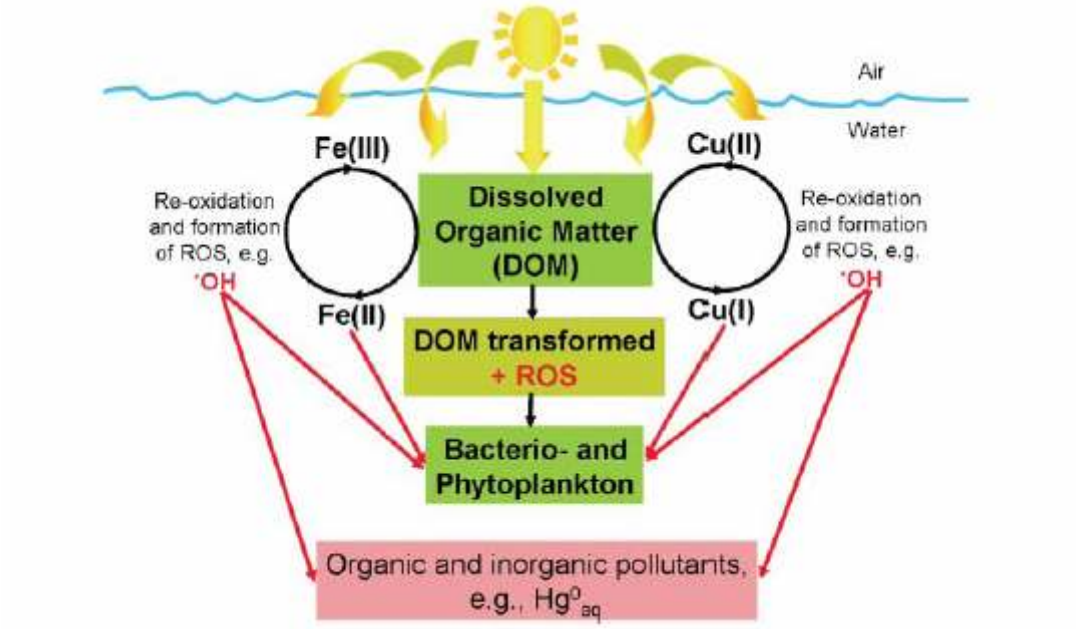

Figure 1.7. Importance of UV radiation in the chemistry of iron and copper in aquatic systems, including its interactions with dissolved organic matter (DOM) and microorganisms. This schematic represents the UV-induced redox cycling of iron and copper, and the concomitant phototransformation of DOM and production of reactive oxygen species (ROS), for example the highly reactive hydroxyl radical (•OH), that can adversely affect bacterio- and phytoplankton and react with organic and inorganic pollutants, for example dissolved gaseous mercury, Hg^0_{aq}. Extracted from Zepp et al (2007).

Coloured dissolved organic matter (CDOM) determines the penetration of UV radiation in aquatic systems, but it is also degraded by solar UV radiation (Hansell et al, 2004; Xie et al, 2004). Future global changes related to increased deforestations and alterations in the patterns of land use will most likely increase stratification of water bodies, that will interact with climate changes to increase the photodegradation of CDOM by UV radiation (Zepp et al, 2002) and, thus, its penetration in aquatic systems. The resultant increase in the transparency of water

bodies may increase UV-B effects on aquatic biogeochemistry at the surface layer of the water column (Häder et al, 2007).

Climate change can alter the exposure of ecosystems to UV-B radiation by influencing processes on the Earth that affect ozone depletion (Häder et al, 2003). Interactions between changing solar UV radiation and climate also influence exchanges of trace gases, such as halocarbons (e.g., methyl bromide), which then influence ozone depletion and sulphur gases (e.g., dimethylsulfide) that oxidize to produce sulphate aerosols that cool the marine atmosphere (Häder et al, 2007). Furthermore, it has been shown that exposure of macroalgae to UV results in significant release of organohalogens that have ozone-depleting characteristics, potentially enhancing the incidence of solar UV (Roleda et al, 2004).

THE SURFACE MICROLAYER AND BACTERIONEUSTON

The air-water interface represents an important ecosystem that plays a major role controlling the exchange of particulate, gaseous and liquid matter, of natural or anthropogenic origin between the hydrosphere and the atmosphere (Hardy, 1997; Franklin et al., 2005; Obernosterer et al, 2005). This interface, so called surface microlayer, or SML, constitutes a common phenomena to most of the freshwater and marine environments (Agogué et al, 2004; Wurl and Obbard, 2004; Obernosterer et al, 2005), inhabited by a community of organisms generically called “neuston” (Zaitsev, 1971). The neuston comprises a vast array of virus, bacteria, algae and several life stages of zooplankton (e.g., larvae and eggs) and fish (Welch, 1935; Zaitsev, 1971). Neustonic organisms have been proposed to contribute to the transformation of toxic compounds, acting the interface as a bioreactor for the detoxification of pollutants (CIESM, 1999). Furthermore, the SML represents an important incubation site for the reproductive and larval stages of several economically relevant fish, including codfish, sole and anchovy (Hardy and Gardiner, 1991). Biotechnological applications have also been suggested for bacterio- and phytoneustonic species, namely in the pharmaceutical and dermo-cosmetic industries (CIESM, 1999).

The bacterial community associated with the surface microlayer is designated as “bacterioneuston”. Due to its peculiar location, it has been attributed to this community a significant role in the dynamics of freshwater ecosystems and the world’s oceans (Zaitsev, 1971, Hardy, 1997). It has also been proposed that bacterioneuston might be involved in the exchange of gases and in mechanisms of transport between the atmosphere and the water column, with a possibly important role in the regulation of in the metabolism of methane and global climate changes (Liss e Duce, 1997).

Even though interfaces are active sites for the development of biological and chemical processes (Hardy, 1982; Hardy et al, 1985; Kuznetsova and Lee, 2001; Agogué et al, 2005a; Franklin et al, 2005), the role of the SML as a determinant ecological niche is still underrated.

THE SURFACE MICROLAYER ENVIRONMENT

The SML has been traditionally defined as the uppermost millimetre of the water column (Liss e Duce, 1997). However, in most investigations it is the depth of the sample layer collected that operationally defines the microlayer, which depends itself on the sampling technique employed (Daumas et al, 1975; Agogué et al, 2004). Its thickness can also vary according to the meteorological conditions and the composition in organic matter (Agogué et al, 2004).

Generically, the SML can be described as a microhabitat comprising several distinct layers which differ by their physical, chemical and ecological properties, with an average depth of 1 to 1000 μm (Hardy, 1991). The literature indicates that an average thickness of 60 μm for the SML can be used meaningfully for the study of physical and chemical properties, while a depth of <1000 μm can be used for the study of the biological properties, though with some variations according to the ecological characteristic being studied (Wurl e Obbard, 2004). The water layer at a depth of >1000 μm must be, thus, referred as the subsurface or underlying water (UW) (Lion e Leckie, 1981).

The surface microlayer is characterized by distinctive physical and chemical properties in respect to the subsurface water layers (Hunter, 1997; Liss and Duce, 1997). This upper layer is formed from biogenic materials produced by plankton at the water column, consisting of complex mixtures of lipids, proteins, sugars and their derivatives as well as many other substances that concentrate at the air-water interface through a series of physical and biological processes, including diffusion, convection, turbulent mixture, bubble or floating particles rising and *in situ* primary productivity (Walczak and Donderski, 2003). Gravity is also responsible for the accumulation of high concentrations of small particles, that being heavier than the air but less dense than the water, accumulate at the interface between both environments, becoming part of the surface microlayer (Liss, 1975; Word et al, 1986; Wakzak e Donderski, 2003; Henk, 2004).

Together with the processes of gravitational deposition, atmospheric precipitation too is important for the enrichment of the SML since it contributes to the falling of different types of aerosols, dust and gases from the atmosphere (Wakzak e Donderski, 2003). The enrichment of the SML has also been found to be intimately associated with the proximity of agriculture fields, industrial plants, and communication lines, for example, from which several chemical substances are transported by the wind and the rain to the hydrosphere (Wakzak e Donderski, 2003). This transport results in the deposition of surfactive organic substances over the water bodies that, depending on its concentration, may represent a source of organic carbon for heterotrophic bacteria or a limiting factor for the development of the microorganisms, due to its direct toxic action and induction of eutrophication of the aquatic systems (Lion e Leckie, 1981). Biological and photochemical mineralization and the vertical transport represent the main loss pathways in the SML (Liss and Duce, 1997).

As a result of the accumulation of materials at the surface, favourable conditions to the development and accumulation of heterotrophic aerobic fauna and flora can be created

(Nauman, 1917, cited by Agogué et al, 2004; Zaitsev, 1971; Williams, et al. 1986; Walczak and Donderski, 2003).

A vast amount of pollutants accumulates at the SML in concentrations up to 10^2-10^4 superior to those occurring at the water column. Some relevant anthropogenic materials that are deposited in the microlayer include plastics, poliaromatic hydrocarbons (PAH's), chlorinated hydrocarbons and potentially toxic metals, such as lead, copper, zinc and nickel. However, it is still unknown how the peculiar processes occurring at the microlayer affect the faith of the anthropogenic substances accumulating at the interface (Liss, 1975; Hardy, 1982).

Thus, the SML works as an important source, or intermediary reservoir, of organic and inorganic compounds that can significantly impact the exchange of materials through the air-water interface (Norkrans, 1980; Obernosterer et al, 2005).

The physical and molecular forces generated at the surface of the hydrosphere, even when considered on their own, represent a considerable challenge to the integrity of the smaller life forms. However, in natural environments, the surface layer is additionally exposed to wide variations of several physical and chemical factors, including radiation, temperature, salt concentration and mechanical disturbance (Liss, 1975; Word et al, 1986; Wakzak e Donderski, 2003; Henk, 2004).

BIOLOGY OF THE SURFACE MICROLAYER

Usually, it is considered that the surface microlayer (Figure 1.8) contains a biological community analogous, though different, of that associated with the water column. This means that organisms whose dimension ranges from virus (nanoneuston or nanoplankton) to plants and fish (macroneuston or macroplankton) exist in both compartments (Hardy, 1982).

Thus, the neustonic community comprises a vast amount of organisms that can be organized according to their order of intervention in the trophic net as: (a) microorganisms, (b) protozoa, the primary consumers, (c) small metazoa (invertebrates), including several species of rotipher, larvae of several species of polychaetes, gastropods and lamellibranchiae molluscs, copepods, cirripides, echinodermes, some species of cladocers, etc., (d) large metazoa, including representatives from Polichaeta, Isopoda, Amphipoda, Cumacea, Mysidacea and Decapoda, and (e) fish eggs and larvae and small fish (Zaitsev, 1971).

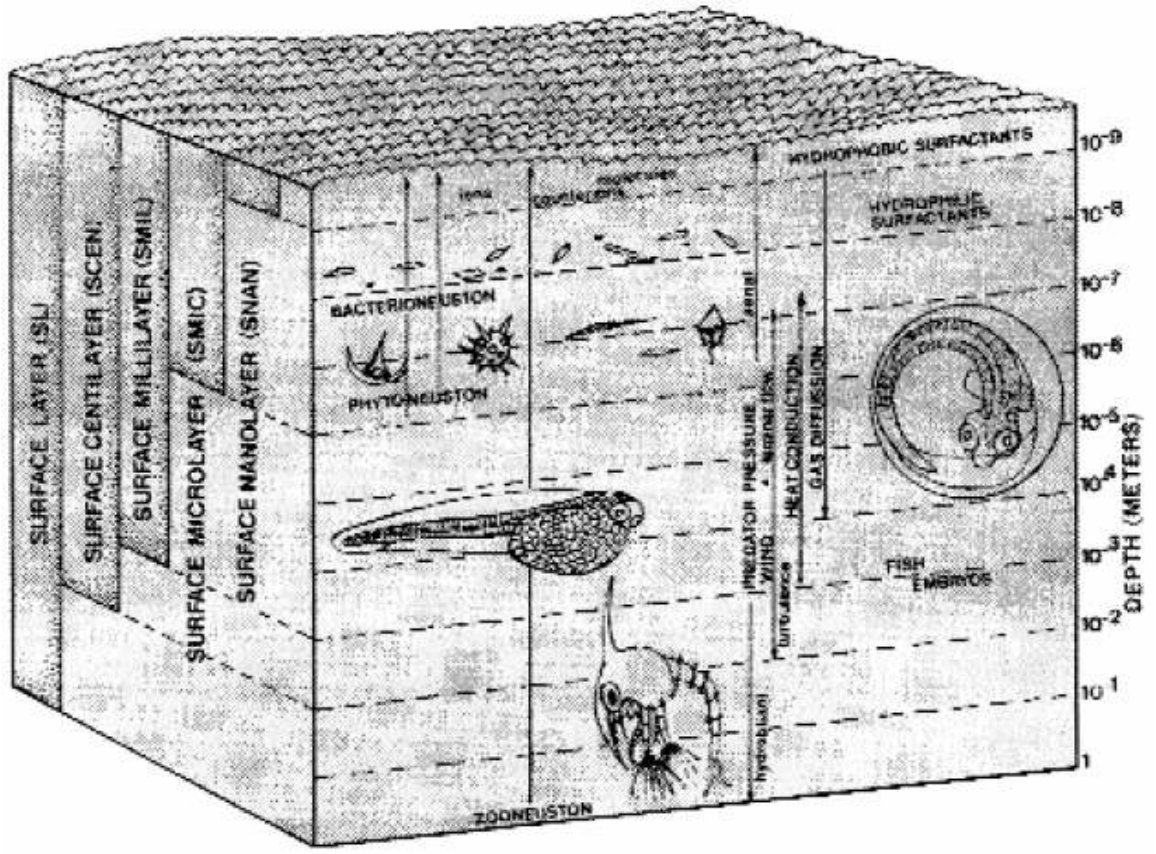

Figure 1.8. Schematic diagram of the aquatic surface layer. Extracted from Hardy and Word (1986).

We can also distinguish between permanent inhabitants of the SML, which include the bacterioneuston, phytoneuston, zooneuston and invertebrates, and temporary inhabitants, such as the eggs and larvae of several species of fish and invertebrates that occupy the surface for most of their larval or embrionary development (Hardy, 1991).

The surface microlayer has been found to contain abundant bacterial communities (Harvey, 1966; Sieburth, 1971; Tsyban, 1971; Sieburth et al, 1976; Münster et al, 1998), with estimates indicating enrichment factors, though variable with the time of the day, ranging from 10^3 to 10^5, in comparison to the underlying water (Bezdek and Carlucci, 1972).

The hydrophobicity of the biological cells probably plays a crucial role in the adhesion of bacteria to the surface microlayer, where the accumulation of great amounts of organic matter offers the nutritive substrate for their growth. However, evidence suggests that the communities at the surface film may, in fact, correspond to heterogeneous groups that include microheterotrophic assemblages, composed of dormant, inhibited or damaged populations, transported from other environments, and viable populations, physiologically adapted or involved in biological processes that occur *in situ* (CIESM, 1999).

Microbial Ecology at the Air-Water Interface

For bacteria, the SML can be an extreme environment, due to exposure to intense solar radiation in the ultraviolet and visible spectra, high concentrations of heavy metals and organic pollutants, temperature fluctuations and salinity changes. However, the SML also offers some advantages to microbial life, including high concentrations of organic and inorganic nutrients (Lion and Leckie, 1981). For example, the SML seems to be a habitat particularly favourable for phototrophic microorganisms because primary producers have unlimited access to carbon dioxide from the atmosphere and light, as well as mineral nutrients and metals that eventually accumulate at the SML (Lion and Leckie, 1981). The high content in organic compounds and particles provides a favourable substrate for the development of heterotrophic microorganisms (Drachev et al, 1965) that also take advantage of the high concentration of available oxygen in the atmosphere (Blanchard and Syzdek, 1970; Baylor et al, 1977; Lyalyuk and Lipnitskaya, 2003).

The enrichment of several heterotrophic communities at the SML, relatively to the UW (Hardy, 1997), is also documented. The enrichment factors vary widely from study to study, according to the specific location, differences in the depth of the sampled layer and type of sampler, among others (Lion e Leckie, 1981; Agogué et al, 2004).

Thus, from a biological point of view, we can considered that the air-water interface is an ecological niche globally hostile that offers, however, rewards to those capable of adapting to life there. Adaptative techniques for life at the surface include physiological adaptations such as chitin floaters, and behavioural adaptations, such as the adhesion to floating residues (David, 1965). Several blue-green algae associated with bloom development use their gaseous vacuoles for floating, so they can concentrate at the SML (Parker and Barsom, 1970). This feature allows them to effectively capture light, absorb gases and avoid hydrossoluble algaecides.

Several periphytic benthic organisms are capable of using surface marine films as habitat, and high concentrations of diatoms, dinoflagelates and green and blue algae can be found (Maynard, 1968). Vast amounts of neustonic choanoflagellates can also adhere to the SML through a protoplast protuberance or using appendages (Norris, 1965).

The microbial community also appears to have developed selective adaptations to life at the air-water interface. Higher frequencies of bacteria resistant to antibiotics and heavy metals at the air-water interface, comparatively with the water column, were initially attributed to the accumulation of toxic compounds in this layer (Hermansson et al, 1987). However, the role of the neuston communities in the transformation of chemical contaminants is still unknown probably due to the current inability to physically isolate the bacterial species able to transform these compounds (CIESM, 1999).

Protective pigmentation can also be a possible physiological mechanism used by microorganisms to allow life at the microlayer. Several pigmented bacterial strains (yellow, yellow-green, orange, brown and red) have been isolated from marine bacterioneuston (Tsyban, 1971). A selective enrichment of pigmented bacteria comparatively with non-pigmented bacteria

has also been observed in aquatic foam (Carlucci and Williams, 1965). Initially, the pigmentation was not considered as a significant factor for the enrichment of the microlayer, but indicative of a different population in the SML. However, further evidence suggested that pigmented cells of the marine bacteria *Serratia marcescens* were more likely (in relation to non pigmented cells) to stay in the surface of the bubbles and, thus, to be more easily transported from the subsurface solution to the microlayer (Blanchard and Syzdek, 1978).

The high abundance of pigmented isolates at the surface has also been interpreted as indicative of an adaptation to the intense solar radiation at the interface, since apparently the neustonic bacterial strains could sustain high metabolic activities, showing higher chances to survive to UVR, than isolates from the water column (CIESM, 1999). However, when bacterioneuston isolates were tested for solar radiation resistance, no correlation between resistance and pigmentation was found (Agogué et al, 2005b).

Adhesion of microorganisms to fractionated particulate materials can also contribute to the enrichment of the SML in bacteria (Harvey and Young 1980). Microscope observations using epifluorescence after acridine orange staining have demonstrated the existence of a large percentage of SML microorganisms associated with the surface of particles (Lion and Leckie, 1981). Apparently, the presence of particulate material in the solution can enhance the transport of bacteria in the bubbles to the surface of the water column (Carlucci and Williams, 1965)

However, there is some controversy about the levels of activity of the microbiota at the interface air-water. Bacterial enrichments of several orders of magnitude have been reported (Marumo et al, 1971), but inferior levels of ATP and heterotrophic activity, assessed by the incorporation of ^{14}C, have also been observed in the microlayer, in comparison to the bulk water (Dietz et al, 1976).

Being the microlayer an adverse environment one could expect, in the light of basic ecological principles, that its diversity should be reduced, being expectable that some bacterial genera would show high concentrations in the microlayer, probably even higher than those at the subsurface waters. However, results are highly contradictory, either supporting higher diversity at the SML (Taguchi and Nakajima, 1971) or at the underlying waters (MacIntyre, 1974). Nevertheless, there is a general agreement about the presence of increased microbial abundances in the populations concentrating at the surface microlayer (Lion e Leckie, 1981).

Thus, a variety of opposing ecological pressures operates at the air-water interface and despite the stressful conditions that occur in this environment, increased levels of organic substrates and nutrients reward the organisms adapted to life in the surface.

DIAGENESIS OF ORGANIC MATTER AT THE SML

The processes of compound (organic matter, inorganic matter and metals) transformation in the water are mainly photochemically driven, being the oceanic biogeochemical flux in their majority determined biologically. However, at the SML, the processes of chemical and biological transformation interact in a complex way, being obvious that this layer plays a crucial role in the interaction between the ocean and the atmosphere (Hardy, 1991). However, our limited understanding of the chemical and biological properties of the SML constraints further insights on the role of the surface microlayer in the biogeochemical fluxes (CIESM, 1999).

The SML accumulates higher concentrations of dissolved organic carbon (DOC) than those occurring in subsurface water layers (Williams et al, 1986; Carlucci et al, 1991), which together with its high bacterial abundance, contributes to the conversion of the solar energy that penetrates in the surface layer in other forms of energy that accumulate in the form of chemical compounds.

The SML shows daily changes of the light intensity passing through it, as well as in its chemical and biological composition, comparatively to the deeper layers of the water column (Zaitsev, 1971; Hardy, 1997). This is directly dependent upon the thickness of the interface, which is itself variable in freshwater and marine habitats, being influenced by the season and the stability of the superior water column (Hardy, 1997). Since the photosynthetic activity is higher during the day, higher concentrations of organic matter are expected to occur at the SML in this period. However, the existence of diel and seasonal patterns of biological activity and organic matter at the SML are still surrounded by some controversy (Lion e Leckie, 1981; Falkowska et al, 2005). Besides stimulating biological processes, the solar energy plays an important role in the processes of photodegradation of organic compounds of natural and anthropogenic origin that accumulate at the surface microlayer (Momzikoff et al, 1983), resulting in the formation of low molecular weight organic substances that can potentially be efficiently incorporated by bacteria (Kieber et al, 1990; Mopper et al, 1991; Obernosterer et al, 1999), but also radicals, such as hydrogen peroxide, that induce oxidative stress, having an inhibitory effect on bacterial activities (Momzikoff et al, 1983; Haag and Hoigné, 1986; Obernosterer et al, 1999).

At the SML, solar radiation, particularly in the UV-B range, has been associated with the inhibition of biological processes, including photosynthesis and respiration (Hardy e Apts, 1989) and alteration of the pools of organic matter. Recent studies have suggested the important role of the SML in the formation of free radicals and other reactive oxygen species (Whitehead and De Mora, 2000). Pronounced gradients of hydrogen peroxide (H_2O_2) have been detected in the surface layers of the water column (Zafiriou et al, 1984; Moffet and Zafiriou, 1990; Moffet and Zafiriou, 1993). Peroxide might interact with the microorganisms at the SML and ultimately lead to the inhibition of microbial activity (McFadzen e Cleary, 1994).

JUSTIFICATION AND THESIS OUTLINE

The surface microlayer is the region where exposure to UV-B radiation is maximum, particularly since photon backscatter from neuston and particules as well as multiple reflections caused by wave action can cause UV-B radiation to be disproportionately high in this layer (Regan, 1992). However, true estimates of the long-term effects of UV-B radiation on aquatic organisms (plankton) are extremely difficult and complicated by such factors as vertical mixing, photo-repair and photoadaptation (Behrenfeld et al, 1993a and b). Enrichment in bacterial numbers at the SML comparatively with the underlying water, suggests that the neuston communities might have developed adaptive strategies to life in this extreme environment, that may include UV resistance mechanisms. This makes it an interesting model-community for studies of UV-associated DNA damage, and mechanisms of protection and repair.

As the surface microlayer is being increasingly recognized as an important compartment governing biogeochemical cycles and impacting climate (CIESM, 1999), it seems crucial to understand how global changes, specially concerning the increase in UVR, will affect the functioning of bacterial communities in the SML and have repercussion on the recycling of organic matter in the aquatic environment.

However, very little is still known on the impacts of ultraviolet radiation on the metabolic activities and community structure of bacterioneuston and no studies have yet been conducted to comparatively assess these impacts on bacterioneuston and bacterioplankton net communities.

The aim of this work is to infer about the impact of solar radiation on the heterotrophic metabolic activities and community structures of bacterioneuston and bacterioplankton from field observations of two diel cycles conducted at Ria de Aveiro, Portugal. Chapter 2 describes this task.

Chapter 3 describes the characterization, quantification and comparison of the impact of artificial UV-B radiation on the heterotrophic metabolism and community composition of bacterioneuston and bacterioplankton from an estuarine system (Ria de Aveiro) and a freshwater system (Lake Vela) by means of microcosm experiments.

In Chapter 4 the results obtained are discussed. The main conclusions and suggestions for future work are also presented.

CHAPTER 2

DIEL VARIATIONS OF ABUNDANCE, HETEROTROPHIC ACTIVITY AND COMMUNITY STRUCTURE OF ESTUARINE BACTERIONEUSTON AND BACTERIOPLANKTON

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Abstract

In order to assess the effects of natural solar radiation on the abundance, bacterial heterotrophic activity (ectoenzyme activity and monomer incorporation rates) and community structure (DGGE) of estuarine bacterioneuston and bacterioplankton, samples were collected during two diel cycles from the surface microlayer (SML) and underlying waters (UW) of Ria de Aveiro (Portugal).
Of all the biological parameters tested, only for lipase activity statistically significant differences ($p<0.05$) were found between samples collected during the day and the night (~40%). These results were supported by DGGE analysis, that revealed fairly constant numbers of ribotypes during the diel cycle and no distinctive clustering of samples from the day or the night.
The absence of diel patterns in microbiological parameters does not allow the inference about the occurrence of mechanisms of light avoidance in bacterial communities. Rather it suggests the uncoupling of estuarine bacteria and primary production, and the probable dependence of microbial assemblages in Ria de Aveiro on allochthonous food sources.
As a proxy to compare the microbial activities between SML and UW during diel cycles, the enrichment factors (EF: ratio between microbiological parameters at the SML to the one on UW) of biological descriptors (total bacterial number, ectoenzyme activity and monomer incorporation rates) were determined. Although an overall tendency for enhanced hydrolytic activity and abundance at the SML was observed, only for β-galactosidase activity and glucose incorporation significant ($p<0.05$) differences were found. Furthermore, the occurrence of strong correlations between microbiological parameters at the SML and UW suggests that the SML may originate from the UW through upward passive transport of microorganisms from the UW. Our results also indicate that the EF is highly variable during the day, pointing out to the importance of the sampling time to comparative studies of bacterioneuston and bacterioplankton properties.

Keywords: bacterioneuston, bacterioplankton, diel cycle

INTRODUCTION

Bacteria are key components of the pelagic food web both in terms of biomass and activity, playing a pivotal role in nutrient cycling and energy flows in aquatic systems (see review by Azam and Malfatti, 2007). The fact that heterotrophic bacterioplankton can consume up to 60% of the total pelagic primary production (Fuhrman and Azam, 1982) has conducted to intensive research about the relations between phytoplankton and bacteria. Besides direct release from photosynthesizing phytoplankton (e.g. Larsson and Hagström, 1982), fixed carbon also becomes available to bacteria through "sloppy feeding" by zooplankton (Eppley et al, 1981; Kuipers et al, 2000) and release from detritus. However, mostly due to problems related with the time scale of the coupling between phytoplankton and bacteria, the relative importance of these

routes is unknown. The assessment of diel patterns might be useful in solving this problem, since tight coupling (i.e. with time scales of minutes to hours) is expected to result in significant diel variations of bacterial activity in response to varying photosynthesis (Falkowska et al, 2005).

A diel pattern on bacterial activity is expected, since phytoplankton exudation and zooplankton grazing activity show a diel periodicity (Fuhrman et al, 1985; Gasol et al, 1998). On the other hand, higher bacterial activity during the night period should occur if bacteria depend on the dissolved organic carbon (DOC) released by zooplankton by "sloppy feeding" (Kuipers et al, 2000).

The existence of a diel periodicity in bacterioplankton activity following with some delay phytoplankton activity has been suggested (Kisand et al, 1998; Van Wambeke et al, 2008).

Diel fluctuations of CO_2, oxygen and DOC in the water column of the tropical Atlantic have been related to diel variations in microbial activity, with DOC accumulating until late afternoon, as a result of phytoplankton extracelular release (Johnson et al, 1983). A similar diel pattern in DOC was found to occur in the upper mixed layer of the northern Adriatic Sea (Herndl and Malačič, 1987) where the highest bacterioplankton activity was also observed in the late afternoon. The natural occurrence of day-night variations in dissolved organic nutrient concentration, whose concentration declines as a result of assimilation by phytoplankton and increases due to regenerative activities by zooplankton and heterotrophic remineralization (e.g. Eppley et al, 1990; Wheeler and Kokkinakis, 1990). Amino acid flux, with higher ratios of amino acid utilization in daylight hours than at night, has also been found to couple with primary production (Carlucci et al, 1984).

Short term/diel variations of heterotrophic bacterial activity have been proposed to occur more intensively in oligotrophic environments (Gasol et al, 1998; Shiah, 1999; Church et al, 2004), due to coupling with photosynthesis (Van Wambeke et al, 2008). In these environments, the diel pattern of bacterial activity was primarily driven by the diel cycles of their nutrient sources (phytoplankton, seagrass, or both), whose production was highly light-dependent (Gasol et al, 1998). However, in coastal waters, consistent diel variations have not been found (Gasol et al, 1998; Shiah et al, 1999), indicating that bacterial activities in natural assemblages are governed by a variety of factors. Physical factors such as tides, temperature and salinity can also have a strong impact on bacterial abundance and activities over tidal and diel cycles, though their effects is often eclipsed by nutrient availability (Kisand et al, 1998).

Surface microlayers (SML) represent a specific interface separating the hydrosphere from the atmosphere (Franklin et al, 2005). Due to its variability in physical, chemical and biological properties compared to those of the underlying water (UW), it is well established that the SML is a unique environment for microbes (Williams et al, 1986). Nutrients, dissolved organic carbon (DOC), amino acids and other dissolved compounds are often enriched in the surface layer as a result of numerous physical and biological processes, including diffusion, turbulent mixing, and transport by rising bubbles or buoyant particles and *in situ* primary production. These conditions likely favour heterotrophic activity (Obernosterer et al, 2005).

However, the air-water interface is also considered to be a stressful environment for microorganisms inhabiting this compartment. Physical properties such as short-term variability in temperature and salinity, exposure to high intensities of solar radiation and a wide range of pollutants (including chlorinated hydrocarbons, organotin compounds, petroleum hydrocarbons and heavy metals) might retard overall metabolic activity in the SML (Wurl and Obbard, 2004). Thus, whether or not the SML represents a habitat of enhanced metabolic activity is determined by the balance between growth promoting and growth-inhibiting processes (Obernosterer et al, 2005).

Diel cycles of biological and related parameters in aquatic systems are frequently the manifestation of the effects of sunlight on biological processes (Cochlan et al, 1991). The SML is particularly exposed to solar environmental radiation, but it also concentrates photoautotrophic biomass, especially in the presence of visible biofilms (Williams et al, 1986) and exhibits enhanced heterotrophic activity (Obernosterer et al, 2005). Bacterial metabolic activities in the SML may be strongly connected to day-night cycles and follow diel patterns, probably in a more significant way at the underlying water. Diel patterns of microbiological parameters can also potentially correlate with behavioural strategies for the avoidance of the most harmful radiation (UV) occurring during the day, and can thus be used as a proxy to assess UV damage in the environment.

In the present study, diel patterns of heterotrophic metabolism, abundance and primary productivity in bacterioneuston and bacterioplankton in terms during diel cycles at Ria de Aveiro, Portugal, were studies with the aim of assessing the importance of sunlight as a driver of bacterial dynamics. The variability of the structure of bacterioneuston and bacterioplankton communities will also be analyzed by the culture-independent methods DGGE and FISH.

MATERIALS AND METHODS

DESCRIPTION OF THE STUDY AREA

Ria de Aveiro (Fig. 2.1.) is a tidal lagoon on the western coast of Portugal, connected to the Atlantic by a narrow opening covering an area of 66 and 83 km^2 at low and high tide, respectively (Silva, 1994). It exchanges with the sea a volume of water of 89 Mm^3 in tides of 1 to 3 m amplitude (Silva, 1994) and receives freshwater from several rivers, with an average water input of 1,8 Mm3 during a tidal cycle (Barrosa, 1985). The Ria has a complex topography, with several channels spreading from the mouth towards the different streams, forming a complex estuarine system.

SAMPLING

Samples were collected during two diel cycles at a fixed point at one of the main channels (Canal de Mira) of the estuarine system (Fig. 2.1). Sampling moments corresponded to high tide of the day (HT-D), low-tide of the day (LT-D), high-tide of the night (HT-N) and low-tide of the night (LT-N). Sampling was conducted with mild weather conditions. The samples

were taken from two water layers: SML and bulk underlying water (UW). SML samples were collected with a Plexiglas plate (Harvey and Burzell, 1972), which collects roughly the upper 60-100 μm water layer. The plate dimensions were 0.25 m wide x 0.35 m long and 4 mm thick. Before the sample collection, the Plexiglas plate was rinsed with ethanol, sterile distilled water and several times with water from the sampling site. The water adhering to the plate was removed from both sides by introducing the plate between two Teflon sheets and collected in a sterilized glass bottle. Samples from underlying water were taken directly into sterile glass bottles from the depth of approximately 20 cm. Samples were kept cold and in the shade during transport to the laboratory where they were processed within the next 2-3 h.

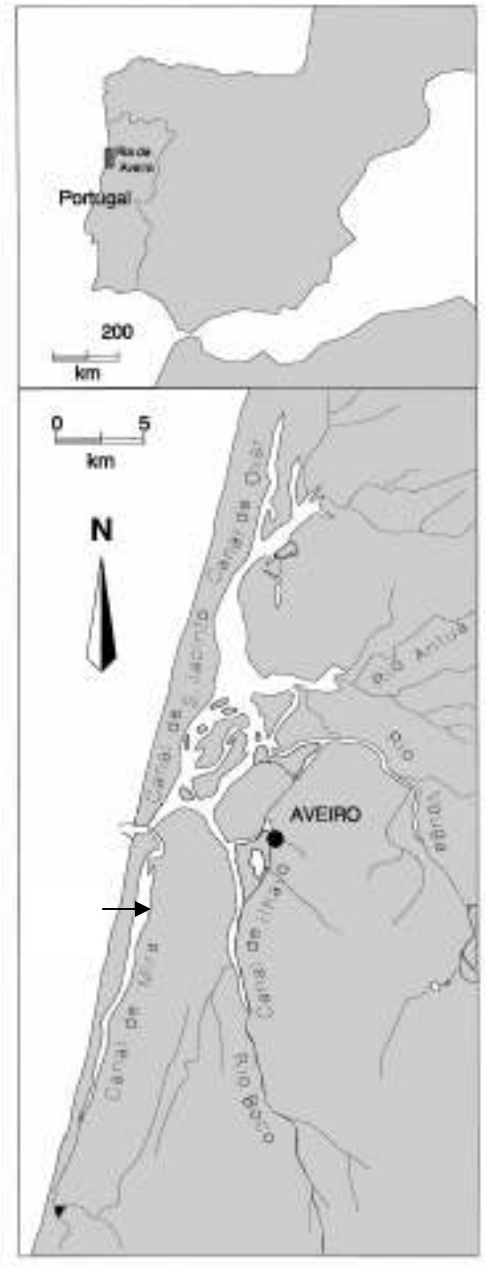

Figure 2.1. Ria de Aveiro (Portugal) with sampling station (CN) indicated by the arrow.

WATER PROPERTIES

Temperature and salinity were measured in the field using a WTW LF 196 Conductivity Meter. Dissolved oxygen expressed as the percentage of saturation, was also determined in the field with a WTW OXI 96 oxygen meter equipped with a WTW BR 190 stirrer. pH was measured in the laboratory, at 20ºC, with a pH probe (Orion, Model 290 A).

Chlorophyll *a* was estimated fluorimetrically (Yentsch and Menzel, 1963) after filtration of 0.05 L triplicate subsamples through Whatman GF/C filters and overnight cold extraction in 90% (v/v) acetone.

TOTAL BACTERIAL NUMBERS (TBN)

Bacterial cells were counted by epifluorescence microscopy using a Leitz Laborlux K microscope. The samples were fixed with 2% (v/v) formaldehyde (final concentration), filtered through 0.2 μm pore black polycarbonate membranes (Poretics), and stained with 0.03% (w/v) acridine orange (Hobbie et al, 1977). At least 200 cells or 20 microscope fields were counted in each of three replicate membranes.

FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

The abundance of α-proteobacteria and β-proteobacteria was quantified by FISH with Cy3-labeled oligonucleotide probes (MWG Biotech) as described by Glöckner et al (1996). Samples were filtered through 0,22 μm polycarbonate filters (GE Osmonics), fixed with 4% paraformaldehyde for 30 min and rinsed with PBS 1X and MilliQ water. The filters were reserved until hybridization.

The probes used in this study were Alf968 for the α-subclass of proteobacteria (Glöckner et al 1999), Bet42a for the β-subclass of proteobacteria (Manz et al, 1996), and a negative control probe for nonspecific probe binding (Karner and Fuhrman, 1997). For each probe a filter piece was placed on a Parafilm-covered glass slide and overlaid with 30 µL hybridization solution with 2.5 ng/µL of probe (final concentration). The hybridization solution contained 0.9 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.01% SDS, and the optimum concentration of formamide for each probe (Eilers et al, 2000; Zarda et al, 1997). Filters were incubated in sealed chambers at 46°C for 90m. After hybridization filters were washed for 20 min at 48°C in wash solution (20 mM Tris-HCl pH 7.4, 5 mM ethylenediaminetetraacetic acid, 0.01% SDS, and the appropriate concentration of NaCl) (Eilers et al, 2000; Zarda et al, 1997). Rinsed and dried filter pieces were counterstained with 2 µg/mL 4', 6-diamidino-2-phenylindole (DAPI) and mounted with Vectashield and Citifluor (1:4). Samples were examined with a Leitz Laborlux K microscope equipped with the appropriate filter sets for DAPI and CY3 fluorescence. At least 20 fields were counted per replicate of sample. All reagents were purchased from Fluka, except when indicated otherwise.

DNA EXTRACTION, PCR AND DGGE

Bacterial cells were collected on 0.2-mm-pore-size filters (Poretics Products Livermore, USA). DNA extraction was performed as described previously (Henriques et al, 2004). The V3 region of bacterial 16S rDNA fragments was amplified using the primers 338F_GC and 518R (Muyzer et al, 1993) as previously described by Henriques et al (2006). The reactions were carried out in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using Taq polymerase, nucleotides and buffer purchased from MBI Fermentas (Vilnius, Lithuania). DGGE was performed on a DCode^{TM} Universal Mutation Detection System (Bio-Rad). Samples containing approximately equal amounts of PCR amplicons were loaded onto 8% polyacrylamide gels (37.5:1, acrylamide/bisacrylamide) prepared in 0.5 x TAE buffer (20 mM Tris-acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM Na_2EDTA). Reagents were purchased from Bio-Rad, unless otherwise indicated. A denaturing gradient ranging from 35% to 60% (100% denaturant contains 7 M urea and 40% formamide) was applied to separate 16S rDNA fragments. Electrophoresis was performed at 60ºC, initially at 20 V (15 min) and then at 80 V (16 h). The gels were stained in an ethidium bromide solution (5 min) and then rinsed in distilled water (20 min). The image was acquired using a Molecular Image FX apparatus (Bio-Rad). The bands occupying the same position in the different lanes of the gels were identified. A binary (1/0) matrix was constructed taking into account the presence or absence of individual bands in

each lane. Cluster analysis was performed using the PRIMER v5 software (Clarke and Gorley, 2001). The binary matrix was transformed into a similarity matrix using the Bray Curtis measure. Dendrograms were generated using the group average method.

ESTIMATION OF ECTOENZYMATIC ACTIVITY

Ectoenzymatic activity was determined fluorometrically (Jasco FP-777 Fluorometer) as the maximum hydrolysis rate (Hm) of model substrates for leucine-aminopeptidase (L-leucine-7-amido-4-methylcoumarin), β-glucosidase (4-methylumbelliferyl-β-D-glucoside), lipase (4-methylumbelliferyl-acetate), β-galactosidase (4-methylumbelliferyl-β-D-galactoside), alkaline phosphatase (4-methylumbelliferyl-phosphtase) and sulphatase (4-methylumbelliferyl-sulphate), added each at the saturation concentration of 1 mM (Hoppe, 1983), chosen after kinetic analysis. Wavelengths for excitation and emission were respectively 380-440 nm for MCA (7-amino-4-metilcoumarone) and 365-460 nm for MUF (4-methyl-umbelliferone). Measurements were made in three replicates for each sample after 2 hours incubations at in situ temperature for lipase and leucine-aminopeptidase, 2-3h for β-glucosidase and around 6h for the remaining enzymes. Calibration was performed by adding a series of 6-8 concentrations of the fluorescent products (0-500 nM for MUF and 0-6 μM for MCA) to a pool of samples of the SML and UW.

HETEROTROPHIC METABOLISM OF MONOMERS (VM)

The heterotrophic metabolism of acetate, glucose and leucine was described by the parameter Vm (maximum uptake velocity) following the procedure described by Gocke (1977). A final saturation of 430 nM of each radiolabeled substrate was added to 5 mL aliquots of water samples. Substrate concentration was chosen after kinetic analysis. Incubations were carried out for 2 hours at in situ temperature. Cells were collected on 0.2 μm Poretics polycarbonate membranes and radioactivity was read in a liquid scintillation counter (Beckman LS 6000 IC) using UniverSol as scintillation cocktail. Radioactive labelled acetate, glucose and leucine (SA 11.5 GBq $mmol^{-1}$, 310 mCi $mmol^{-1}$) were obtained from Amersham.

STATISTICS

SigmaStat 3.5 was used for data analysis. Simple correlation analysis (Pearson coefficient of correlation) was used to calculate general correlations between prokaryote abundance (TBN), ectoenzyme activity, monomer incorporation rates and physical-chemical parameters. The occurrence of differences between water properties and microbiological parameters was assessed by 1-way ANOVA, using either Holm-Sidak or Tukey test.

RESULTS

WATER PROPERTIES

The range values of parameters describing water properties are summarised in Table 2.1.

Salinity ranged between 26.4 and 36.7 during the first diel cycle and 25.2 and 36.5 during the second diel cycle. Significant differences were not found between salinity at both compartments and during the day and the night (Table 2.1). Salinity was significantly higher at high tide (on average up to 40%, $p<0.001$, $n=8$).

Temperature varied between 16.3 and 23.7ºC during the first sampling moment and 15.7 and 22.1 ºC during the second sampling, being similar during the day and the night (Table 2.1). Temperature was ~20% higher ($p<0.05$; $n=8$) at low tide.

The concentration of dissolved O_2 ranged between 50.5 and 92.2 mg L^{-1} in the first sampling campaign and between 51.3 and 70.3 mg L^{-1} during the second diel cycle. During the day, the concentration of dissolved oxygen was only slightly (>10%) higher than at night (Table 2.1). Dissolved oxygen concentration was similar in both tidal conditions.

Table 2.1. Average values of salinity, pH, chlorophyll a concentration and total bacterial number (TBN) during two diel cycles at the SML and UW and average values of water temperature and dissolved oxygen concentration.

	1st Diel Cycle		2nd Diel Cycle	
	Day	**Night**	**Day**	**Night**
Temperature (ºC)	21.1	19.9	18.5	18.9
O_2 Concentration (mg L^{-1})	71.3	59.0	60.6	57.0
SML				
Salinity	32.0	31.7	30.5	30.8
pH	7.9	7.9	7.8	7.7
Chlorophyll a (μg L^{-1})	2.4	2.3	5.5	1.8
TBN (x10^5 cells mL^{-1})	18.3	16.6	10.2	8.6
UW				
Salinity	36.0	36.0	35.4	36.5
pH	7.8	7.8	7.9	7.9
Chlorophyll a (μg L^{-1})	3.5	2.2	9.4	3.3
TBN (x10^5 cells mL^{-1})	12.6	8.5	6.4	6.6

The values of pH were very similar in both sampling moments and between compartments, ranging from 7.7 to 7.9. pH was also similar during the day and the night (Table 2.1). No differences were found in the pH value between tides. Chlorophyll a concentration ranged between 1.6 and 3.5 μg mL^{-1} during the first diel cycle and between 1.1 and 9.4 during the second one. Chlorophyll a concentration was significantly higher ($p<0.001$) during the day (up to 2.5 times), reaching its maximum values during the second campaign. When comparing the averaged value of chlorophyll a in both compartments, significant differences were not found (Table 2.1). Chlorophyll a concentration was ~20% higher at low tide.

TOTAL BACTERIAL NUMBERS (TBN)

Total bacterial number ranged between $5.0x10^5$ and $2.2\ x10^6$ cells/mL. On average it was up to twice as high at the SML ($p<0.01$), and up to 20% higher during the day. TBN was on average 30% higher during low tide (Table 2.1). Total bacterial number was on average 40% higher in the second diel cycle ($p<0.05$).

FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

The results of the FISH analyses of α and β-proteobacteria are presented in Fig. 2.2. The relative abundance of α-proteobacteria (expressed as the percentage of total DAPI counts) ranged between 2.7 and 19.0%. The relative proportion of α-proteobacteria was ~25% higher at the SML and up to 40% higher during high tide ($p<0.05$). Significant differences between the abundance of α-proteobacteria during the day and the night were not found.

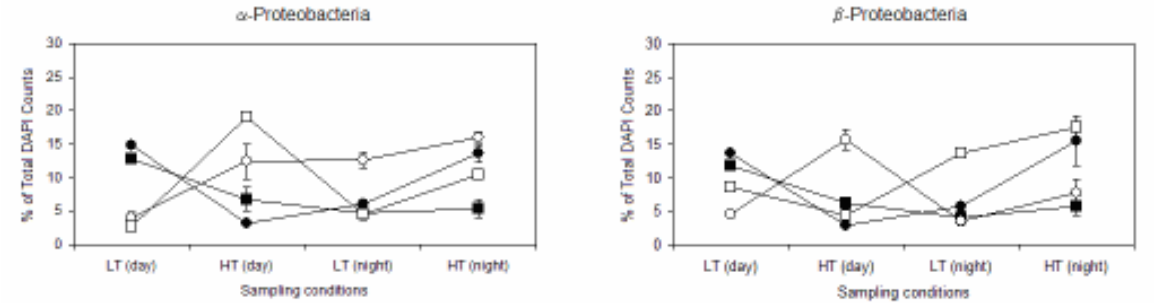

Figure 2.2. Relative abundance of α and β-proteobacteria (% of total DAPI counts) at the SML and UW in Ria de Aveiro at high (HT) and low (LT) tide during the two diel cycles. (● SML, 1 st. diel cycle; ■ UW, 1 st. diel cycle; ○ SML, 2 nd. diel cycle; □ UW, 2nd. diel cycle).

The proportion of β-proteobacteria ranged between 2.9 and 17.6%, being similar during the day and the night and at both the SML and UW. During low tide, the relative abundance of β-proteobacteria was up to 20% higher in comparison with high tide.

DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

Bacterial community structure was examined by comparing DGGE profiles of the PCR-amplified V3 region of the 16S rDNA from bacterioneuston and bacterioplankton collected during the different sampling moments. Bray-Curtis similarity index ranged between ~25% and ~90%, varying widely between sampling moments. In general, a distinctive grouping was not observed between samples collected during the day and the night.

Cluster analysis of the band patterns obtained from DGGE analysis (Fig. 2.3) revealed the occurrence of strong similarity (up to ~85%) between bacterioneuston and bacterioplankton collected at the same time. However, some exceptions were observed. Samples from bacterioplankton collected at different moments (high tide at nigh and low tide during day, both during the second diel cycle) were closely grouped (<60%). In bacterioneuston, one of the samples collected at night in high tide (second diel cycle) showed the lowest similarity with the rest (~20%).

The number of bands shared between bacterioneuston and bacterioplankton was widely variable, but in general higher in the second diel cycle (~20), than in the first cycle (~14) during the first diel cycle. The highest resemblance between the DGGE profiles was observed during the morning, while in the afternoon a lower number of bands shared between the two compartments was observed.

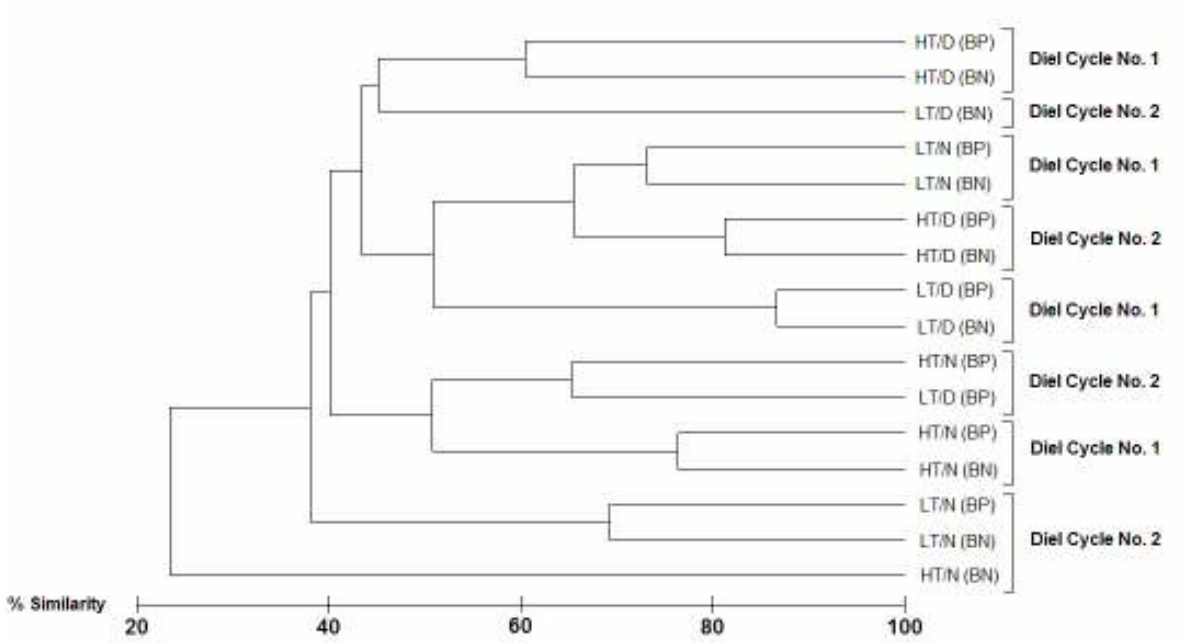

Figure 2.3 Dendrogram generated from the pattern of bands obtained by DGGE for bacterioneuston (BN) and bacterioplankton (BP) at the different sampling moments (HT, high tide; LT, low tide) of the day (D) and of the night (N) during the two diel cycles.

ECTOENZYMATIC ACTIVITY

Of all the hydrolases tested, aminopeptidase Hm (Fig. 2.4A) showed the highest values of activity varying between 1155.4 and 4269.5 nmol $L^{-1}h^{-1}$. Significant differences between aminopeptidase activity at the SML and UW were not observed. Aminopeptidase Hm values were on average 20% higher during the night and up to twice as high at low tide ($p<0.05$).

β-Glucosidase activity (Fig. 2.4B) rates (27.3-371.8 nmol L^{-1} h^{-1}) varied according to distinct profiles in each diel cycle, being significantly higher ($p<0.05$, Tukey Test) in the second diel cycle (on average up to 4 times higher). Overall rates showed that β-glucosidase activity was up to 30% higher at the SML and 15% higher during the day, though these differences were not statistically significant.

Lipase activity (Fig. 2.4C) exhibited the second highest maximum hydrolysis rates (Hm), ranging from 747.8 to 2851.3 nmol L^{-1} h^{-1} and averaged rates of activity were significantly higher during the night ($p<0.05$, Holm-Sidak Test), and on average up to 30%, and up to 40% higher during the second diel cycle. Significant differences were not found between the SML and UW.

β-Galactosidase activity (Fig. 2.4D) showed a wide variation between sampling conditions, ranging from 29.2 to 357.7 nmol L^{-1} h^{-1}. The overall rates of activity were similar during both diel cycles. The rates of β-galactosidase activity were significantly higher ($p<0.05$, Tukey Test) at the SML (on average up to 60%).

Phosphatase activity (Fig. 2.4E) also varied widely (2.0-216.7 nmol $L^{-1}h^{-1}$) during the diel cycles. Variability was stronger at the SML, showing variations of up to 60 times between sampling moments. On average, phosphatase activity was ~10% higher during the first diel cycle ($p<0.05$), 25% higher during the night and up to 50% higher at the SML.

Sulphatase Hm (Fig. 2.4F) ranged from 7.0 to 94.6 nmol L^{-1} h^{-1} being On average 40% higher during the day and at the SML and similar between the two diel cycles.

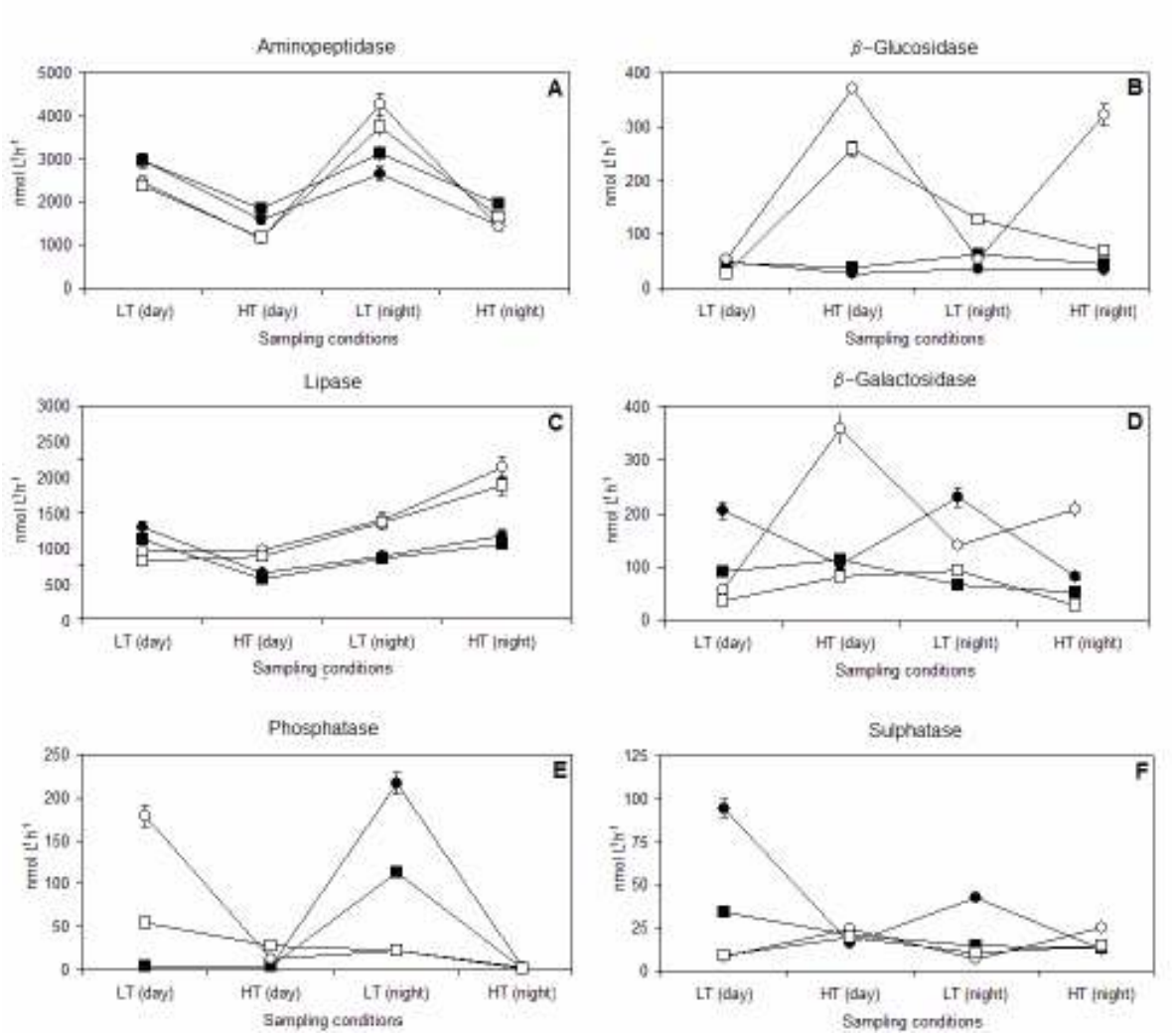

Figure 2.4. Variations in ectoenzyme activity at the SML and UW in Ria de Aveiro at high (HT) and low (LT) tide during the two diel cycles.(● SML, 1 st. diel cycle; ■ UW, 1 st. diel cycle; ○ SML, 2 nd. diel cycle; □ UW, 2nd. diel cycle).

MONOMER INCORPORATION

Acetate incorporation Vm (0.8-250.3 nmol L^{-1} h^{-1}) showed an overall similar trend during the two diel cycles, though a pronounced variation (up to 11 times) was observed at the UW, during the second diel cycle. On average, acetate incorporation was up to ~70% times higher at the UW and 60% higher during the day (Fig. 2.5A).

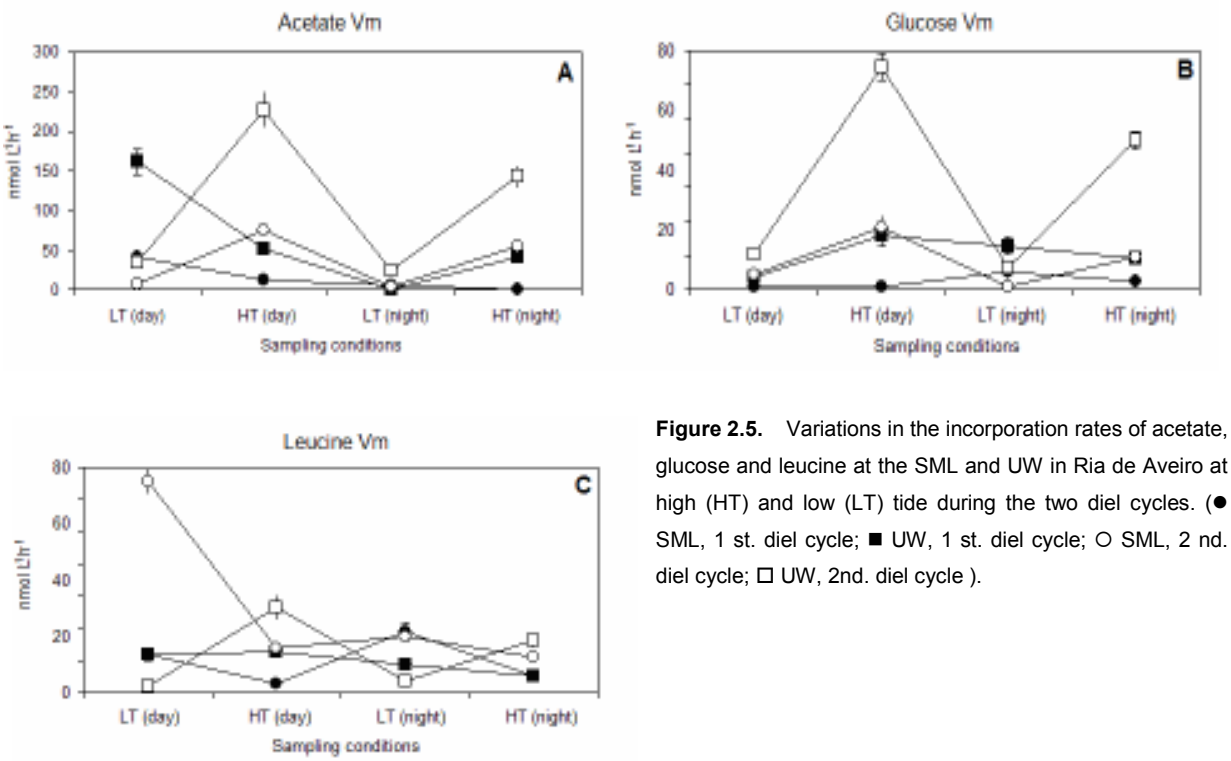

Figure 2.5. Variations in the incorporation rates of acetate, glucose and leucine at the SML and UW in Ria de Aveiro at high (HT) and low (LT) tide during the two diel cycles. (● SML, 1 st. diel cycle; ■ UW, 1 st. diel cycle; ○ SML, 2 nd. diel cycle; □ UW, 2nd. diel cycle).

Glucose incorporation (0.6-65.3 nmol L^{-1} h^{-1}), in accordance to what was observed for acetate, showed a similar pattern of variations during both diel cycles. The wider temporal variation was registered for the UW (10-fold variation). Glucose incorporation at the UW was up to 4 times higher than at the SML ($p<0.05$), and on average ~25% higher during the day, for both compartments (Fig. 2.5B).

The tidal variation of leucine incorporation (2.1-65.76 nmol L^{-1} h^{-1}) showed similar pattern of variation in both diel cycles. Differently from what was observed with the other monomers, leucine incorporation was 40% higher at the SML and 40% higher during the day (Fig. 2.5C).

CORRELATION BETWEEN MICROBIOLOGICAL PARAMETERS

Pearson product moment correlations between bacterial heterotrophic activities in both compartments are represented in Table 2.2. Relationships between SML and UW were examined on the basis of the whole data set for each parameter, revealing highly significant and positive correlations for all parameters except for the relative abundance of α- and β-Proteobacteria, β-Glucosidase, β-Galactosidase and leucine incorporation.

Table 2.2: Correlation coefficients between biological parameters measured in the SML and UW (Spearman's rank correlation). *p<0.05, **p<0.01, ***p<0.001. Proteo., Proteobacteria; Leu-AMP, leucine-aminopeptidase, β - Glc, β-Glucosidase; β-Galact., β-Galactosidase.

UW / SML	TBN	α-Proteo	β-Proteo	Chl a	Hm Leu-AMP	Hm β-Glc	Hm Lipase	Hm β-Galactosidase	Hm Phosphatase	Hm Sulphatase	Vm Acetate	Vm Glucose	Vm Leucine
TBN	0.738*	-	-	-	-	-	-	-	-	0.174*	-	-	-
α-Proteo	-	-	-	-	-	-	0.929***	-	-0.174*	-	-	-	-
β-Proteo	-	-	-	-	-	-	-	-	-	-	-	-	-
Chl a	-	-	-	0.929***	-	-	-	-	-	-	-	-	-
Hm Leu-AMP	-	-	-	-	0.958***	-	-	-	-	-	-	-	-
Hm β-Glc	-	-	-	-	-	-	-	-	-	-	-	-	-
Hm Lipase	-	-	0.738*	-	-	-	0.993***	-	-	-	-	-	-
Hm β-Galactosidase	-	-	-	-	-	0.810***	-	-	-	-	-	-	-
Hm Phosphatase	-	-	-	-	-	-	-	-	0.930***	-	-	-	-
Hm Sulphatase	0.905***	-	-	-	-	-	-	-	-	0.874*	-	-	-
Vm Acetate	-	0.762*	-	-	-	-	-	-	-	-	0.964***	-	0.810***
Vm Glucose	-	-	-	-	-	-	-	-	-	-	-	0.953***	-
Vm Leucine	-	-	-	-	-	-	-	-	0.786*	-	-	-	-

Correlations between physical and microbiological parameters were also assessed (Table 2.3). At the UW, aminopeptidase activity was found to negatively correlate with salinity ($r=-0.839$; $p<0.001$). pH negatively correlated with the incorporation of all the substrates tested ($-0.732 < r < -0.767$; $p<0.05$). At the SML, aminopeptidase activity also correlated negatively with salinity ($r=-0.860$; $p<0.01$). pH correlated positively with TBN ($r=0.765$; $p<0.05$) and negatively with β-glucosidase activity ($r=-0.814$; $p<0.05$). Finally, aminopeptidase activity at the SML was also found to correlate negatively with salinity at the UW.

Table 2.3: Significant correlations between physical parameters and microbiological descriptors at the SML and UW. Asterisks denote significant levels, where *p<0.05, **p<0.01, ***p<0.001

UW	**UW Salinity**	**pH**
Salinity		0.769*
pH		
TBN		
Hm Aminopeptidase	-0.839**	
Hm β-Glucosidase		
Vm Acetate		0.731*
Vm Glucose		0.740*
Vm Leucine		0.767*
SML		
SML		
Salinity		
pH		
TBN		0.765*
Hm Aminopeptidase	-0.860**	
Hm β-Glucosidase		0.814*
Vm Acetate		
Vm Glucose		
Vm Leucine		
UW		
SML		
Salinity		-0.783*
pH		0.757*
Hm Aminopeptidase	-0.828*	
Hm β-Glucosidase		
Vm Acetate		
Vm Glucose		
Vm Leucine		

DISCUSSION

Total Bacterial Number, Relative Abundance of Specific Prokaryote Groups and Community Composition

The variation of total bacterial abundance during the diel cycles in both the SML and UW was followed in order to detect signs of high avoidance strategies in bacterial communities. A distinctive diel pattern was not observed for bacterial abundance, though a decrease (~20%) of the averaged TBN from late afternoon to the night was observed, probably resulting from the

depletion of food sources to bacteria during the night (Gasol et al., 1998). The slight morning increase in TBN (~30%) is probably consequence of the increase in the DOM supply rate during the morning.

To study the existence of a diel pattern of the occurence of specific bacterial groups, the relative abundances of α-proteobacteria and β-proteobacteria was determined by FISH. These groups had been identified in previous studies as major groups in the bacterioplankton of the Ria, namely, dominating at the high and low salinity sections of the estuary, respectively (Henriques et al., 2006). Results from the variation of α- and β-proteobacteria during the sampling period, did not show significant differences between samples collected during the day or the night.

DGGE fingerprints of PCR amplified 16S rDNA were used as a tool to provide insights into temporal and vertical (surface vs subsurface samples) changes of estuarine bacterial communities because of its ability to compare a large number of samples with high reprodutibility. The samples used in this study were not subjected to pre-filtration, so templates other than bacterial DNA, such as plastids from phytoplankton, may have been amplified by PCR.

Bacterial community structure was examined by comparing DGGE profiles of the PCR-amplified V3 region of the 16S rDNA from bacterioneuston and bacterioplankton collected during the different sampling moments. The relevance of this technique to explore the structure of bacterial communities of Ria de Aveiro had already been demonstrated previously (Henriques et al, 2004; Henriques et al, 2006) and estimated by several preliminary tests. During this study, a consistent diel pattern did not emerge from DGGE data, which can indicate that the ribotypes responsible for active degradation of POM are probably constant throughout the day (Ghiglione et al, 2007). Further studies possibly envolving 16S rRNA analysis should be useful for further analysis of diel variation of bacterial assemblages (Moeseneder et al, 2001).

DIEL FLUCTUATION OF BACTERIAL HETEROTROPHIC ACTIVITY

In this study, leucine-aminopeptidase showed the highest activity rates of all the ectoenzymes tested (average = 2313.3 nmol L^{-1} h^{-1}), in accordance to what was previously reported for the Ria de Aveiro (Cunha et al, 2000) and other brackish water systems (Hoppe, 1983; Rego et al., 1985; Chróst and Velimirov, 1991; Hoppe et al., 1996, 1998). A consistent pattern of fluctuation of ectoenzymatic polymer hydrolysis and monomer incorporation could not be clearly established with this study, with the possible exception of lipase activity which was significantly higher during the night ($p>0.05$).

Of all the hydrolases tested, lipase showed the second highest potential activity level (average = 1494.55 nmol L^{-1} h^{-1}), pointing out to the relevance of lipid metabolism in estuaries (Mudryk and Skorczewski, 2004). Lipid compounds sometimes dominate in samples collected from polluted waters (Mudryk and Skorczewski, 2004) , and this can explain the high levels of lipidic activity. This can be the case of the estuarine site visited in this study which is exposed to a strong anthropogenic influence, mainly due to fishery related activities. Phytoplankton (either

alive or dead), zooplankton and macroflora are the main sources of lipids (Siuda et al., 1991; Nagata and Kirchman, 1992) and lipase activity correlates with lipid concentration. The higher levels of lipase activity during the night could result from lower abundance of other labile substrates available, such as polysaccharides, so that heterotrophic bacteria divert their metabolism to the most available substrate. The lipid pool can itself experience diel fluctuations in composition (Ghiglione et al., 2007). Lipids from phytoplankton origin (pigment and glycolipids) decrease from day to night, while lipids from zooplankton origin (triacylglycerols) increase (Ghiglione et al., 2007). The marked shift in bacterial heterotrophic metabolism towards lipid degradation from day to night may indicate that phytoplankton material was undergoing degradation in samples collected at night. Furthermore, at night zooplankton produce a large quantity of faecal pellets by intensive feeding on phytoplankton, resulting in an increase in the concentration of lipid originating from zooplankton that can be rapidly consumed by bacteria (Ghiglione et al., 2007).

Diel patterns of uptake of organic compounds have been described, namely for carbohydrates (Sieburth et al., 1977; Burney et al., 1982; Johnson et al., 1983), amino acids (Carlucci et al., 1984) and nitrogen (Wheeler et al., 1989). In this work we failed to detect distinctive and statistically significant diel patterns for the uptake of any of the substrates tested. Nevertheless, trends of increased monomer incorporation (up to 60%) during the day were noticed, probably reflecting the contribution of phytoplankton exudates to the low-molecular-weight DOM pool.

Although in open oceans, the main source of dissolved organic matter (DOM) is primary production, so tight relationships between phytoplankton and heterotrophic bacteria are expected, in coastal waters bacteria often process organic carbon originated mainly inland (Opsahl and Benner, 1998). In Ria de Aveiro, allochthonous organic matter has been shown to contribute with a significant fraction to the bacterial production even during the warmer season (Almeida et al., 2001). This variety of mostly allochthonous organic matter sources that, opposing phytoplankton primary production, does not show distinctive diel patterns, can possible account for the absence of consistent diel variations of heterotrophic bacterial activity. Similar results have also been shown by several other authors (Riemann and Sondergaard, 1984; Turley and Lochte, 1986; Gocke et al., 1987), almost exclusively in coastal areas.

COMPARATIVE ANALYSIS OF BACTERIAL DYNAMICS AT THE SML AND UW

Total bacterial abundance was significantly higher at the surface microlayer. Differences between the relative abundance of α- and β-proteobacteria in bacterioneuston were not statistically significant and DGGE showed a high resemblance between the dominant bacterial assemblages at the SML and UV, as already indicated by previous works (Agogué et al., 2005).

The results of this study indicate that there are differences in the levels of potential ectoenzymatic activity between enzymes in the SML and bulk water. In general, the activity of the studied ectoenzymes was higher at the surface than in UW, probably due to stimulation of extracelular enzyme hydrolysis by high concentrations of organic matter in surface waters

(Williams et al., 1986; Maki, 1993; Münster et al., 1998; Mudryk, 1998;). However, this study also revealed that this trend can vary during the day. The occurrence of enhanced or depleted heterotrophic activity at the SML can be related to the time of sampling (Obernosterer et al., 2005). These changing patterns of hydrolytic potential in both compartments can result from shifts in the availability of organic matter during the day (Carlucci et al., 1986, Williams et al., 1986, Agogué et al., 2004) and could help explain the contrasting results observed by several authors, in addition to the bias associated with the use of different sampling devices (Hardy, 1997; Agogué et al., 2004).

The rates of monomer incorporation were in general lower at the SML, though a statistically significant difference was only observed for glucose incorporation ($p<0.05$).

Pearson product moment correlation revealed strong positive correlations between microbiological parameters in the two compartments. The highest correlation was found between the values of lipase Hm ($r=0.993$; $p<0.001$). These strong correlations point to a high metabolic dependence between communities at both compartments (Joux et al., 2006).

REGULATION OF BACTERIAL DYNAMICS BY PHYSICAL AND CHEMICAL PARAMETERS

Physical and chemical factors may impose distinct temporal patterns on bacterioplankton dynamics. Temperature, as an abiotic factor, can be an important regulator of bacterial activity, especially below 10ºC (Wiebe et al., 1993). During this study, the temperatures observed were always much higher than this value and only few and poor correlations were observed between temperature and microbiological parameters. This suggests that temperature is probably a minor driver of bacterial dynamics during tidal and diel cycles.

The negative effect of salinity on bacterial heterotrophic activity may determine the shape of the tidal profiles of variation (Murrel et al, 1999). In this study, the negative relation with bacterial activity was only significant for aminopeptidase. Since this activity is generally considered rather tolerant to salinity, this relation may indicate that the supply of proteinaceous material is associated with low salinity water masses reaching the high salinity sections through tidal transport during ebbing.

Solar radiation, particularly UV radiation, can impose a chronic stress on marine bacteria, resulting in significant inhibition of DNA and protein synthesis, monomer incorporation and ectoenzyme activities (Herndl et al., 1993; Karentz et al., 1994). UV affects bacterioplankton more than phytoplankton (Jeffrey et al., 1996) and leads to an accumulation of DNA damage during the period of intense solar radiation.

Heterotrophic bacteria depend, at least in part, on primary productivity, which exhibits a distinctive diel pattern. Thus, the variation of bacterial heterotrophic activity depends on the balance of primary production and the direct and indirect effects of solar radiation on phytoplankton and bacterioplankton. In this study, a significant correlation between chlorophyll a and bacterial activity was not found. This suggests that bacterial communities may be using mostly allocthonous nutrient sources. On estuaries like Ria de Aveiro, the concentration of

allochthonous organic mater, which does not follow a diel pattern, is important in substrate supply for bacterioplankton (Almeida et al., 2001). Thus, if bacteria depend on allochthonous organic matter rather than authocthonous sources, bacterial heterotrophic activity does not necessarily follow a diel pattern.

The detection of diel cycles in bacterial activities can also be compromised by several factors, in addition to the uncoupling between phytoplankton primary production and bacterioplankton metabolism. Photolytic cleavage of DOM by UV produces a whole suite of compounds (Scully et al., 2003; White et al., 2003), which can be efficiently taken up by bacterioplankton, leading to enhanced bacterial activities (Lindel et al, 1996). This indirect positive effect of solar radiation may induce increased monomer incorporation (especially acetate and glucose). Light can also induce a negative effect caused by the production of detrimental photoproducts of DOM light-induced degradation that can result in reduced bacterial activity (Helz et al., 1994). In this work, detrimental effects of light were probably responsible for the decrease in the averaged ectoenzyme activity (lipase, phosphatase and leucine-aminopeptidase) during the day.

Ria de Aveiro is a tidal estuary and in this work a tidal cycle is superimposing on the diel cycle. Tides have obvious effects on bacterial activity and abundance. In this work, significant differences between tides ($p<0.05$) were observed for some microbiological parameters (relative abundance of α-proteobacteria, alkaline phosphatase and aminopeptidase activity), that probably compromised the detection of consistent diel patterns in these parameters.

Some other microbiological descriptors (most notably the relative abundance of α and β-proteobacteria, the rate of leucine incorporation and lipase activity) did not vary according to a consistent tidal pattern, suggesting that the outcome of the combination of diel and tidal cycles may be highly variable and different for the kind of community atributes that is described by each parameter.

CONCLUSIONS

The results from this work show that the detection of diel patterns of bacterioplankton and bacterioneuston abundance and activity, as an indication of the relative importance of light as an ecosystem driver, is difficult in Ria de Aveiro. The allocthonous inputs of organic matter can decouple bacterial metabolism from primary production. Furthermore in tidally affected areas, like Ria de Aveiro, diel and tidal cycles combine, difficulting the discrimination between them. However, the existence of averaged day-time maxima for several of the biological parameters tested, suggests that bacteria may respond more strongly to the direct and indirect effects of light intensity than to changes in tidal conditions.

The heterotrophic activity and abundance of bacterioneuston and bacterioplankton is highly variable throughout the day, probably responding to changes in nutrient availability in both compartments. However, the occurrence of strong, positive correlations between the values of the biological parameters (Hm and Vm) in the SML and UW suggests that metabolic

activities in both compartments could be closely interdependent. One important aspect that arises from these investigations is the relevance of the moment of sampling to the comparison between bacterioneuston and bacterioplankton communities, since their relative abundance and activity may vary considerably during the day.

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CHAPTER 3

EFFECTS OF ARTIFICIAL ULTRAVIOLET RADIATION ON ABUNDANCE, DIVERSITY AND ACTIVITY OF BACTERIONEUSTON AND BACTERIOPLANKTON

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ABSTRACT

The impact of ultraviolet-B (UV-B) radiation on the abundance, ectoenzymatic activity and monomer incorporation was assessed in bacterioneuston and bacterioplankton communities from Ria de Aveiro (Portugal) and compared to those of freshwater bacterial communities from Lake Vela (Portugal).
Exposure to artificial UV-B radiation (0.4 W m^{-2}) for 9 hours led to 16-90% declines in ectoenzymatic activity ranging from 16 to 90% and a 80 to 100% decrease in monomer incorporation. A stimulation of lipase activity upon exposure to UVR was observed, probably related to the production of more labile compounds through the phototransformation of DOM.
The ability of estuarine bacteria to recover from UV-induced stress was also assessed by incubating the samples in the dark, after UV irradiation. Following irradiation, ectoenzymatic activity showed recovery rates ranging from 10 to 100%, while for monomer incorporation this recovery was only 40%.
In general, bacterioneuston was more affected by UVR exposure than bacterioplankton. However, following the phylogenetic diversity of bacterial communities from surface (SML) and underlying waters (UW) by DGGE showed that upon the same period of exposure to UVR, the reduction of the number of OTUs (operational taxonomical units) in bacterioplankton was much higher than in bacterioneuston. This can suggest that bacterioneuston may be more adapted to high intensities of UV radiation occurring in the environment.

Keywords: ultraviolet radiation (UVR), bacterioneuston, bacterioplankton

INTRODUCTION

Most studies of UV effects on aquatic systems have been focused on primary producers (Helbling et al, 1992; Helbling et al, 2001; Villafañe et al, 2003). However, the information on the role of UV radiation on the microheterotrophic components of the aquatic food webs is still scarce, though evidence suggests that UV-R may impose a chronic stress on bacteria (Herndl et al, 1993; Lesser, 2006). Some studies also show that heterotrophic bacterioplankton are more sensitive than phytoplankton to UV-stress, due to their small size and short generation times (Garcia-Pichel, 1994) and the absence of UV absorbing pigments (Karentz et al, 1994).

UV radiation of different wavelengths is responsible for distinct types of damage. UV-A radiation (320 to 400 nm) results in indirect damage to cellular DNA, proteins, and lipids through the formation of chemical intermediates such as reactive oxygen species (ROS) (Tyrrell, 1992). UV-B (280 to 320 nm) radiation is responsible for direct DNA damage by inducing the dimerization of DNA bases, leading to the formation of DNA photoproducts, being the cyclobutane pyrimidine dimers and the pyrimidine-pyrimidone (6-4) photoproduct the most common (Mitchell and Karentz, 1993; Häder and Sinha, 2005).

Experimental exposure of marine bacteria to natural solar UV radiation results in direct effects ranging from reduced total cell abundance (Müller-Niklas et al, 1995; Pakulski et al, 1998), decreased amino acid uptake (Bailey et al, 1983) and oxygen consumption (Pakulski et al, 1998), to inhibition of DNA (Aas et al, 1996), protein synthesis (Herndl et al, 1993) and ectoenzyme activities (Herndl et al, 1993; Müller-Niklas et al, 1995). UV-R may also affect the activity of bacteria responsible for biogeochemical transformations in carbon, nitrogen and phosphorus cycles (Hooper and Terry, 1974; Horrigan et al, 1981).

UV-R may also impose indirect effects on aquatic bacteria, due to the differential sensitivity of bacterioplankton grazers (Sommaruga et al, 1996). Photoaltered bioavailability of dissolved organic matter (DOM) (Obernosterer et al, 1999; Obernosterer et al, 2001) can also affect aquatic bacteria, since upon exposure to solar radiation, DOM may form many different products including low molecular weight compounds (Kieber et al, 1989), which are efficiently taken up by bacterioplankton (Lindel et al, 1995; Wetzel et al, 1995). In fact, enhanced bacterial activity in several aquatic environments has been reported has a result of phototransformations of DOM (Kieber et al, 1989; Lindell et al, 1995; Benner and Biddanda, 1998), and a combination of photochemical and microbial processes may increase the degradation of DOM up to three times (Miller and Moran, 1997). However, the photolysis of DOM can also result in the production of reactive radicals, such as oxygen and hydroxyl radicals (Miller, 1994; Zepp et al, 1995) leading to an overall retardation in the growth of bacterioplankton. Hence, in the aquatic ecosystems the action of UV radiation on DOM present in the surface water layer modifies the pool of organic matter through the photochemical production of compounds that promote and inhibit the growth of bacterioplankton (Kaiser and Herndl, 1997).

Due to its peculiar location, the bacterioneuston inhabiting the top layer of the water surface is naturally exposed to high levels of solar radiation, particularly in the UV spectra. However, the surface microlayer has been reported to have higher microbial abundances and enhanced microbial activity in comparison to the underlying waters (Norkrans, 1980; Agogué et al, 2004; Hardy, 1982). This suggests a possible adaptation to hostile conditions, including UV radiation.

The objective of this work is the characterization and quantification of the effects of artificial UV radiation on bacterioneuston and bacterioplankton described in terms of abundance, diversity and heterotrophic activity (ectoenzymatic activity and monomer incorporation). A comparison of the differential responses of freshwater and marine communities to artificial UV-B radiation was also attempted.

MATERIALS AND METHODS

DESCRIPTION OF STUDY AREA

Ria de Aveiro (Fig. 3.1) is a 45 km long and 10 km large estuarine system located at the northwest Atlantic coast of Portugal (40°5´N, 8°8´W). The main freshwater supply is provided by two main rivers: the Antuã river (5 m^3 s^{-1} average flow) and the Vouga river (50 m^3 s^{-1}) (Dias et al, 1999). The Ria has a very irregular and complex topography, with long and narrow channels

The deepest areas of the lagoon correspond to the inlet channel and to small areas close to its mouth, at the western boundary of the lagoon, where the depth may reach values of the order of 30 m. Elsewhere, at the inner parts of the lagoon, the depths, in general, >3m, and most frequently close to 1 m, namely at the upper sections of the lagoon. The estimated lagoon tidal prism is 136.7×106 m^3 for maximum spring tide and 34.9×106m^3 for minimum neap tide (Dias et al, 1999).

Lake Vela (Fig. 3.1), a temperate shallow lake (mean depth = 0.9 m, maximum depth = 2.4 m) with a total volume of 70 × 104 m^3, is located in the littoral-centre of Portugal (40°5´N8°8´W, 45 m elevation). It is surrounded by agricultural fields and some human settlements in its East margin and by a forest road in the West margin, separated from the lake by a pine tree (*Pinus pinaster*) and acacia (*Acacia* spp.) forest. It is a eutrophic system, mainly because of regular nutrient inputs from agriculture (Abrantes et al, 2006). It is characterised by a permanently turbid water column and blooms of cyanobacteria are frequently observed, including some toxic species, such as *Microcystis aeruginosa* (Vasconcelos et al, 1993; Barros, 1994; Fernandes, 1999).

SAMPLING

Sampling took place weekly during the month of November at estuarine site CN (Fig. 3.1), and at the northwestern section of Lake Vela (LV), near the arboreal vegetation area (Fig. 3.1). SML (surface microlayer) samples were collected with a 0.25 m wide x 0.35 m long and 4 mm thick Plexiglas plate (Harvey and Burzell, 1972), which removes roughly the upper 60-100 μm water layer. Samples were collected either in the early morning (08:00) or at noon (13:00). Samples from underlying water (0.2 m depth) were collected by submerging an acid cleaned 2L dark glass bottle. Water samples were kept cold and in the shade during transport to the laboratory, where experiments were performed within 1 hour after collection.

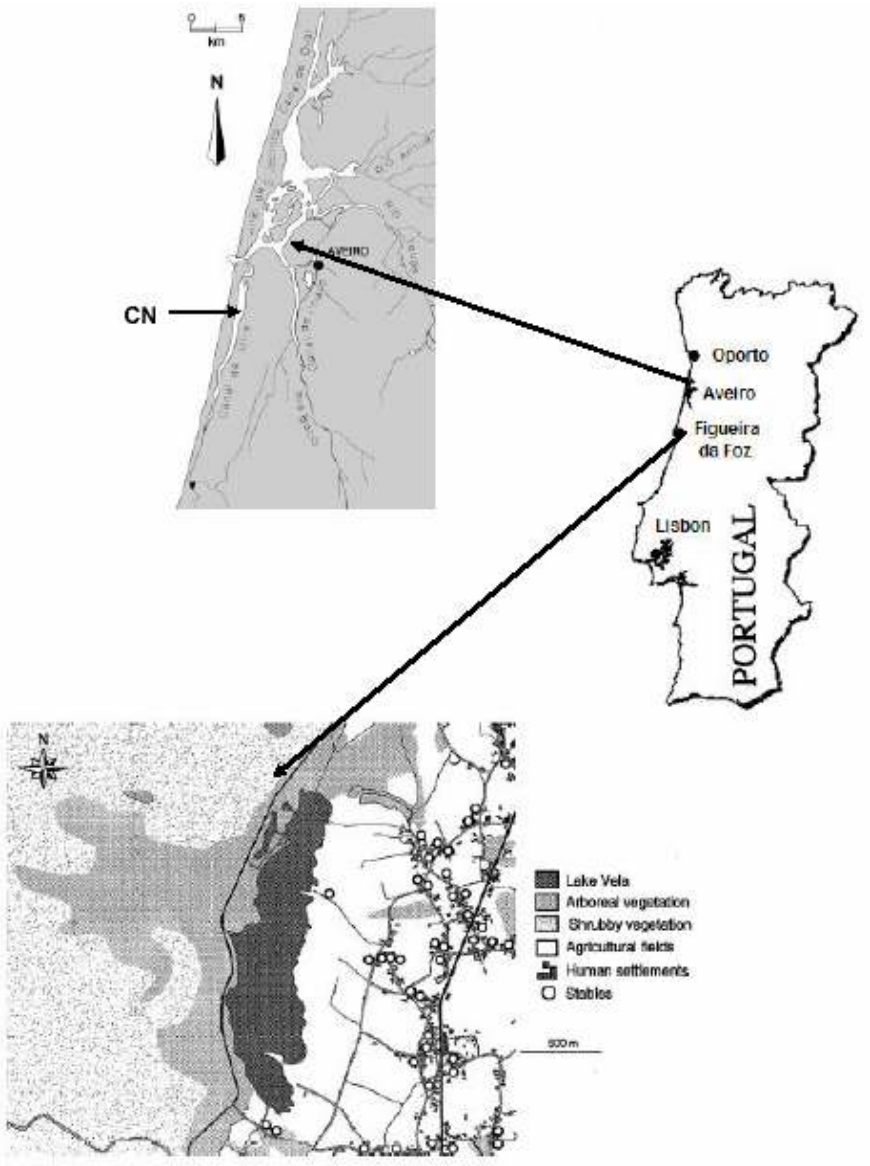

Figure 3.1. Ria de Aveiro lagoon, with sampling site CN indicated by the arrow and Lake Vela (Portugal).

Temperature and conductivity were measured in the field using a WTW LF 196 Conductivity Meter, while dissolved oxygen concentration was measured with a WTW OXI 320 oxygen meter.

Chlorophyll *a* was estimated fluorimetrically (Yentsch and Menzel, 1963) at the laboratory after filtration of 0.05 L triplicate subsamples through Whatman GF/C filters and overnight cold extraction in 90% (v/v) acetone.

EXPERIMENTAL SETUP

For the assessment of the UV-effects on estuarine and freshwater bacterioneuston experiments were conducted in a transparent Plexiglas container with an overall capacity of approximately 3 L. Whole water samples were placed under a UV-B lamp (Philips, UV-B TL 100 W/01; emission peak at 311 nm; intensity of 0.4 W m^{-2}) suspended 20 cm above the water

surface and experiments were conducted at room temperature. Before the beginning of each experiment the lamp was pre-burned for 1h to ensure light emission stability.

Three different irradiation experiments were conducted for estuarine samples: (i) 9-hour irradiation with artificial UVR; (ii) 6-hour irradiation with artificial UVR of pre-adapted samples and (iii) 6-hour irradiation followed by 6-hour dark recovery. For freshwater samples only condition (i) was tested. For experiment (i) samples collected in the early morning were exposed to artificial UVR during 9 hours and sample aliquots were collected every 3 hours and analysed for microbiological descriptors. For experiment (ii) samples collected at noon were exposed for artificial UVR as described for experiment (i). For experiment (iii) samples were exposed to UVR during 6 hours, followed by a 6 hour period of incubation in the dark, and aliquots were collected every 2 hours. Dark controls were included in each experiment.

DETERMINATION OF ECTOENZYMATIC ACTIVITY (HM)

The maximum hydrolysis rates (Hm) of model substrates was determined fluorometrically (Jasco FP-777 Fluorometer) for lipase (4-methylumbelliferyl-β-acetate), β-glucosidase (4-methylumbelliferyl-β-D-glucoside) and leucine-aminopeptidase (L-leucine-7-amido-4-methylcoumarin) after adding substrates at the saturation concentration of 1 mM (Hoppe, 1983), determined after kinetic analysis. For MCA the wavelengths for excitation and emission were respectively 380-440 nm (7-amino-4-metilcoumarone) and 365-460 nm for MUF (4-methyl-umbelliferone). For each sample, measurements were done in triplicate after 2 hours incubations at *in situ* temperature. A series of 6-8 concentrations of the fluorescent products (0-500 nM for MUF and 0-6 μM for MCA) were added to a pool of samples of the SML and UW to calibrate the procedure.

HETEROTROPHIC METABOLISM OF MONOMERS (VM)

The maximum rate of incorporation (Vm) of acetate, glucose and leucine was Was conducted according to the procedure described by Gocke (1977) in 3 replicates and 1 blank of each sample, using a final saturation concentration of 430 nM of labelled 14C-monomers (Amersham SA 11.5 GBq $mmol^{-1}$, 310 mCi $mmol^{-1}$) added to 5 mL aliquots. The saturating concentration was chosen after kinetic analysis. Incubations were carried out for 2 hours at *in situ* temperature, in the dark, without agitation. Cells were collected on 0.2μm Poretics polycarbonate membranes and radioactivity was read in a liquid scintillation counter (Beckman LS 6000 IC) using UniverSol as scintillation cocktail.

TOTAL BACTERIAL NUMBER (TBN)

Total bacterial number (TBN) was determined by cell counting under epifluorescence microscopy after fixation of water samples with 2% formaldehyde (final concentration). The samples (3 replicates) were then filtered through 0.2 μm black polycarbonate membranes (Poretics) and stained with 0.03% acridine orange (Hobbie et al 1977). Bacterial cells were counted by epifluorescence microscopy using a Leitz Laborlux K microscope.

DNA EXTRACTION, PCR AND DGGE

Bacterial cells collected on 0.2-mm-pore-size filters (Poretics Products Livermore, USA). Total DNA from water samples was extracted after filtering 150 mL of the sampled water through 0.22-mm polycarbonate filters Collected cells were resuspended in 2mL of TE buffer [10mM Tris HCl, 1mM ethylenediamine tetraacetic acid (EDTA), pH 8.0] and centrifuged. After resuspension in 200 mL TE, 1 mg mL^{-1} lysozyme solution was added to induce cell lysis and incubated at 37ªC for 1 h according to the procedure described by Henriques et al (2004). DNA extraction was performed using the genomic DNA purification kit (MBI Fermentas). DNA was resuspended in TE buffer and stored at –20ºC.After staining with ethidium ethidium bromide solution (5 min) and then rinsed in distilled water (20 min), gel images were acquired using a Molecular Image FX apparatus (Bio-Rad).

The impact of artificial UV-B radiation on the diversity of bacterial communities was assessed by determination of the number of bands, i.e., operational taxonomic units (OTU), in DGGE images from samples before and after the irradiation period of either 6 or 9 hours.

RESULTS

PHYSICAL AND CHEMICAL PARAMETERS

Results of the physical and chemical characterization of the water samples are presented in Table 3.1. At the estuarine sampling site CN, conductivity varied between 30.5 and 45.3 mS cm^{-1}. Dissolved oxygen concentration ranged from 3.9 to 7.5 mg/L, temperature between 16.0 and 17.7ºC and pH between 7.6 and 7.9. Chlorophyll a concentration varied between 2.86 and 3.99 $\mu g\ L^{-1}$.

Table 3.1. Conductivity, temperature, pH, dissolved oxygen concentration and chlorophyll a concentration at the SML and UW during the sampling period at the estuarine (CN) and freshwater (LV) sites averaged from the several sampling moments. Experiment (i) refers to 9-hour irradiation with artificial UVR; (ii) 6-hour irradiation with artificial UVR of pre-adapted samples and (iii) 6-hour irradiation followed by 6-hour dark recovery.

Experiment	Origin	Layer	Conductivity (mS cm^{-1})	Temperature (ºC)	pH	O_2 Concentration (mg L^{-1})	Chl a ($\mu g\ L^{-1}$)
(i)	Estuarine	SML	45.3	16.25	7.6	7.45	3.83
		UW	44.5		7.6		3.61
	Freshwater	SML	444.4	14.25	8.7	9.15	17.65×10^4
		UW	448.8		8.7		7.5×10^4
(ii)	Estuarine	SML	40.4	17.50	7.6	3.90	3.19
		UW	38.3		7.7		2.86
(iii)	Estuarine	SML	32.6	16.55	7.9	6.75	3.99
		UW	38.3		7.8		3.37

At the freshwater site LV (Table 3.1) pH varied between 8.3 and 9.6 mg L^{-1}, the dissolved oxygen concentration between 7.9 and 9.2 mg L^{-1} and the temperature between 14.7 and 13.9. Conductivity ranged from 431.6 and 462.3 mS cm^{-1} . The concentration of chlorophyll a ranged from $7.5x10^4$ and $17.65\ x10^4\ \mu g\ L^{-1}$.

9-HOUR EXPOSURE TO ARTIFICIAL UV-B

The experimental exposure of estuarine bacteria to artificial UV-B radiation led to a decrease in bacterial abundance (expressed as percentage of the dark control) of 33% and 24% for the surface and subsurface community, respectively, after a 9-hour irradiation period (Fig. 3.2A). Exposure of freshwater bacteria to a 9-hour irradiation period led to a decrease in TBN of ~32% in both communities (Fig. 3.2B).

UVR exposure was accompanied by a substantial reduction of bacterial community diversity as revealed by the number of bands present in DGGE profiles (Table 3.2) was observed. A 9-hour irradiation period of estuarine bacterioplankton led to a decrease of ~70% in diversity expressed as number of ribotypes), comparatively with the dark control. The effects on estuarine bacterioneuston were less marked with a reduction of diversity of ~30%. The exposure of freshwater bacterioplankton and bacterioneuston to 9 hours of artificial UV-B radiation led to a reduction of ~50% in diversity, in both communities.

Table 3.2. Summary of the number of OTUs detected by DGGE in experiment (i).

Experiment	Origin	Layer	T_0	Irradiation	Dark Control
(i)	Estuarine	SML	36	24	36
		UW	35	11	35
	Freshwater	SML	47	24	47
		UW	37	20	37

Exposure of estuarine and freshwater bacteria to artificial UV-B (0.4 W m^{-2}) led to an overall reduction in ectoenzymatic activity. For estuarine bacterioneuston (Fig. 3.2C), β-glucosidase and leucine-aminopeptidase decreased by 45 and 90%, respectively, after 9 hours of irradiation. Lipase activity decreased by 23% after a 3-hour exposure, but increased more than 50% relatively to the initial value, during the remaining irradiation period (Fig. 3.2C).

When exposed to UV-B, β-glucosidase activity of estuarine bacterioplankton (Fig. 3.2D) declined by 16% after 3 h, while the same period of exposure led to a decrease of 90% in leucine-aminopeptidase activity. Upon exposure to UV radiation, lipase activity increased by up to 24% after 9 h of irradiation, in relation to the initial value.

Freshwater bacterioneuston (Fig. 3.2E) showed a reduction of enzymatic activity for all the ectoenzymes tested. After 9 hours of exposure to artificial UV-B radiation lipase decreased by 46%, while glucosidase and leucine-aminopeptidase decreased by 82 and 92% of the initial value, respectively.

Freshwater bacterioplankton (Fig. 3.2F) decreased by 35, 57 and 68% in relation to the initial value for lipase, glucosidase and aminopeptidase, respectively, after 9 hours of exposure.

Exposing estuarine bacterioneuston to artificial UV-B radiation resulted in a significant decrease in the monomer incorporation rates (acetate, glucose, lipase). After a 9-hour irradiation period, acetate, glucose and leucine incorporation were inhibited by 99% (Fig. 3.2G).

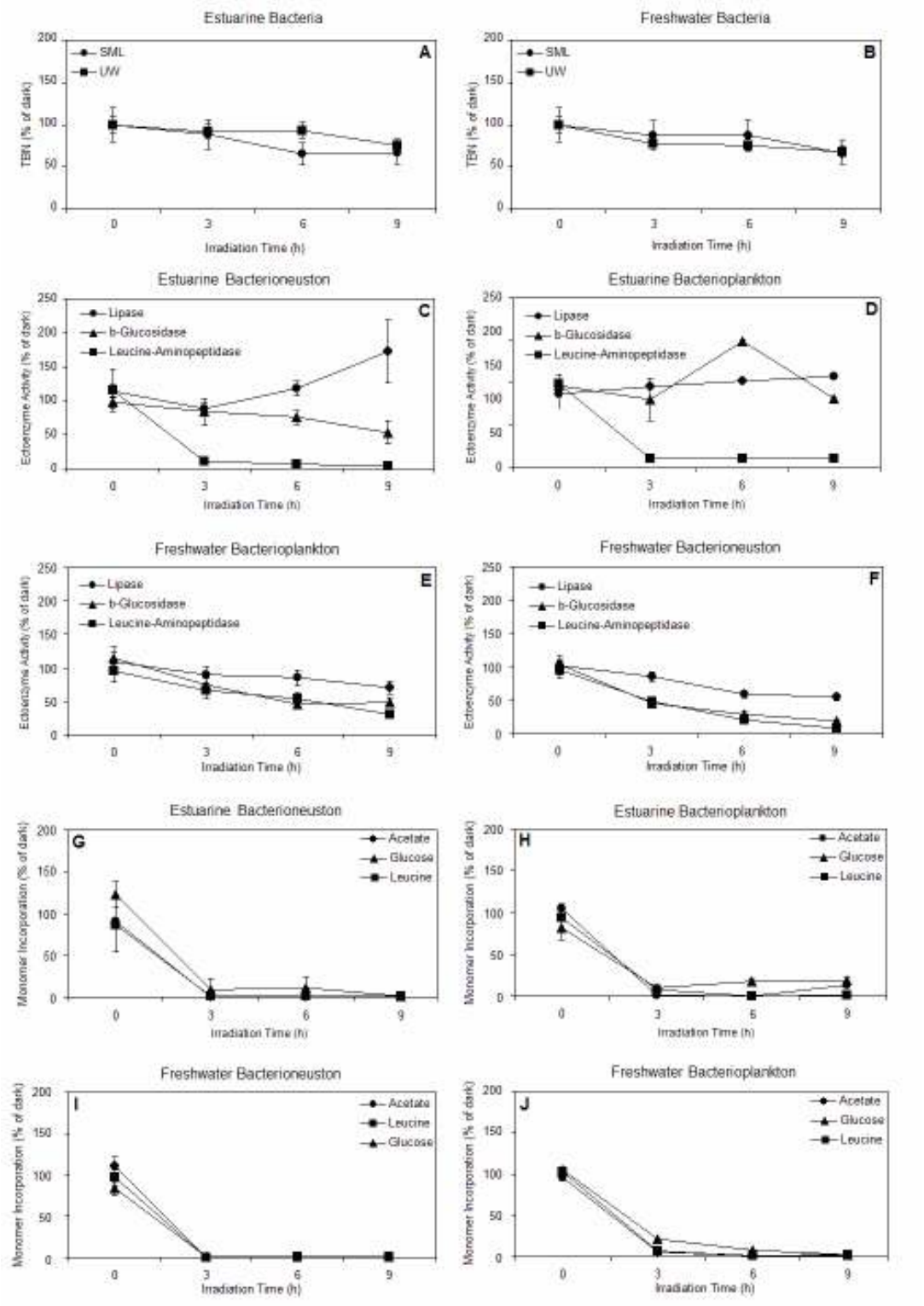

Figure 3.2. Effects of artificial UVR on total bacterial number (TBN), ectoenzymatic activity and monomer incorporation rates in freshwater and estuarine bacterioneuston and bacterioplankton.

For estuarine bacterioplankton (Fig. 3.2H), a 3-hour exposure led to a decrease of 99, 88 and 91% in acetate, glucose and leucine incorporation, being leucine incorporation completely inhibited after a 9-hour period of exposure. However, a 9-hour period of irradiation actually resulted in an increase of around 11% in monomer incorporation for acetate and gglucose incorporation, in respect to the value observed at 3h of irradiation.

After 3 hours of irradiation of freshwater bacterioneuston (Fig. 3.2I), the incorporation of all the monomers showed an inhibition of >98%. The effect in bacterioplankton was similar with a reduction in monomer incorporation of >97% (Fig. 3.2J).

6-HOUR EXPOSURE OF PRE-ADAPTED SAMPLES

Samples collected around noon rather than at early morning and therefore pre-exposed to solar radiation, were considered as pre-adapted and used in 9-hour exposure of artificial UVR irradiation.

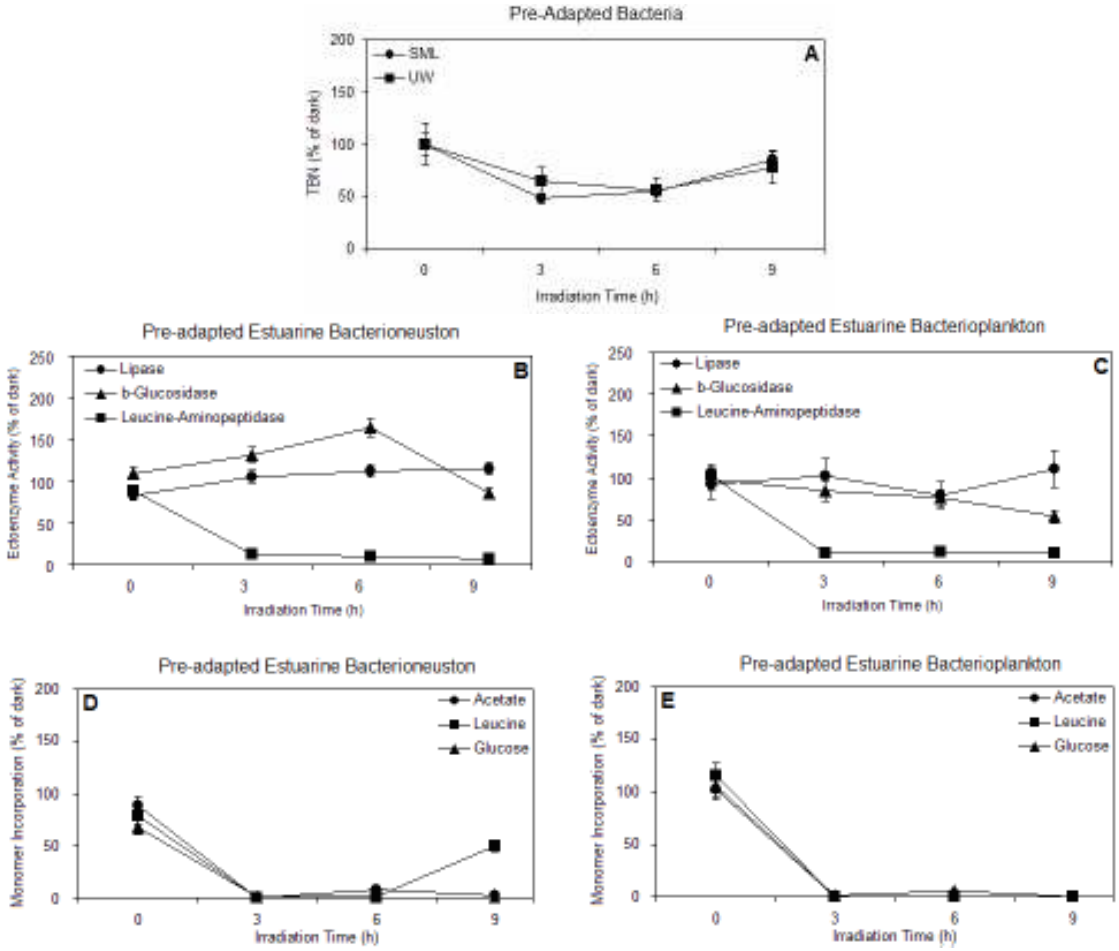

Figure 3.3. Effects of artificial UVR on total bacterial number (TBN), ectoenzymatic activity and monomer incorporation rates in estuarine pre-adapted bacterioneuston and bacterioplankton.

Exposure of pre-adapted bacterial communities to UV-B artificial radiation for 3h led to a reduction in bacterial abundance of 52 and 36% for bacterioneuston and bacterioplankton, respectively. However, after a 9h exposure, a slight increase in bacterial abundance (38% and 13% for bacterioneuston and bacterioplankton, in relation with the initial value) was observed (Fig. 3.3A).

The UV irradiation of pre-adapted bacterioneuston resulted in a decrease in diversity, assessed by the number of bands, of ~30%, while for estuarine bacterioplankton the decrease was more than 90% (Table 3.3).

Table 3.3. Summary of the number of OTUs detected by DGGE in experiment (ii).

Experiment	Layer	T_0	Irradiation		Dark Control
			6h	9h	Control
(ii)	SML	34	-	27	34
	UW	35	-	5	35

The effects of ectoenzymatic activity were heterogeneous. After a 9-hour exposure to UV-B, lipase activity of pre-adapted bacterioneuston (Fig. 3.3B) increased by 38% while β-glucosidase declined by 22% and leucine-aminopeptidase declined by 91%, in relation to the initial value. Pre-adapted bacterioplankton (Fig. 3.3C) also showed a 19% increase in lipase activity, while for β-glucosidase and leucine aminopeptidase, 7% and 90% reductions were observed, respectively.

Exposure of pre-adapted bacterioneuston to a 3-hour UV irradiation led to a >99% decrease in monomer incorporation rates (Fig. 3.3D). However, further irradiation led to a 62% increase of leucine incorporation rates, in relation to the value detected after 3 hours of irradiation. Pre-adapted bacterioplankton (Fig. 3.3E) showed an almost complete inhibition (>99%) in the incorporation of all the monomers tested throughout the irradiation period.

DARK RECOVERY

In the dark recovery experiments, a period of 6-h exposure to UVR was followed by a 6-hour period of incubation in the dark. The exposure of bacteria to artificial UV-B radiation for 6 h led to a reduction in bacterial abundance of 48% and 60% in bacterioneuston and bacterioplankton, respectively. During the dark recovery period, bacterial abundance increase to 94 and 81% of the initial abundance, for the SML and UW communities, respectively (Fig. 3.4A).

Table 3.4. Summary of the number of OTUs detected by DGGE in experiment (iii).

Experiment	Layer	T_0	Irradiation	Dark	
				Control	Recovery
(iii)	SML	34	27	34	34
	UW	33	13	33	17

The variation of the composition of bacterial communities during the dark recovery period was also followed by DGGE (Table 3.4). The initial exposure of estuarine bacterioplankton to

artificial UV-B light for 6 h, led to a decrease of ~60% in diversity, but after the dark incubation only ~30% of the bands detected at the initial moment were absent. Estuarine bacterioneuston was less affected. Initial irradiation caused a ~20% reduction in bacterial diversity, but ribotypes were totally recovered after the dark incubation.

After 6 h of exposure to artificial UV-B (0.4 W m^{-2}), the ectoenzymatic activity in estuarine bacterioneuston dropped by 31% for lipase, 44% for β-glucosidase and up to 100% for leucine-aminopeptidase, relatively to the initial value (Fig. 3.4B). During the dark recovery period lipase and β-glucosidase activity recovered to 60 and 12% of the initial values, respectively, while recovery did not occured for leucine-aminopeptidase activity (Fig. 3.4B).

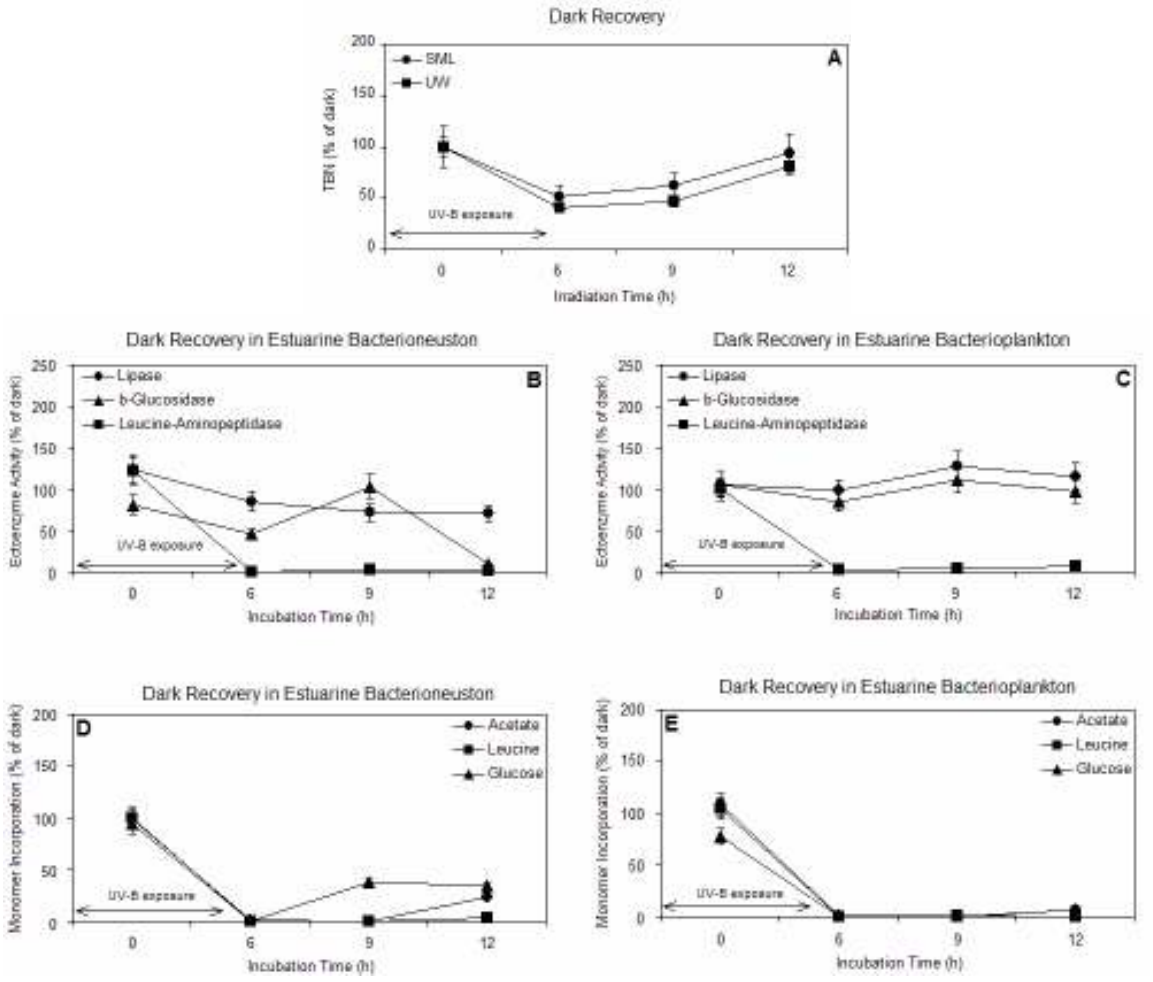

Figure 3.4. Effects of artificial UVR on total bacterial number (TBN), ectoenzymatic activity and monomer incorporation rates in estuarine bacterioneuston and bacterioplankton incubated in the dark, after a 6-h exposure to artificial UVR.

The recovery experiments performed with bacterioplankton revealed that irradiation caused a decrease in lipase activity of 7%, while β-glucosidase and leucine-aminopeptidase showed a 20%

and 97% reduction, respectively. After the dark incubation period, lipase and β-glucosidase activity showed recoveries to 100 and 92% of the initial values, respectively, while recovery of aminopeptidase activity did not occur (Fig. 3.4C).

In bacterioneuston, the incorporation of monomers was severelly inhibited (>99% decrease) after a 6-hour irradiation period (Fig. 3.4D). During the following dark incubation for 6 h, acetate and glucose incorporation recovered to 24 and 37% of the initial value, respectively, while leucine incorporation remained very low.

Bacterioplankton responded to UV exposure in a similar way (Fig. 3.4E). Irradiation caused a >99% decrease in incorporation of monomers, but during the dark incubation period, the recovery was less pronounced than for bacterioneuston. Acetate incorporation recovered to less than 10% of the initial value. Recovery was not observed for the incorporation of glucose nor leucine.

DISCUSSION

EFFECTS OF UV RADIATION ON AQUATIC PROKARYOTE COMMUNITIES

The experiments performed in the laboratory were designed with the aim of siolating UVR effects on bacterial communities from other environmental sources of regulation. The intensity used corresponded to natural dose (0.4 W m^{-2} of UV-B), similar to that which can be measured in the field on clear sky days, and that has already been used by several authors (Herndl et al, 1993; Müller-Niklas et al, 1995; Kaiser and Herndl, 1997).

Decreases on bacterial abundance upon exposure to UVR were observed, but may be under-estimated because the epifluorescent enumeration of orange acridine stained bacteria, allows the quantification of both viable (active) and non-viable cells (dead or dormant).

The impacts of artificial UV radiation in bacterial diversity were assessed by DGGE, which revealed that upon exposure, the number of bands detected was considerably reduced, by as much as 60%. This opposes other results in the literature (Winter et al, 2001), that had reported only minor changes in bacterioplankton community composition. The present study demonstrates that environmentally occuring levels of UVR can significantly affect the structure of bacterial assemblages at the upper layers of the water collumn.

Exposure of aquatic bacteria to artificial UV radiation resulted in significant decreases in heterotrophic activity. Aminopeptidase activity was the ectoenzyme most severelly affected with a reduction in activity of up to 100%. This strong impact of UV exposure on ectoenzymatic activity is probably related to the photolytical cleavage of ectoenzymes (Müller-Niklas et al, 1995), being the enzyme expression only inhibited by UV radiation to a minor extent (Herndl et al, 1993).

However, lipase activity increased by as much as 50% in estuarine bacterial communities upon irradiation. Ectoenzymatic activity responds to the availability of substrates at a given moment (Chróst and Rai, 1993; Sinsabaugh et al, 1997) and can be used to infer which classes of macromolecules are relevant to bacterial assemblages (Christian and Karl, 1995; Sala et al, 2001).

Enhanced lipase activity suggests increased availability of lipidic materials. The increase in the concentration of lipids during the irradiation period can be related to photoinduced damage of phytoplankton and zooplankton present in our samples, most likely resulting in the release of triacylglicerols (Lee et al, 1971).

Monomer incorporation was also severely affected by UV radiation. The inhibition of processes at the membrane level can explain the inhibition of substrate transport. The accumulation of photooxidation products, including free radicals, during irradiation may also explain the inhibition of bacterial activities (Kjelleberg and Håkansson, 1977).

The incorporation of glucose showed a slight increase, latter in the irradiation period, after a strong initial inhibition. Membrane damage is not accompanied by cell death (Kelland et al, 1983; Jeffrey et al, 1996), as suggested by the results of the recovery experiments. The late increase in glucose incorporation is possibly a consequence of the photolytical cleavage of recalcitrant dissolved organic matter (DOM), mainly of celulosic origin, to low-molecular weight compounds, that are efficiently taken up by bacteria, counteracting with the direct negative effect of UV-B on bacteria (Herndl et al, 1997; Lindell et al, 1995, 1996; Wetzel et al, 1995; Bano et al, 1998; Benner and Biddanda, 1998; Chróst and Faust, 1999). By reducing bacterial ectoenzymatic activity and simultaneously photolytically cleaving macromolecules, UV-B radiation substitutes bacterial enzymatic activity in the processing of dissolved organic matter (DOM) (Müller-Niklas et al, 1995), explaining the increase in glucose incorporation uncoupled from β-glucosidase. The observation that monomer incorporation was more affected that ectoenzymatic activity can also indicate that, upon exposure to UV-B, membrane systems are more affected than the expression of proteins, some of which are mostly periplasmic.

EFFECTS OF ULTRAVIOLET-B RADIATION ON FRESHWATER AND ESTUARINE BACTERIAL COMMUNITIES

UV exposure during 9-hour experiments impacted freshwater bacterial communities more strongly than the estuarine ones, suggesting that these communities are more exposed or more sensitive to the effects of UV radiation, in terms of heterotrophic activities.

Organic molecules of the DOC pool are main factors involved in the attenuation of the the penetration of UV radiation in natural waters (Williamson et al, 1996; Morris and Hargreaves, 1997), protecting bacterial assemblages from harmful doses (Chróst and Faust, 1999). Since freshwater systems tend to exhibit a higher concentration of DOM, UV penetration in these environments is usually much lower than in marine systems (Scully and Lean, 1994). A crude estimation of the higher concentration of organic matter in the freshwater sampling station can be inferred from the concentration of chlorophyll a, much higher (>$50x10^4$ times) at the freshwater site.

Mixing events in the freshwater site are scarce when compared to the occurence of physical mixing in estuarine systems. This accounts for the reduced efficiency of the systems repair of DNA

lesions in freshwater communities, when compared to marine or estuarine counterparts (Kaiser and Herndl, 1997).

The effects of UVR on community diversity, measured from the averaged numbers of OTUs, were similar in freshwater and estuarine samples.

EFFECTS OF UVR ON BACTERIONEUSTON AND BACTERIOPLANKTON

In estuarine samples collected at early morning, the reduction of activity upon irradiation was stronger in bacterioneuston than in bacterioplankton. The accumulation of organic compounds and pollutants at the microlayer may result, upon exposure, in the production of noxious compounds (e.g., free radicals), whose toxicity to bacterial cells is accentuated in an enclosed microcosm. This results in strong inhibition of bacterial activities. Lipase activity in the bacterioneuston, substantially stimulated by irradiation exposure, constitutes an exception. Since the SML is often described as highly enriched in lipidic compounds (Lion and Leckie, 1981), bacterioneuston communities might have adapted to take advantage of light-induced lipid hydrolysis.

Constranstingly, DGGE analysis of bacterial communities exposed to UV irradiation during a 9 hour period revealed a decrease in the number of operational taxonomic units (OTU) of 70% for estuarine bacterioplankton but only 25% for bacterioneuston. UV radiation can influence the composition of bacterial communities by several ways. Differential sensitivity among various bacterial strains can induce changes in bacterial community composition (Joux et al, 1999; Arrieta et al, 2000; Agogué et al, 2005). Surface communities have been reported to be as sensitive as deep water communities, suggesting that bacterial assemblages do not respond to solar exposure with increased UV resistance (Herndl et al, 1993). However, experiments performed with samples from the same sampling sites show that bacterioneuston maintains a higher culturable fraction after exposure to artificial UV-B radiation (unpublished). Thus, UV resistance can be in fact involved in determining the composition of bacterial communities upon irradiation.

Freshwater communities from the SML and UW were found to be similarly affected by UV exposure in terms of ectoenzymatic activity and monomer incorporation rates, though the inhibition was stronger for bacterioneuston. The effects of UV exposure on community composition were also similar for both compartments.

DARK RECOVERY

Substantial recovery of bacterial abundance and heterotrophic activity was observed in bacterioneuston and bacterioplankton, pointing to the importance of dark recovery mechanisms. A similar conclusion emerged from diel studies conducted on the estuarine sampling site (unpublished) that suggested repair of DNA lesions at night after diurnal exposure to sunlight.

In the dark recovery experiments, the rates of monomer incorporation were more affected in bacterioneuston than in bacterioplankton. However, the rates of recovery (up to 40% in respect to

the initial value) were also much higher in bacterioneuston than in bacterioplankton (<10% in respect to the initial value). UV exposure induced decreases in the number of OTUs of ~60% for estuarine bacterioplankton and of ~20% for estuarine bacterioneuston. After the dark recovery period, bacterioplankton diversity recovered by 30% and the recovery was almost absolute for bacterioneuston. This might indicate more efficient mechanisms of recovery (possibly DNA repair mechanisms) in bacterioneuston than in bacterioplankton, that enables it to endure the high levels of UV radiation naturally occurring at the surface microlayer (Agogué et al, 2005). However, the opposite trend was observed for ectoenzymatic activity, found to recover more in bacterioplankton than in bacterioneuston. Ectoenzymatic activity is tightly dependent on the array of substrates available (Chróst and Rai, 1993). In the initial period of UV irradiation, substantial ectoenzyme destruction and lesions at the membrane may occur, inhibiting hydrolytic activity and monomer incorporation. At the same time, UV induces photolysis of DOM, and the different behaviour of bacterioplankton and bacterioneuston may reflect the differences in their nutritional environment. DOM in the SML is naturally more exposed to UV and therefore more enriched in labile substrates (monomers) derived from its photodegradation, contributing to the fast recovery of the monomer incorporation capacity in bacterioneuston, once UV-exposure is terminated. Ectoenzymatic activity mat be inhibited by a catabolite repression-like mechanism (Debroas et al, 1999), thus explaining lower rates of recovery of ectoenzymatic activity in bacterioneuston. More recalcitrant DOM in UW, results in the opposite effect, which is reflected by higher rates of recovery for ectoenzymatic activity and decreased recovery of monomer incorporation, relatively to the SML (Lion and Leckie, 1981).

Overall, the results of this work show that monomer incorporation responds more rapidly to changing conditions, such as UV irradiance, than ectoenzymatic activity. This can be a result of either indirect effects of UV radiation on the DOM pool or a direct effect of UV radiation on ectoenzymes and cellular membrane transport processes.

Experiments using bacterial communities collected around noon showed similar responses, suggesting that bacterial assemblages occuring in the top layers of the water column at noon may not be more resistant to UVR than those occuring in the morning. However, for acetate incorporation, a significant increase was observed during the irradiation period in pre-adapted noon samples. The reduction of diversity in bacterioneuston was similar in samples from early morning and from noon, but in bacterioplankton the loss of diversity with irradiation was stronger in adapted (noon) samples.

These observations can indicate a possible photoadaptation of bacterioneuston exposed to the higher intensities of UVR occurring at noon. The occurrence of this process in natural samples is, however, a matter of controversy (Pakulski et al, 1998; Joux et al, 1999). Besides efficient DNA repair mechanisms, several strategies have been related to the enhanced resistance of bacterioneuston to high levels of solar radiation. Exopolysaccharides secreted by bacteria, algae and other marine organisms that accumulate at the surface (Sieburth et al, 1976; Maki, 1993) may

provide protection from environmental stress such as UV radiation (Elasri and Miller, 1999). High concentrations of chomophoric DOM and POM (particulate organic matter), comparatively to underlying waters (Harvey and Young, 1980; Carlson, 1982; Whitehead and Vernet, 2000; Obernosterer et al, 2005) may also provide *in situ* protection to bacterioneuston.

Pigmentation could have a potential role in UV protection in bacterioneuston. Recent investigations on the resistance of bacterioneuston to solar radiation showed that pigmented bacteria were not more resistant to solar radiation than non-pigmented strains (Agogué et al, 2005). In the samples used in this study, it was observed that upon irradiation for more than 15h, plating in solid media resulted in ~60% of the colonies being yellow or orange (unpublished results), suggesting a possible role of pigmentation in UV resistance that needs further clarification.

CONCLUSION

From this work we conclude that, in general, the heterotrophic metabolism of bacterioneuston is more affected by exposure to UVR than that of bacterioplankton, probably because of enhanced oxidative stress in the surface microlayer environment. The interactions between primary producers and bacteria can also be a critical factor in the outcome of the exposure to UV radiation on bacterial activities and community structure. In this work, the experiments were conducted without removing either phytoplankton or zooplankton, since true estimates of UV-radiation effects in the environment require all components of the ecosystem to be examined simultaneously. The measured responses of bacteria to UVR are thus the result from a combined effect of the responses of UVR at several trophic levels. Algae can also pose a potential protecting effect, though not directly assessed in this study.

In general, the activity of freshwater bacterial communities was more affected than its estuarine counterparts, which can indicate a higher sensitivity of bacteria in these environments to increased UVR levels reaching the Earth, as a result of ozone depletion.

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CHAPTER 4

DISCUSSION

UVR AVOIDANCE IN AQUATIC BACTERIAL COMMUNITIES

To assess the occurrence of UVR avoidance in bacterial communities from the surface microlayer (SML) and underlying waters (UW), bacterial numbers, diversity and activity were followed during diel cycles, since these are primarily a manifestation of the relation between sunlight and marine biota (Fuhrman et al, 1985).

Pronounced diel patterns of both bacterial abundance and heterotrophic activity have been found in open ocean samples (Burney et al, 1982; Johnson et al, 1983; Gasol et al, 1998; Van Wambeke, 2008), in slope waters (Fuhrman et al, 1985) and some coastal waters (Sieburth et al, 1977; Meyer-Reil et al, 1979; Hagström and Larsson, 1984), indicating tight coupling between phytoplankton and bacterioplankton, i.e., the daily dynamics of phytoplankton is the major force driving the daily dynamics of bacterioplankton metabolism. Being heterotrophic bacteria tightly coupled to variations in primary production, a conspicuous diel cycle in bacterial growth should be observed, with peaks of activity around noon.

However, in this study a consistend diel pattern of variation of bacterial abundance and activity was not observed, supporting results from previous studies that had already indicated that in coastal areas, where bacterial assemblages may be more dependent on allochthonous carbon, diel cycles of microbiological parameters may not occur (Gasol et al, 1998).

CELL ABUNDANCE AND COMMUNITY STRUCTURE

A distinctive diel pattern was neither observed for bacterial abundance nor for the relative abundance of α-proteobacteria and β-proteobacteria during the assessment of diel cycles. However, higher bacterial abundance was observed during the day (~20%), in particular during the morning period, as also reported for other ecosystems (Fuhrman et al, 1985; Kuipers et al, 2000), probably due to increased DOM supply associated with increased phytoplankton biomass (>60%) during the day. Afternoon and evening decreases in bacterial cell abundance are probably associated with increased grazing by microflagellates that migrate upward during this period (Fuhrman et al, 1985), in addition to lowered DOM flux, due to decreased photosynthesis during this period (Fuhrman et al, 1985).

In this study, solar radiation was not found to impose a significant effect on bacterial diversity, since less than approximately 10% of all the taxa were found to vary during the day. This was reflected by the inexistence of a grouping between the bacterial diversity of samples collected during the day and the night upon construction of the respective dendrogram. Information on how

solar radiation affects bacterial community composition is scarce. The few studies conducted in aquatic ecosystems have shown highly contrasting results, ranging from only minor (Winter et al, 2001; Norris et al, 2002) to clear changes in bacterial community composition (Kadivar and Stapleton, 2003; Van Mooy et al, 2004) as a result of radiation effects. Changes in bacterial community structure upon exposure to solar radiation is a result of the detrimental effects of UV on bacteria (Herndl et al, 1993; Arrieta et al, 2000), or triggered indirectly by factors such as changes in the DOC bioavailability induced by photochemical reactions (Lindell et al, 1995; Wetzel et al, 1995; Tranvik and Bertilsson, 2001) or differences in the development of phytoplankton (Pinhassi et al, 2004).

Overall, these results show the difficulty in detecting the importance of light as a driver of bacterial communities of estuarine systems, such as Ria de Aveiro. Nevertheless, the reduced influence of solar radiation on the composition of bacterial communities in the Ria de Aveiro can be explained by the fact that even sensitive species are never exposed to a dose of radiation high enough to lead to their complete disappearance under the conditions prevailing in mid-latitude, mesotrophic areas, like Ria de Aveiro, where organic matter and wind-induced mixing can provide at least some protection (Winter et al, 2001). Slight changes in community composition occured between sampling moments corresponding to different phases of the tidal cycle. These observations suggest that short term variations in the descriptors of bacterial metabolic activity mostly reflect changes in the relative level of activity of the bacterial assemblages, but at least in part are a result of changes in its composition.

Microcosm experiments were conducted with the aim of clarifying the impact of UV-B radiation in controlled condiction, and to further explained the field observations. For that, samples from the SML and UW were exposed to artificial UV-B radiation, with an intensity of 0.4W m^{-2}. This intensity is realistic corresponding to that occuring in cloudless days during the summer in mid-latitude areas (Müller-Niklas et al, 1995; Kaiser and Herndl, 1997).

Exposure of bacterial assemblages to artificial UV-B radiation led to substantial reductions in prokaryote abundance (ranging from 14 to 50%), similar to those previously observed upon the exposure of bacterial communities from the Northern Adriatic Sea to artificial UV-B radiation of 0.4 W m^{-2} (Müller-Niklas et al, 1995). Decreased bacterial numbers after irradiation are probably due to direct damage to DNA resulting in the formation of CPDs that can block DNA polymerase, hampering bacterial replication and resulting in cell death (Mitchell and Karentz, 1993). However, these results should be considered with caution since the method applied to quantify bacterial cells we used, i.e., the epifluorescent enumeration of orange acridine stained bacteria, considers both viable (active) and non-viable cells.

Only few studies have addressed the direct effects of UV on the composition of bacterial communities (Winter et al, 2001; Norris et al, 2002; Van Mooy et al, 2004; Kadivar and Stapleton, 2003). In this study, the effects of artificial UV-B exposure in the composition of bacterial assemblages were followed by DGGE in microcosm experiments. Artificial UV-B radiation imposed

strong changes in bacterial diversity, resulting in decreases of phylogenetic units up to 90%. This strong effect suggests differences in the sensitivity to UV-B or in the repair efficiency of the UV-induced damage among different bacterial strains (Joux et al, 1999; Arrieta et al, 2000; Agogué et al, 2005).

Changes in bacterial diversity upon exposure to UV radiation are not only associated with the direct effects of UV radiation, but can also be attributed to indirect effects, including the impact of radiation in phytoplankton composition and biomass (Pinhassi et al, 2004). Photochemical induced changes in the availability of dissolved organic carbon (DOC) to bacteria can also result in changes in bacterial community composition (Tranvik and Bertilsson, 2001), favouring bacterial community members both specialized on the photochemically modified DOC compounds (growth promoting), and more resistant to oxidative stress (growth inhibiting) (Langenheder et al, 2006).

These results clearly differ from those obtained in the field, where significant differences were not found between bacterioneuston and bacterioplankton, and can possibly be related to the different setups used in each experiment (i.e., macrocosm vs microcosm). Probably, in the in situ study the detection of direct detrimental effects of radiation on bacterial communities was impaired by the natural dynamics of the estuary (most likely tides), as well as occasional inputs of allochthonous organic matter and also by the vertical movement of water masses. In this conditions, bacteria are never exposed to levels of radiation sufficient to induce clear changes in bacterial diversity. In microcosms, the confinement of communities allows prolong exposure to induce noticeable changes in the community structures. This is further enhanced by the limited vertical movements in this setup (see below for further discussion of experimental setups).

UVR EFFECTS ON BACTERIAL HETEROTROPHIC ACTIVITY

ECTOENZYMATIC ACTIVITY

In this study, the absence of clear diel patterns in bacterial heterotrophic activity, though phytoplankton abundance (assessed by chlorophyll a concentration) exhibited a distinctive diel pattern, indicates uncoupling of bacterial activity to primary production (Opsahl and Benner 1997). In Ria de Aveiro, bacterioplankton do not strictly depend upon phytoplankton primary production for carbon supply, with allochthonous carbon contributing a significant fraction of the bacterial production even during the warm season (Almeida et al, 2001).

These low amplitude or inconsistent (unpredictable) diel patterns, also reported in other coastal systems, have been related to the trophic status of the environment (Riemann and Søndergaard, 1984; Riemann et al, 1984). Largest diel variations in bacterial activity are found under oligotrophic conditions (Zohary and Roberts, 1992; Zweifel et al, 1993; Gasol et al, 1998), while under more eutrophic conditions the diel patterns in bacterial activity are not that pronounced (Zweifel et al, 1993; Simon, 1994). Thus, a complex interaction of different facts may cause

uncoupling of the bacterial activities and the phytoplankton production, difficulting the detection of distinctive diel cycles at estuaries.

Contrasting with the generalized absence of diel patterns in bacterial abundance and activity, lipase showed a distinctive diel pattern, being in average up to 30% higher ($p<0.05$, 1-way ANOVA) during the night. Lipids from phytoplankton origin (pigment and glycolipids) have been found to decrease, whereas lipids from zooplankton origin (triacylglycerols) increase from day to night (Ghiglione et al, 2006). The marked shifts in bacterial heterotrophic metabolism towards lipid degradation from day to night suggest that the phytoplankton organic material was undergoing degradation when collected at night. These results can indicate the dominance of organic matter of zooplankton origin at night, when zooplankton produce a large quantity of faecal pellets by intensive feeding on phytoplankton and a large increase of lipid originating from zooplankton (triacylglycerols) occurs in this period (Ghiglione et al, 2006).

Statistically significant day-night differences were not detected between ectoenzymatic activities of the remaining enzymes tested, including aminopeptidase, showing the highest Hm of all the ectoenzymes tested. On average aminopeptidase activity was ~20% higher during the night, possibly as a result of the release of proteinaceous material from heterotrophs that migrate upward at night (Fuhrman et al, 1985). The high levels of leucine-aminopeptidase detected in the diel study (1115.4 and 4269.5 nmol L^{-1} h^{-1}) reflects the importance of proteinaceous material in Ria de Aveiro (Cunha et al, 2001), probably related to high degrees of eutrophication. High protein results in increased activity of leucine aminopeptidase (Jacobsen and Rai, 1991; Martinez and Azam, 1993; Foreman et al, 1998; Mallet and Debroas, 1999; Lamy et al, 1999; Thompson and Sinsabaugh, 2000). The high level of eutrophication detected in the Ria also accounts for the decoupling between primary production and bacterial activities, that impairs the detection of distinctive diel patterns.

A distinctive diel pattern was not detect for β-glucosidase activity, but it occured in the concentration of chlorophyll a. Positive correlations of β-glucosidase activity with phytoplankton biomass and dissolved monomeric carbohydrates (DMHCO) have been documented (Karner et al, 1992), but in this study a diel coupling between photosynthesis and polysaccharide hydrolysis was not detected. This effect could be related to direct inhibition of bacterial activities by UVR during the day, including the possible destruction of ectoenzymes by solar radiation (Herndl et al, 1993) that would inhibit the use of polimeric carbohydrate compounds (Chróst and Faust, 1999). A similar effect may have occured with other ectoenzymes.

The results obtained in this study show relatively low levels of β-glucosidase activity (27.25 – 371.84 L^{-1} h^{-1}) comparatively to aminopeptidase and lipase activities. Carbohydrates contribute with 10-20% and > 25% to the DOM and POM, respectively, in aquatic systems (Riley, 1970). In Ria de Aveiro the high levels of protein material provide enough substrate for bacteria, so that bacterial metabolism is diverted towards protein hydrolysis.

Upon a 9-h exposure to artificial UV-B radiation in the microcosm studies, ectoenzymatic activity was substantially inhibited (16 to 90%, relatively to the initial values). This decline can be attributed to a combined effect of photolytic cleavage of ectoenzymes (Herndl et al, 1993; Müller-Niklas et al, 1995) and inhibition of enzyme expression when CPDs block mRNA transcription (Karentz et al, 1991; Herndl et al, 1993; Karentz, 1994). Ectoenzatic activity responds directly to the array of macromolecules (polymers) present in the environment at a given moment (Chróst and Rai, 1994). So, the effects of UVR on ectoenzymatic activity are also indirectly mediated by the effects of UVR on DOM and DOM producers, especially zooplankton and phytoplankton. By photolytically cleaving polymers in monomers, UVR substitutes bacterial ectoenzymatic activity in DOM processing. As these monomers accumulate in the environment, bacteria probably imply a resource management strategy that involves a catabolite repression-like metabolism, i.e., the occurrence of a repression/derepression control mechanism of enzyme synthesis by the final product of the hydrolysis reaction (Chróst, 1990; Chróst, 1991; Debroas et al, 1999).

In some experiments, stimulation of ectoenzymatic activity by UVR occured, most noticeably for lipase activity (up to 50% of the initial value), but also for β-glucosidase. As UV exposure photolytically cleaves ectoenzymes and induces DNA lesions that can inhibit enzyme expression, increased enzyme activity during the irradiation period can only be attributed to increased substrate availability. Probably, exposure to UVR induces the death of zooplankton and phytoplankton, that it accompanied by the release of significant amounts of lipids, on which bacterial lipolytic enzymes act, resulting in the increased lipase activity observed in the microcosm experiments. Stimulation of β-glucosidase activity upon exposure can be related to the release of polymeric carbohydrates origin by stressed algae, that is known to be greater than the release by healthy, nonstressed algae (Ridal and Moore, 1993).

Further insights of the mechanisms involved in the control of enzyme synthesis and activity are difficult considering that the determination of substrate concentrations was not attempted in this study.

MONOMER INCORPORATION

The study along diel cycles revealed that the rates of incorporation of acetate and leucine were up to 60% higher during the day than at night, though the enzymes responsible for the hydrolysis of the correspondent polymers did not show the same pattern. Daily maxima of uptake of organic compounds have been described, namely for carbohydrates (Sieburth et al, 1977; Burney et al, 1982; Johnson et al, 1983), amino acids (Carlucci et al, 1984) and nitrogen (Wheeler et al, 1989), as a result of the diel cycles of production of the correspondent polymers, and probably reduced zooplankton predation during the day (Gasol et al, 1998). The decoupling between monomer incorporation and polymer hydrolysis under UV irradiation may reflect the existence of free dissolved substrates that do not require the hydrolysis. Daily changes in the spectra of

biologically labile materials in the aquatic systems have been described described, namely for cAMP (Ammerman and Azam, 1981), DOC and DFAA (Meyer-Reil et al, 1979), dissolved carbohydrates (Sieburth et al, 1977; Burney et al, 1979). The diel changes in concentration are assumed to be driven by heterotrophic activity. Light induced degradation of refractory DOC (Kieber et al, 1989; Wetzel et al, 1995) could also account the occurrence of higher levels of monomer incorporation during the day that was observed in this study.

The incorporation of glucose did not describe a consistent diel pattern. However, chlorophyll a was significantly higher during the day (>60%, $p<0.05$, ANOVA) and fluctuated according to a consistent diel pattern. Although DOC potentially accumulates as phytoplankton photosynthesis occurs, bacteria in the upper layers of the water column are not able of completely consuming this labile DOC upon release, possibly because of direct inhibition by UVR. About half of the euphotic layer is exposed to potentially harmful UVR, which affects bacterioplankton more than phytoplankton (Jeffrey et al, 1996a) and leads to an accumulation of DNA damage in bacterioplankton cells during the period of most intense solar radiation. Later in the afternoon, when solar radiation decreases in intensity and the wavelength spectrum in the water column is shifted towards long, less harmful wavelength of radiation, DNA repair may take place. This would result in a rate of glucose incorporation during the night similar to that occurring during the day observed in this study that actually corresponds to a delayed response of bacteria to the levels of photosynthetate that accumulated during the day.

Experiments with artificial UV-B radiation showed a dramatic inhibition of monomer incorporation, sometimes up to 100%, upon exposure. Monomer incorporation was always more affected by radiation than ectoenzymatic activity. The effects of UV radiation on monomer incorporation of natural samples are mostly due to the direct impact of exposure in the bacterial membrane (Sommaruga et al, 1997), inducing lesions that inhibit the incorporation of monomers (Kubitschek and Doyle, 1981; Moss and Smith, 1981; Jeffrey et al, 1996b; Fernandéz and Pizarro, 1996). In a few cases a slight stimulation of monomer incorporation rates, especially acetate, was observed during UVR exposure. This indicates that UV-induced damage at the membrane level is not accompanied by cell death (Porter and Feig, 1980; Jeffrey et al, 1996a). The effect of UVR is actually dependent on the dose received, with sublethal doses producing only a transient growth inhibition due to cell membrane damage and alterations in membrane permeability. Higher doses of UVR result in cell death probably caused by reactive oxygen species (Pizarro and Orce, 1988; Pizarro, 1995).

Some stimulation of monomer incorporation may eventually occur by the increased availability of growth-supporting compounds by UV induced photolysis of DOM (Mopper et al, 1991). Bio-assays conducted using bacterial communities from Ria de Aveiro have shown an increase in heterotrophic activity (assessed by the incorporation of ^{3}H-leucine) by as much as 10 times, when cells were inoculated on previously irradiated water samples (Santos, personal communication). This positive effect of radiation is at least partly compensated by the concurrent

UV-mediated production of growth inhibiting substances, such as free radicals (Zafiriou et al, 1984; Palenik et al, 1991). The balance between the formation of bioavailable low molecular weight compounds supporting bacterial activity and compounds inhibiting bacterial activity determines the net effect of the photochemically altered DOM for bacterioplankton (Herndl et al, 1997).

Although, a consistent diel pattern of heterotrophic metabolic activity could not emerge from the results of this work, strong short-term variability in the values of the microbiological descriptors (abundance, ectoenzymatic activity and monomer incorporation) was observed, probably reflecting the changing availability of organic matter, more than changes in the bacterial assemblages present at a given moment.

COMPARATIVE ANALYSIS OF BACTERIONEUSTON AND BACTERIOPLANKTON

Comparing diel changes in descriptors of bacterial heterotrophic activity, only the averaged activity of β-galactosidase and the averaged rate of incorporation of glucose were significantly higher in the SML (Tukey test, $p<0.05$). Although a tendency for higher averaged values of ectoenzymatic activity at the SML was observed, consistent and significant differences could not be established. Enhanced bacterial activity at the SML can be attributed to higher availability of resources, that accumulate in the SML through vertical transport. The higher degree of oxygenation of the SML also stimulates heterotrophic metabolism (Obernosterer et al, 2005). Lower loss processes in the SML due to inhibition of predators (viruses, heterotrophic protozoa, and metazoa) (Carlucci and Bezdek, 1972) may also account for more active bacterioneuston communities.

The variation of microbiological parameters at the SML mostly paralel to that in the UW and a strong correlation between them occured (Pearson correlation, $r^2>0.99$), as observed in other studies (Joux et al, 2006). This seems to imply a non-selective accumulation of bacteria in the SML, found to be twice as abundant as in the underlying layer. This accumulation is mainly driven by physical processes such as flotation, bubble scavenging of surface active organic solutes and particulate matter (Carlucci and Bezdek, 1972). The general structure of the communities is maintained as inferred from the small differences (often inferior to 5%) between the diversity of bacterioneuston and bacterioplankton. These results indicate that the avoidance of solar radiation, in particular UVR, in bacterioneuston communities cannot be directly demonstrated.

Our results show that during the day the pattern of heterotrophic metabolism of bacterioneuston can change from being enriched to depleted, comparatively with the underlying waters. So, in addition to the sampling device used (Hardy, 1997; Agogué et al, 2004), heterotrophic activity enrichments or depletions at the SML are also influenced by the time of sampling. Nevertheless, trends of enriched ectoenzymatic activity in surface waters were generally observed.

The laboratorial assessment of the effects of artificial UV-B on bacterial activity and diversity revealed parallel patterns of inhibition in bacterioneuston and bacterioplankton. Although

the values of EF (enrichment factor calculated as the ratio between the values of the parameter at the SML and in bulk water) were highly variable (0.3-5.5) (Table 4.1) between stations and sampling moments, indicating different levels of activity in bacterioneuston and bacterioplankton, the effects of UV were similar in both communities. This confirms the tight connection between the two communities. The most significative differences ($p<0.05$, 1-way ANOVA) between compartments were observed at the estuarine site, in samples collected in the morning, while for the freshwater site, at least for ectoenzymatic activity, EF was ~1, probably as a result of the inexistance of a distinctive surface biofilm in the field.

Table 4.1. Summary of enrichment factors (EF), calculated as the ratio between microbiological parameters at the SML and UW, at the beginning of the experiments.

Experiment	Origin	Hm Lipase	Hm β-Glc	Hm Leu-AMP	Vm Acetate	Vm Glucose	Vm Leucine
9-hour irradiation	Estuarine	1.2	0.8	1.3	5.5	0.5	0.3
	Freshwater	1.0	0.9	0.8	0.7	0.6	0.5
6-hour irradiation + dark repair	Estuarine	0.3	0.7	0.8	0.9	2.6	1.42
9-hour irradiation after pre-adaptation	Estuarine	1.2	1.2	0.6	1.4	0.9	1.0

Although the profile of variation of heterotrophic activity upon exposure to UVR was usually similar in the SML and UW, the inhibition was stronger in bactrerioneuston. The exception was again lipase activity which was more stimulated by UV exposure in the SML.

DGGE profiles revealed that UV radiation exerted a much stronger effect on the diversity of bacterioplankton, than that of bacterioneuston. This indicates that at the SML, UV inhibition of ectoenzymatic activity does not compromise survival and radiation is replacing ectoenzymes in the production of labile monomer, that can then be readily incorporated. On the other hand, in bacterioplankton, although ectoenzymes are less affected by the exposure to UV radiation, the inhibition of the incorporation of monomers may be related to the reduction in diversity. Thus, it seems that bacterioneuston communities could be more adapted to high levels of UV radiation than bacterioplankton, even if immediate effects on the rates of activity may indicate otherwise, due to the fact that they are naturally exposed to high levels of solar radiation, including in the UV range (Hardy, 1982; Zuev et al, 2001).

PHOTOADAPTATION AND DARK RECOVERY

Dark Recovery

Differences in bacterioneuston and bacterioplankton were also found when assessing recovery after UV exposure. Monomer incorporation recovered more effectively in bacterioneuston. Dark recovery was also assessed for the bacterial community composition. The reconstitution of diversity was almost complete for bacterioneuston, but of only 30% (assessed in terms of OTUs) for

bacterioplankton. These results can suggest the occurrence of more efficient mechanisms of recovery (possibly DNA repair mechanisms) in bacterioneuston comparatively with bacterioplankton that enables the former to resist high levels of UV radiation naturally occurring at the surface water layer. Constrastingly, the recovery in ectoenzymatic activity was stronger in bacterioplankton (up to a complete recover), than in bacterioneuston.

Overall, the results from the dark recovery experiments indicate that the main effect of UV radiation in bacterioneuston is at the rates of polymer degradation, while in bacterioplankton, damage at the membrane may predominate, precluding the incorporation of monomers. This may point to a complementary role of bacterioneuston and bacterioplankton in the turnover of DOM upon exposure to natural UV radiation in the environment, already suggested by the results of the studies along diel cycles.

Photoadaption

The experimental assessment of the effects of UV-B artificial radiation were also performed with samples collected around noon, under higher radiation intensity, and thus expected to be pre-adapted to some degree of UV-B exposure. The moment of sampling did not significantly affect the responses to UVR. However, stimulation of lipase activity was more intense in morning samples. If increased lipase activity is related to UV-induced phytoplankton mortality, lower stimulation of lipase activity of pre-adapted samples during the irradiation period can be justified with lower phytoplankton abundance around noon, assessed by chlorophyll a concentration. This can be related to the fact that bacterial assemblages sampled at noon may contain bacteria that have accumulated lesions during the previous exposure period (morning).

Following bacterial community composition by DGGE showed that in bacterioneuston, the reduction in the number of phylotypes (OTUs) induced by UV exposure was similar (~30%) in morning and noon samples. In bacterioplankton, the reduction of diversity was higher in noon samples. These results can reflect the potential of bacterioneuston for photoadaptation, since it is naturally exposed to higher levels of UVR than bacterioplankton (Agogué et al, 2005). Wether photoadaptation occurs in the environment is still a controversial matter. However, the selection of light-tolerant strains upon exposure to sunlight was found in a subtropical reef bacterial community (Pakulski et al, 1998).

UVR AS AN ENVIRONMENTAL DRIVER OF BACTERIAL DYNAMICS

Light, as other physical factors, may be important in the regulation of bacterioplankton dynamics. Temperature as an abiotic factor can be important, especially below 10ºC (Heinänen, 1992; Pomeroy et al, 1991). During this study, temperature was always above 10ºC and only poor significant correlations were found between temperature and microbiological descriptors, namely

aminopeptidase activity at the UW ($r=0.756$, $p<0.05$, $n=8$) and glucose incorporation at the SML ($r=-0.732$, $p<0.05$, $n=8$). This indicates that temperature is probably a minor driver of bacterial dynamics during diel cycles.

There are also some reports of a negative impact of the salinity on the regulation of ectoenzymatic activities (Murrel et al, 1999), especially for β-glucosidase activity. However, leucine-aminopeptidase activity showed the strongest negative correlations with salinity, at the UW ($r=-0.839$) and at the SML ($r=-0.860$, $p<0.05$, $n=8$). Salinity at the UW was even found to negatively correlate with aminopeptidase activity at the SML ($r=-0.828$, $p<0.05$, $n=8$). During this study, marked tidal variations were observed only for leucine-aminopeptidase activity. The other ectoenzymes tested did not show a distinctive pattern of tidal fluctuation. For monomer incorporation these patterns were not consistent between samplings. This occurrence of non-equivalent oscillations (i.e., 6-hour patterns) suggests that bacterial metabolism could be responding to the diel cycle, more than to the tidal fluctuations.

EFFECTS OF UVR ON THE RECYCLING OF DOM IN ESTUARINE AND FRESHWATER SYSTEMS

In terms of sensitivity to UVR radiation, samples from the freshwater site were more strongly impacted than estuarine samples exposed to the same amount of radiation. Stimulatory effects of bacterial activities were not observed in freshwater samples. Shading protection by the highly abundant phytoplankton did not seem to compensate for direct deleterious effects on bacterial communities (Sommaruga et al, 1997). The observed results suggest a stronger direct effect of UV radiation on ectoenzymes. Phytoplankton excretion of low molecular weight compounds, enhanced under UV stress (Ridal and Moore, 1993), can also inhibit ectoenzymatic activity and repress the synthesis of extracellular enzymes in bacteria (Chróst, 1990, 1991). The enhanced negative impact of UVR on freshwater bacterial communities could correspond to a shift of the equillibrium between growth promotor and growth inhibitor substances that results from exposure of DOM to UVR. This shift can be associated with the formation of free radicals in highly eutrophyzed water of Lake Vela. Different DOM sources can also explain the distinct magnitude of UV impact between estuarine and freshwater sites.

The phototransformation of phytoplankton derived DOM (young DOM) is likely to result in less labile DOM and reduced bacterial activity (Pausz and Herndl, 2002), while originally refractory DOM (mainly of terrigenous origin) may become more labile and stimulate bacterioplankton in surface waters (Lindell et al, 1995). Considering that the fraction of refractory DOM is probably higher in the estuarine sites and on the contrary, “young” labile DOM dominates at the freshwater site, the inhibitory effect of UVR should be stronger in freshwater bacterial communities. The rate of vertical mixing of the water column may also influence the expression of UVR effects on heterotrophic bacterial activity.

The turnover of DOM in the upper layers of the water column results from the combined effects of the photolysis of DOM, direct effects of irradiation on bacterioplankton and the magnitude of mixing processes (Fig. 4.9). Close to the surface, high radiation levels photolytically cleave DOM resulting in easily available for bacterial uptake, but at the same time inhibit the uptake of substrate by bacterioplankton due to the damage of macromolecules such as DNA and ectoenzymes. Only upon mixture into deeper layers (especially during the night) of the water column bacteria can efficiently repair damage by using the longer UV-A radiation and the short PAR. The compounds which were photolytically cleaved in the surface layers are then efficiently taken up by the bacterioplankton (Kaiser and Herndl, 1997).

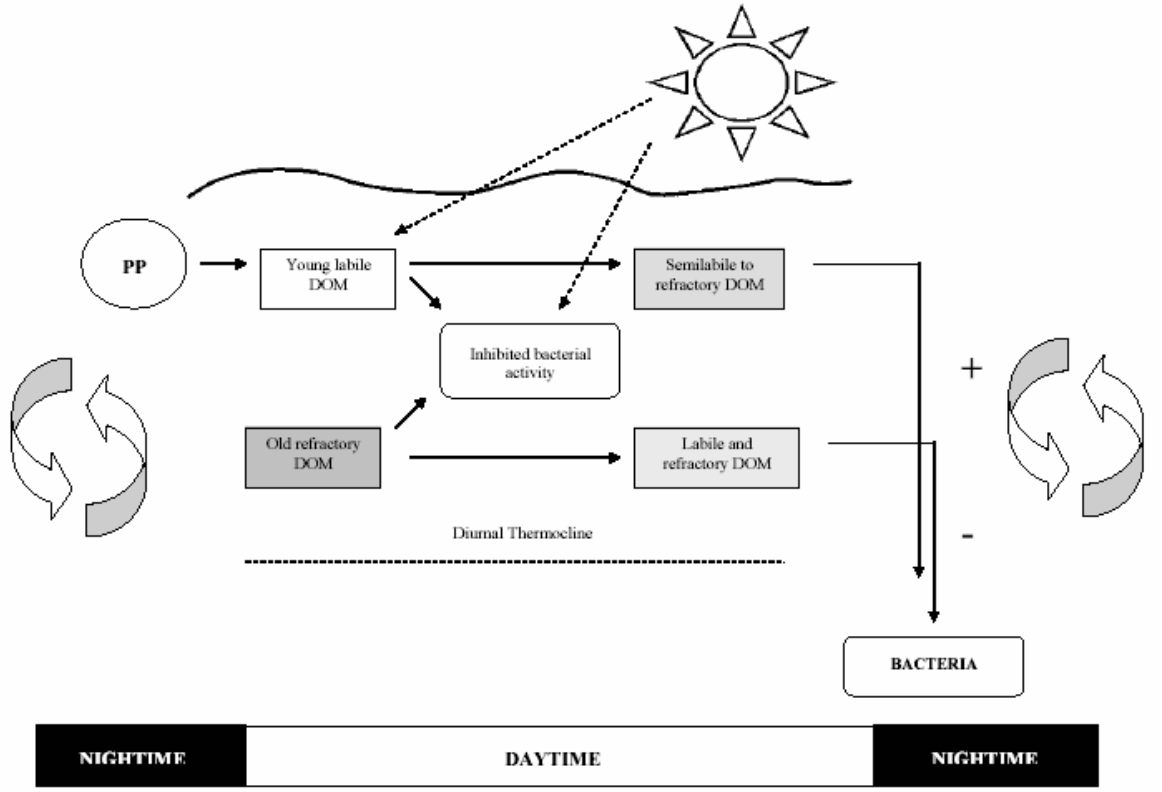

Figure 4.1. Scheme of the interaction between diurnal stratification and the action of solar radiation on the DOM pool and on bacterioplankton in stratified surface layers. Adapted from Herndl et al (1997).

The fact that Lake Vela is not tidally affected and mixing events are rare, with the exception of occasional wind-induce mixture, can affect the efficiency of the repair mechanisms on which bacteria depend to resume metabolic activities after inhibition by UVR. Contrastingly, Ria de Aveiro is subjected to strong hydrodynamism either by tides or by wind-induced mixing, waves and currents that stimulate vertical circulation of water masses (Dias, 2001; Lopes et al, 2006). The results from this study indicate that globally, the freshwater system Lake Vela may be more vulnerable to UVR effects on DOM cycling than the estuarine system Ria de Aveiro.

METHODOLOGICAL CONSIDERATIONS

METHODOLOGICAL CONSIDERATIONS OF STUDYING DIEL CYCLES

In the course of this work we decided a strategy of sampling at one fixed location at each site, since it is logistically the easiest to perform.

A practical aspect of studying diel cycles involves the choice of optimum sampling times. The rates of photosynthesis vary throughout the day and it is of major importante to carefully choose sampling and incubation times for primary productivity measurements. However, it is not as obvious whether sampling and incubation times are equally important for measurements of bacterial activity. Constrained by logistic difficulties, most of the previous studies of the distribution of bacterial biomass and activity at sea involving ship cruises (e.g. Ferguson & Palumbo 1979, Fuhrman et al 1980) have taken samples whenever sampling is possible at a particular site rather than waiting for a particular time of day for “comparable” measurements.

Studies of diel cycles usually follow 3 basic approaches: (1) samples are kept in containers and subsampled over time (Eppley et al, 1981); (2) sampling is repeated at a fixed point (e.g. anchored ship, beach) over time (LeBouteiller and Herbland, 1982); (3) some attempt is made to follow a defined water mass which is sampled over time (Ryther et al, 1971).

Each approach has advantages and disadvantages. Confinement in containers or microcosms has the advantage of ensuring that the same water is being subsampled over time, but there is the disadvantage of not knowing if the confined communitiy is representative of that of the natural system from which it was collected.

Following a natural mass of water with time has the advantage of sampling the same planktonic populations without confinement artefacts. The disadvantages are related to the requirement of previous knowledge of the physics of water masses and some means of following the water (i.e. a drogue and a ship). Conceptual problems arise because it is not clear to what extent a water mass remains a single entity over time as it is turbulently mixed with adjacent water.

The approach selected in this work, i.e., sampling at a fixed-point (e.g. Meyer-Reil et al, 1979) has the advantage of dealing with truly natural communities and is fairly easy to perform. However, water keeps flowing, and different populations may be sampled at different times (Ferguson and Palumbo, 1979). This may be particularly pronounced in a tidally influenced system such as the Ria, where apparent repeating patterns (6 or 12 h periodicities) could be mistaken for diel variations of a single population. The disadvantage was noticeable in the results obtained with this study. Some microbiological descriptors (e.g. leucine-aminopeptidase) followed clear tidal patterns rather than diel fluctuations, hampering the assessment of changes in bacterial activity that could be attributed directly to the effect of solar radiation.

Methodological Considerations of the Experimental Assessment of UV-R Effects on Aquatic Bacterial Communities

Over the last decade, our knowledge about the effects of UVB radiation on organisms in the laboratory has improved significantly. However, it is still not possible to predict the changes that might occur in the natural environment. Extending laboratorial results to a whole ecosystem perspective requires that some fundamental criteria are met in the experimental design.

First, the organisms used in the experiment must be representative of natural conditions, in terms of abundance and sensitivity to UV-B radiation (Ochs, 1997). For example, organisms cultured in laboratory in the absence of molecular sunscreens, both coloured and colourless (Garcia-Pichel and Castenholz, 1993), can show reduced repair capabilities and thus be more susceptible to UV-B damage than natural organisms (Paerl et al, 1985).

Secondly, the net impact of UV-B radiation on the ecosystem is determined by the relative sensitivity of the different members of the community. Although physiological experiments and UV-B dose-response curves for a particular group of organisms from a single trophic sensitivity level may elucidate UV-B damage and repair processes, different organisms (e.g., predator and prey, Bothwell et al, 1994) can show different sensitivity levels to UV-B radiation and the results of these experiments cannot be applied directly to the natural environment, which is governed by a complex web of trophic interactions.

Thirdly, experimentally enhanced UV-B doses must be representative of plausible natural conditions. Besides measurements of the dynamics of several trophic levels, changes in the dynamics of environmental variables such as nutrients and temperature should also be investigated in enhanced UV-B studies.

Finally, in the pelagic system, the fate of planktonic organisms is closely linked to mixing dynamics (Neale et al, 1994). The euphotic zone of the pelagic system is heterogeneous with respect to UV-B response, permitting periodic damage at the surface as well as periodic repair in deeper waters. Good experimental design will ensure that mixing is uniform, reproducible and representative of the natural conditions.

Theoretically, we find the microcosm used in these experiments in accordance with most of the requirements. Whole water samples contain organisms representative of those naturally occurring in the environment at the different levels of the trophic chain, since the exposed samples were not subjected to any treatment to selectively remove predators, as performed by some authors (e.g. Chróst and Faust, 1999). The interactions of primary producers and bacteria appear to be a critical factor influencing the outcome of UV radiation exposure. Thus, when using whole water samples, the measured response for bacteria actually represents a combined effect of bacterial inhibition and effects of UV stress or photo-inhibited phytoplankton. Removing phytoplankton has been found to decrease the nutrient pool available for bacteria. Therefore, although understanding the direct effects of UV radiation on bacteria is best accomplished in alga-free incubations, a true estimate of UV radiation effects in the environment necessary requires whole water studies to be conducted (Aas et al, 1996).

Furthermore, the levels of UV-B radiation used in the studies hereby reported are representative of those occurring in the nature and that have been used by other authors studying the effects of artificial UV-B radiation on bacterioplankton metabolism (e.g., Herndl et al, 1993; Müller-Niklas et al, 1995; Kaiser and Herndl, 1997). We also provided mechanic stirring to the system, theoretically simulating physical mixing occurring in the environment. However, the results suggest that the confinement of the samples in the system was potentially leading to the accumulation of inhibitory photoproducts that could exacerbate damage to bacteria, besides that directly induced by UVR. Thus, a more correct approach would probably be the use of mesocosm studies, rather than a microcosm, that could allow the diffusion of these growth-inhibitor substances.

INTEGRATING FIELD AND LABORATORY EXPERIMENTS TO ASSESS THE IMPACT OF ULTRAVIOLET RADIATION ON BACTERIAL COMMUNITIES

Several different models and experimental approaches have been used to study the effects of UVR on bacterial assemblages (see e.g. Arrieta et al, 2000; Chatila et al, 2001). However, the multifactorial nature of the process makes it very difficult to generalize response patterns (Peachey, 2005). Experimental models, such as microcosms, can help simulating mechanisms of net DNA damage and repair considering variables such as dissolved organic matter (liability/composition), spectral irradiance and vertical mixing of the water column (Huot et al, 2000), but the results must be integrated with field studies to achieve a realistic perspective of the effects of increased UVR levels on bacterial activities and composition.

The intensity of the UV-B radiation used in our studies is comparable to levels in the solar spectrum naturally occurring during the summer. However, the results obtained by experimentally manipulating UVR exposure do not parallel the results obtained in the field, during the assessment of diel cycles. This can be attributed to complex interactions that are established between DOM and bacteria in coastal areas that make it difficult to discriminate the direct effect of solar radiation. Some artefacts introduced by the experimental setup can also enhance the detrimental effects of UVR.

Repair processes occur in the environment and can compensate the direct negative effects of solar UV-B radiation. Photoenzymatic repair of DNA damage is one of the most important repair processes occurring in the nature, and probably is more important for bacterioplankton than the dark repair (Kaiser and Herndl, 1997). This repair pathway is activated by the longer UV-A wavelength range (360 to 400 nm) and by blue light (400 to 430 nm) (Friedberg, 1985). These wavelengths are naturally present in solar radiation, but were not incorporated in our microcosm experiments and thus their importance was not assessed.

In the environment, bacterial assemblages are naturally transported between surface and the deeper water layers, where UV-B radiation is selectively attenuated more rapidly than UV-A and than blue light. This allows recovery to take place (Kaiser and Herndl, 1997). The microcosm approach basically traps bacterial communities in a layer where radiation incides with high intensity and that does not allow stratification. The suppression of at least the most UV

sensitive bacterial strains (Winter et al, 2001) may occur. The upper layer of the water column is involved in mixing processes and therefore it constitutes an environment in which radiation conditions are rapidly changing (Kaiser and Herndl, 1997). Also, damage to bacteria by reactive oxygen species resulting from UV-induced photoxidation of DOM may be exacerbated by enclosed experimental systems (Sommaruga et al, 1997).

Finally, in the environment several factors contribute to the attenuation of the incident radiation including cloud coverage, atmospheric pollution and ozone concentration and the angle of incident radiation (Paul and Gwynn-Jones, 2003), factors that were not present in the microcosm study and that could also help to explain the high levels of inhibition observed.

Future work will be directed to the molecular mechanisms involved in solar and UV-resistance, its implications to the cycling of DOM at the SML and the consequences of increased UVR associated with ozone depletion to the function of this peculiar ecological niche.

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