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Effects of solar radiation on marine bacterial and phytoplankton heterotrophic activities

(Efectos de la radiación solar sobre las actividades heterotróficas del bacterioplancton y fitoplancton marinos)

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En Barcelona, a de de

La Doctoranda	El Director	El Codirector
Clara Ruiz González	Josep M. Gasol	Rafel Simó

A mis padres,
A Marta,
A Juancho, el mejor apoyo,
Y a mis cuatro incondicionales
acompañantes en esta "tesis con Alegría"

Contents

Abbreviations

List of the most common abbreviations and acronyms used in this thesis:

BA: Bacterial abundance
BHP: Bacterial heterotrophic production
Bcdt: *Bacteroidetes*
CARD-FISH: Catalyzed reporter deposition-fluorescence in situ hybridization
Chl *a*: Chlorophyll *a*
DMS: Dimethylsulfide
DMSP: Dimethylsulfoniopropionate
DOC: Dissolved organic carbon
DOM: Dissolved organic matter
Eub: *Eubacteria*
Gam: *Gammaproteobacteria*
HB: Heterotrophic bacteria
HNF: Heterotrophic nanoflagellates
LIR: Leucine incorporation rates
MAR-CARD-FISH: Microautoradiography combined with CARD-FISH
PAR: Photosynthetically active radiation (400-700 nm)
PFA: Paraformaldehyde
Ppeuk: Photosynthetic picoeukaryotes
Prochl: *Prochlorococcus*
Ros: *Roseobacter*
Syn: *Synechococcus*
TCA: Trichloroacetic acid
UVA: Ultraviolet radiation A (320-400 nm)
UVB: Ultraviolet radiation B (280-320 nm)
UVR: Ultraviolet radiation (280-400 nm)

Resumen

Desde el hallazgo, hace dos décadas, de que la radiación ultravioleta (UVR, 280-400 nm) penetraba en el océano más de lo que se creía hasta el momento, se ha avanzado significativamente en el conocimiento de sus efectos sobre diversos procesos y organismos de las capas superficiales del océano; sin embargo, aún son muchos los aspectos que desconocemos acerca de las interacciones entre la radiación solar y las comunidades oceánicas. Las bacterias marinas, consumidoras primarias de la materia orgánica disuelta (DOM) en el agua, se consideran entre los organismos del plancton más sensibles a la UVR debido a su pequeño tamaño y a la falta de pigmentos. Nuestro objetivo fue el estudio de los efectos de la radiación solar sobre la actividad heterotrófica de las bacterias marinas, aunque ocasionalmente también nos concentramos en su impacto sobre la osmoheterotrofía del fitoplancton. Para ello combinamos medidas de asimilación total de compuestos orgánicos con técnicas de resolución individual como la microautoradiografía a fin de examinar tanto los efectos de la luz a nivel de comunidad como las respuestas particulares de distintos grupos de microorganismos. Nuestro trabajo subraya el papel de la radiación solar como moduladora de los flujos de DOM, si bien las respuestas observadas variaban dependiendo no sólo de los cambios en la luz, sino también de la exposición previa de las muestras, la identidad de los organismos implicados, los sustratos considerados y la similitud entre nuestras condiciones de exposición y la radiación recibida por las muestras in situ. Mientras que la exposición a la UVR a menudo redujo la incorporación de leucina por los organismos, la radiación fotosintéticamente activa (PAR, 230-400 nm) provocó una estimulación ocasional de la toma de este aminoácido, discutiéndose, entre otras posibilidades, una potencial fotoheterotrofía. Por otro lado, la asimilación del compuesto orgánico dimeltilsulfoniopropionato (DMSP) generalmente aumentaba en presencia de luz, particularmente en las fracciones no bacterianas. Nuestros resultados indican por tanto que la osmoheterotrofía del fitoplancton, también influida por la luz, es más común y está más extendida de lo que se pensaba, especialmente en las comunidades de aguas polares de verano tan ricas en DMSP. Esta tesis ilustra la complejidad de las interacciones entre la luz y los microorganismos y señala la necesidad de estudiar en más detalle sus efectos sobre los distintos componentes de las redes tróficas microbianas.

Summary

Since the discovery, 20 years ago, that solar ultraviolet radiation (UVR, 280-400 nm) penetrates much deeper into the ocean's water column than previously thought, knowledge on its effects on diverse surface processes and organisms has significantly increased, yet further research is needed for a good understanding of sun-organism interactions. Marine bacteria are considered some of the most susceptible to sunlight damage due to their small size and lack of pigments. Since they are primary consumers of dissolved organic matter (DOM) in seawater, one might expect that any effect on them will ultimately have implications for carbon and nutrient cycling. In this work, we contributed to increase the knowledge on the effects of sunlight on the heterotrophic uptake of DOM by bacteria, although we also devoted some effort to assess its impact on the osmoheterotrophic activity of phytoplankton. For that purpose, we combined bulk activity measurements with a single-cell approach involving microautoradiography for identification of both the responses at the community levels and particular responses and sensitivities to sunlight of different microorganisms. Our work highlights the role of solar radiation as a significant modulator of DOM fluxes through differentially affecting the diverse components of the planktonic food webs. The observed responses were shown to vary not only at different scales depending on changes in the light conditions, but also owing to the previous light-exposure history, the identity of the organisms involved, the substrates considered and the accuracy of our light incubation conditions. Whereas exposure to sunlight often led to a reduction in the amount of leucine incorporated, incubation under photosynthetically active radiation (PAR, 400-700 nm) conditions sometimes stimulated its uptake, and potential photoheterotrophy is discussed among other possibilities. In contrast, bulk assimilation of the organic compound dimethylsulfoniopropionate (DMSP) was often increased upon light exposure, particularly within the non-bacterial fraction. We also found that algal osmoheterotrophy seems to be widespread and more common than previously thought, particularly within the phytoplankton assemblages inhabiting the DMSP-rich summer polar waters, and that this behaviour was highly dependant on the light levels. The present thesis sheds light on the complexity of sun-microbe interactions and provides evidence of the need to afford the study of solar radiation effects on the different compartments of the microbial food webs.

GENERAL INTRODUCTION

SUNLIGHT BENEATH THE OCEAN'S SURFACE

Light as the power of the planktonic food web

Although the vast majority of the world ocean's total volume is plunged into darkness, the processes that take place within the relatively narrow illuminated surface layer (i.e. the photic layer) are of enormous significance to the global biosphere. The visible region of the solar spectrum (so-called photosynthetically available radiation, PAR, 400-700 nm) reaching this photic layer fuels around half of the photosynthetic creation of organic material of the planet, supporting both, all the other inhabitants of the ocean's surface and most denizens from deeper waters. As a consequence, most of the oceanic biomass is concentrated in this 3% of the total oceanic volume, and organisms inhabiting there interact creating a complex framework of relationships that shape the planktonic food web.

Until 1974, the marine food web was believed to fit reasonably well with the classical paradigm of primary producers, secondary producers and decomposers; however, several new discoveries led scientist to think that such a textbox description in fact achieved only a small fraction of the total flow of energy. In 1974, Lawrence Pomeroy proposed that the unseen microbes were far more important and diverse than previously assumed, and suggested than the classical paradigm had to be redrawn towards a more comprehensive model where bacteria played a key role in remineralizing the large oceanic dissolved organic carbon (DOC) pool thus making it available to higher trophic levels. Since then, new findings such as the regulation of bacterial populations by grazers (Borsheim, 1984; Fenchel, 1982; Pedrós-Alió and Brock, 1983) or viral infection (Bratbak *et al.*, 1992; Bratbak *et al.*, 1994) have added extra layers of complexity to the so-called microbial loop (after Azam *et al.*, 1983). This complexity of the trophic structure in the ocean is summarized in Fig. 1

Sunlight was thought to enter the system mainly via phytoplankton cells thus acting as a major modulator of the fluxes of DOM throughout this complex framework, since any variations in light availability would ultimately alter the amount of organic matter generated by photosynthesis. Nonetheless, the relatively recent consideration of solar ultraviolet radiation (UVR, 280-400 nm) has further challenged the assumed role of light in marine planktonic communities.

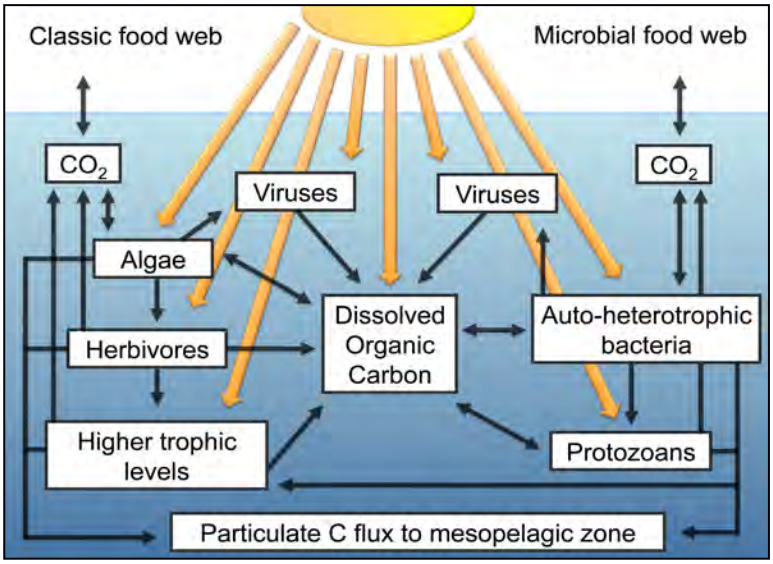

Fig. 1. Schematic diagram of the pelagic marine food web illustrating the flow of carbon and energy through the system and some of the compartments directly affected by sunlight (see below). Adapted from Häder *et al.* (2007).

UV radiation in marine ecosystems: should we care about it?

By the mid 1980s, concerns about ozone depletion and resulting increases in UVB (280-320 nm) reaching the Earth's surface prompted urgent research to examine the effects of this energy at the organism level. Subsequent discoveries, such as the evidence that this potentially harmful fraction of the solar spectrum penetrates much deeper into the ocean's water column that previously thought (Karentz and Lutze, 1990; Worrest and Häder, 1989) began to throw light on the fact that UVR might significantly modulate the cycling of organic matter in the sea by affecting not only algae, but all the components of the microbial loop.

Much progress has been achieved since that discovery, yet our present knowledge of the role of solar UVR on the total biogeochemical fluxes is still incomplete. Moreover, even thought stratospheric ozone depletion has now stabilized and its concentrations are beginning to increase again following decreases in the emissions of ozone-depleting substances, current projections indicate that the return to pre-1980 ozone levels may not occur for several decades, with the consequently elevated UVR sustained over that period (McKenzie *et al.*, 2007). In

addition, since UVR is influenced by many factors other than ozone, including cloud cover, aerosols and air pollution, all of which are in turn affected by global change, unpredictable interactions between ozone and climate change make the future still rather uncertain (e. g. Andrady *et al.*, 2007).

Solar radiation is now known to affect several processes in marine systems and, depending on the wavelengths or regions of the solar spectrum, these effects may vary. Although, on a photon basis, UVA radiation (320-400 nm) contains less energy than UVB, the greater fraction of solar radiation in the UVA spectrum may result in a significant source of biological damage (Karentz *et al.*, 1994).

Initially, most studies focused on phytoplankton and abundant literature exists on the sensitivity of these organisms to solar UVR in aquatic environments ranging from polar to tropical (see refs in (Xue *et al.*, 2005). High levels of solar radiation have been shown to not only inhibit photosynthesis (Smith *et al.* 1992, Neale *et al.* 1994, Cullen & Neale 1997,Villafañe *et al.* 2004, Yuan *et al.* 2007), but also affect motility, growth and development, pigment content, respiration, nutrient uptake and metabolism of algal cells (see refs. in Rai and Mallick, 1998; Xue *et al.*, 2005). Instead, much less is known about UVR effects on other components of the microbial food webs, yet an increasing number of studies are showing that it can also damage aquatic viruses (Jacquet and Bratbak, 2003; Regan *et al.*, 1992; Suttle and Cheng, 1992; Wilhelm *et al.*, 2003), heterotrophic flagellates (Ochs, 1997; Ochs and Eddy, 1998; Sommaruga *et al.*, 1996) and heterotrophic bacteria (Gustavson *et al.*, 2000; Herndl *et al.*, 1993; Jeffrey *et al.*, 1996a; Kaiser and Herndl, 1997). Further, besides this inhibitory effect on the different organisms, UVR can also indirectly modify the fluxes of carbon and nutrients trough the trophic web by enhancing or decreasing the bioavailability of dissolved organic matter (DOM) for microbes (Benner and Biddanda, 1998; Herndl *et al.*, 1997; Morán and Zepp, 1997; Obernosterer *et al.*, 1999; Obernosterer *et al.*, 2001; Tedetti *et al.*, 2009; Tranvik and Kokalj, 1998; Wetzel *et al.*, 1995), or by tuning the interactions between viruses and hosts (Jacquet and Bratbak, 2003; Maranger *et al.*, 2002), prey and predators (Scott *et al.*, 1999; Van Donk and Hessen, 1995), symbionts (Dionisio-Sese *et al.*, 2001), and so on. Hence, depending on the vulnerability of each trophic compartment, a given UVR negative impact might be compensated, or even reversed, by an indirect stimulatory UVR-driven effect (e.g. damage on bacteria vs. bacterial stimulation due to photochemical transformation of DOM into more biolabile forms). Finally, PAR and UVA wavelengths might be involved in the photoenzymatic repair of DNA (Sancar and Sancar, 1988), so, depending on the relative

exposures to UVB versus longer wavelengths, the balance between damage and repair will vary (Herndl *et al.*, 1997; Kaiser and Herndl, 1997), further complicating any attempt of modeling or prediction.

UVR modulates DOM fluxes throughout the marine food web

Among all these potential targets of UVR penetrating the ocean, heterotrophic bacteria have received increasing attention. Since they are major players in the cycling of nutrients and energy, any impact on them could significantly modify the fluxes of DOM channeled to higher trophic levels.

Due to their lack of pigments and small cellular volume, marine heterotrophic bacteria are considered among the groups of plankton more susceptible of sunlight damage (García-Pichel, 1994). Rising research on this topic has demonstrated that short-term exposure of bacteria to both artificial or natural UV doses can significantly reduce the incorporation of radiolabelled substrates such as ^{3}H-leucine or ^{3}H-thymidine (Aas *et al.*, 1996; Alonso-Sáez *et al.*, 2006; Herndl *et al.*, 1993; Kaiser and Herndl, 1997; Müller-Niklas *et al.*, 1995; Sommaruga *et al.*, 1997), showing inhibition percentages as high as 75% of the corresponding dark control (Conan *et al.*, 2008). Although, in general, the damaging effect of UVR increases towards shorter wavelengths, this is not always the case: significant (or even dominant) inhibition due to both PAR and UVA has also been reported (Aas *et al.*, 1996; Morán *et al.*, 2001; Pakulski *et al.*, 2007; Sommaruga *et al.*, 1997). This is believed to occur through the formation of reactive oxygen species or free radicals that can interact with DNA or other cellular components (Harrison, 1967; Mitchell, 1995). In many cases, though, the wavelength dependence of the observed effects is not well understood and varying responses of bacterial activity have been detected as a function of sampling location, depth, time of the day, substrate considered, light intensities and other variables. For a general view, the existing literature on light effects upon bulk marine bacterial heterotrophic activity (measured as ^{3}H-leucine or ^{3}H-thymidine uptake, the most commonly used tracers) is summarized in Table 1, illustrating the remarkable variability among the reported results.

Table 1. Compilation of literature on the effects of sunlight on marine bacterial heterotrophic activity with respect to dark treatments. Black and white arrows indicate inhibition and stimulation of activity, respectively. Only ^{3}H-leucine or ^{3}H-thymidine incorporation data are included for simplification since these are the most commonly used tracers.

Authors	Year	Location	Light source	Wavelenghts	Time of exposure	Main reported effects Substrate incorporation	
						Leucine	Thymidine
Herndl et al.	1993	Adriatic Sea	Artificial	UVB	30 min/4h	↓	↓
			Sunlight	PAR+UVR	4h		↓
				PAR+UVA	4h		↓
				Darkness	4h-8h		Recovery
Müller-Niklas et al.	1995	Adriatic Sea	Artificial	UVB	6-12h		
				Darkness			
			Sunlight	PAR+UVR	4h		↓
				Darkness			Recovery
Aas et al.	1996	Gulf of Mexico	Sunlight	PAR	1-11h	↓⇧	↓⇧
				PAR+UVA		↓⇧	↓⇧
				PAR+UVR		↓	↓
		St. Rosa Sound (Florida)		PAR	2-10h	↓⇧	↓
				PAR+UVA		↓	↓
				PAR+UVR		↓	↓
Kaiser & Herndl	1997	N Adriatic Sea	Artificial	UVB	2-4h		↓
				UVA			Recovery
				PAR			Recovery
				Darkness			No recovery
			Sunlight	PAR+UVR	3h	↓	↓
				PAR+UVA		Recovery	Recovery
				Darkness		Recovery	No recovery
Sommaruga et al.	1997	N Adriatic Sea	Sunlight	PAR	3-4h	↓	↓
				PAR+UVA		↓	↓
				PAR+UVB		↓	↓
Pakulski et al.	1998	Pickles Reef (Florida)	Sunlight	PAR+UVR	2 days	↓	↓⇧
Shiah	1999	Kuroshio (Taiwan)	Sunlight	PAR	1day		↓(day)
			Artificial	PAR	4-6h		↓
Visser et al.	1999	Caribbean Sea	Sunlight	PAR	3h	↓	↓
				PAR+UVA		↓	↓
				PAR+UVR		↓	↓
Gustavson et al.	2000	Gullmar Fjord (Sweden)	Sunlight	*respect to PAR+UVA*	1-11 days		
				PAR+UVR			↓⇧
			+artif. UVB	PAR+UVR+UVB			⇧
Ziegler & Benner	2000	Laguna Madre (Texas)	Sunlight	PAR	1h	⇧	
				PAR+UVR		⇧	
Chatila et al.	2001	St. Lawrence estuary (Québec)	Sunlight	PAR	7 days		-
				PAR+UVR			-
			+artif. UVB	PAR+UVR+UVB			↓
Morán et al.	2001	NW Mediterranean	Sunlight	PAR	2h	↓	
			artif.UVB	PAR	2h	↓⇧	
		N Atlantic	Sunlight	PAR	3-6h	↓⇧	
Pausz & Herndl	2002	North Sea	Artificial	PAR+UVR	4h	↓⇧	
				PAR+UVA		Recovery	
				PAR		Recovery	
				Darkness		Recovery	
Visser et al.	2002	Caribbean Sea	Sunlight	PAR+UVR	8h	↓	↓
Church et al.	2004	North Pacific	Artificial	PAR	1-2h	⇧	
Alonso-Sáez et al.	2006	NW Mediterranean	Sunlight	PAR	4h	↓	
				PAR+UVA		↓	
				PAR+UVR		↓	
Hernández et al.	2006	Coliumo Bay (Chile)	Sunlight	PAR	4-11 h	↓	↓
				PAR+UVA		↓	↓
				PAR+UVR		↓	↓
Hernández et al.	2007	Coliumo Bay (Chile)	Sunlight	PAR	5-20h	↓⇧	↓⇧
				PAR+UVA		↓⇧	↓⇧
				PAR+UVR		↓⇧	↓⇧
Michelou et al.	2007	N Atlantic	Sunlight	PAR	1h, 6h	⇧	
Pakulski et al.	2007	E Pacific	Sunlight	PAR	4h	↓	↓⇧
				PAR+UVA		⇧	↓⇧
				PAR+UVA(370nm)		↓⇧	↓⇧
				PAR+UVR		↓	↓⇧
Conan et al.	2008	New Caledonia	Sunlight	PAR	6h	↓	
				PAR+UVA		↓	
				PAR+UVR		↓	
Pakulski et al.	2008	Palmer Station (Antarctica)	Sunlight	PAR	12h	↓	
				PAR+UVR		↓	
Joux et al.	2009	NW Mediterranean	Sunlight	PAR	9-10h	-	-
				PAR+UVR		↓	↓
Santos et al.	2010	Ria de Aveiro (Portugal)	Artificial	UVB	9h	↓	

A careful look at Table 1 shows that the effect of light on bacterial activity is not always negative. How could we explain, for example, the consistent PAR-driven increases in bacterial production found by Church *et al.* (2004), or the enhancement in both ^{3}H-leucine and ^{3}H-thymidine incorporation due to every light treatment reported by Pakulski *et al.* (2007)? Besides the possibility of some indirect light-driven effects such as increased photosynthate production or higher bioavailability of photolysed DOM, recent research has suggested that mixed metabolic strategies, collectively known as photoheterotrophy, might be rather common among marine bacteria and widespread in oceanic waters, potentially contributing a considerable fraction of the measured bacterial production in surface waters, (Béjà *et al.*, 2000; Kolber *et al.*, 2001; Kolber *et al.*, 2000; Zubkov *et al.*, 2003).

Photoheterotrophy in the ocean: can everyone do everything?

Unlike our view of most terrestrial ecosystems where taxonomically-defined plants and animals are considered to ecologically behave as such, the limits among marine microbes are not so well defined. Most microbes can alternate or simultaneously use different modes of metabolism so that, depending on the environmental conditions, they may use different energy and carbon sources. In this regard, three major types of mixotrophic strategies can be distinguished: proteorhodopsin (PR)- containing bacteria, bacteriochlorophyll-containing bacteria (so-called aerobic anoxygenic phototrophs, AAnPs), and prokaryotic or eukaryotic phytoplankton capable of phagotrophy or osmoheterotrophic uptake of DOM. Additionally, a kind of acquired phototrophy involving algal endosymbiosis or plastid retention from ingested preys seems to be widespread among radiolaria, foraminifera and ciliates, although its relevance in aquatic ecosystems is still less recognized (Porter, 1988; Stoecker, 1998; Stoecker *et al.*, 2009; Stoecker *et al.*, 1987).

Proteorhodopsin-containing bacteria

Before 2000, most marine phototrophic metabolisms were thought to be based on chlorophyll-like molecules, a paradigm that was challenged that year with the discovery of bacterial rhodopsin-like proteins (proteorhodopsins; PR) in the ocean. These simple

photosystems, long known to occur in some halophilic archaea (Oesterhelt and Stoeckenius, 1973), structurally resemble the rhodopsin proteins of the retina of high eukaryotes (such as humans), but have a different evolutionary origin. They are retinal-containing integral membrane proteins that function as light-driven proton pumps, and were first shown to be widely harbored by SAR 86, an abundant marine *Gammaproteobacteria* clade (Béjà *et al.*, 2000), a fact that brought about the name 'proteorhodopsins'. Shortly after this discovery, these PRs started to be identified in many bacterial groups and now they are known to occur within *Alpha- Beta- and Gammaproteobacteria, Bacteroidetes, Actinobacteria* and *Planctomycetes*, as well as in some archaeal taxa (Béjà *et al.*, 2000; Giovannoni *et al.*, 2005; McCarren and DeLong, 2007; Sharma *et al.*, 2008; Stingl *et al.*, 2007). This spread suggests a significant role of these proteins in the life of bacteria inhabiting the photic zone of oceans and lakes. Nonetheless, there is a striking lack of knowledge concerning the magnitude of the role of PR in natural communities: so far, only a few reports have provided direct evidence that PRs actively function as a light-harvesting pigment in seawater (Béjà *et al.*, 2001; Giovannoni *et al.*, 2005), and just another pair have reported on the physiological advantages that PRs confer to marine bacterial isolates, promoting either growth (Gómez-Consarnau *et al.*, 2007) or survival (Gómez-Consarnau *et al.*, 2010). However, in spite of this lack of evidences, some very recent results about increased PR expression upon light exposure (Béjà and Suzuki 2008; Gómez-Consarnau *et al.*, 2007; Lami *et al.*, 2009; Poretsky *et al.*, 2009) suggest an active (although greatly unknown) role in natural ecosystems.

Aerobic anoxygenic photosynthetic bacteria (AAnPs)

It was initially believed that bacteriochlorophyll *a* (Bchl*a*)- containing bacteria, also known as purple bacteria, required anoxic conditions for photosynthesis (Pfennig, 1967), until (Shiba *et al.* (1979) and Shiba and Simidu (1982) showed that an obligate aerobic bacterium (*Erythrobacter longus*) produced their photosynthetic apparatus in the presence of oxygen and light. Later on, these authors found high proportions of these bacteria in the coasts of Australia, and suggested that this metabolic versatility may help them to prevail under certain conditions (Shiba *et al.* 1991). However, it was not until 2000, when Bchl*a* containing bacteria were found to be abundant in the surface ocean (Kolber *et al.*, 2001; Kolber *et al.*, 2000), that it started to be considered a metabolism of certain significance on a global scale. Since then, diverse environmental data have confirmed the ubiquitous

presence of Bchl*a* among different members of the bacterial community such as *Alpha-*, *Beta-*, *Gammaproteobacteria* and *Bacteroidetes*, although they have mostly been found in the alphaproteobacterial *Roseobacter* group (Cottrell *et al.*, 2006; Rusch *et al.*, 2007; Sieracki *et al.*, 2006; Venter *et al.*, 2004; Yutin *et al.*, 2007).

As for PRs, very little information is available about the biological advantages of harboring Bchl*a*. Although none of the existing isolates has yet been grown autotrophically, light stimulation of CO_2 uptake has been detected in several species (Kishimoto *et al.*, 1995; Shiba, 1984; Shiba and Harashima, 1986; Suyama *et al.*, 2002) but at too low rates for strictly autotrophic growth. However, it might be a way of harvesting additional energy to supplement their otherwise purely heterotrophic metabolism, a mechanism potentially adjustable under certain conditions such as low DOC concentrations (Suyama *et al.*, 2002). An increase in knowledge about the physiology and ecology of these organisms is needed since very little is understood about their contribution to phototrophic processes.

Phytoplankton osmoheterotrophy and phagotrophy

Unlike the relatively recent discovery of the previous two groups of photoheterotrophs, the uptake and utilization of DOM was demonstrated many years ago for a wide variety of algal cultures (see references in Amblard, 1991; Neilson and Lewin, 1974), yet it was initially thought to be ecologically irrelevant due to the commonly low substrate concentrations found in natural environments (Hellebust, 1970; Wright and Hobbie, 1965; Wright and Hobbie, 1966). However, some studies have shown that diverse phytoplankton species are capable of actively taking up a broad diversity of organic substrates from the dissolved pool so that they might be good competitors with bacteria (Allen, 1971; Kamjunke *et al.*, 2008; Kamjunke and Tittel, 2008). Together with the phagotrophy described for many algal groups (Jones, 1994; Raven, 1997), this suggests that algae may play more diverse roles in aquatic biogeochemical cycles besides that of primary production and the supply of heterotrophs with autotrophically synthesized organic matter. Again, however, very little is still known about the potential role of phytoplankton heterotrophy in natural marine communities.

On the other hand, even though cyanobacteria are often described as photoautotrophic, the uptake of DOM by some genera is well described in the literature (e.g. Chen *et al.*, 1991; Collier *et al.*, 1999; Paerl, 1991; Rippka, 1972; Zubkov *et al.*, 2003; Zubkov and Tarran, 2005).

Recent studies on the abundant marine cyanobacteria *Prochlorococcus* and *Synechococcus* have unveiled a major role of these groups in the cycling of different organic compounds (Malmstrom *et al.*, 2005; Zubkov *et al.*, 2003; Zubkov and Tarran, 2005). This potential for partial heterotrophy may lead to overestimation or misinterpretation of bacterial production measurements using e.g. the leucine incorporation method (Kirchman *et al.*, 1985) since, for example, *Prochlorococcus* may account for up to 25% - 30% of leucine incorporation rates (Michelou *et al.*, 2007; Zubkov and Tarran, 2005).

In this regard, some inevitable questions arise: to what extent does light influence all these photoheterotrophic behaviors? Can this potential stimulation of heterotrophic activities overshadow the inhibitory effects of UVR in the uptake of substrates by different microorganisms? Could light exposure lead to a more efficient competition of photoautotrophic organisms against their purely heterotrophic counterparts for the uptake of DOM?

To answer these and other issues, one main idea becomes obvious: knowledge of the plankton community composition becomes essential to properly understand the occurring processes that drive the observed bulk ecosystem responses to light.

Identity analysis: the importance of the individual roles

Whereas, as previously stated, the effects of UVR on bulk bacterial communities have been widely studied in the last 20 years (Table 1), we know very little of whether the effects of PAR and UVR on cellular components or activity are uniformly distributed within natural bacterioplankton assemblages. By exposing marine bacterial isolates, some studies have evidenced interspecific variability not only in the accumulation of DNA damage (Joux *et al.*, 1999), but also in their viability after exposure, specific activities, or DNA damage repair capabilities (Agogué *et al.*, 2005; Arrieta *et al.*, 2000; Helbling *et al.*, 1995). However, since most bacteria are not easily cultivable, such isolates may be only minor components of natural bacterial populations and the observed responses may not be representative of those of nartural bacterial communities (Amann *et al.*, 1995). Only a small number of studies have addressed this issue with natural assemblages yet unveiling that the more we advance in knowledge, the larger the complexity that seems to arise.

In this regard, using PCR-DGGE analysis based on 16S rDNA, (Winter *et al.*, 2001) found a few UVR sensitive phylotypes in UVR-transparent mesocosms, whereas Santos *et al.* (2010) observed that exposure to UVR caused a considerable reduction in freshwater and estuarine bacterial diversity in the coast of Portugal, pointing to a potential role of UVR in shaping microbial communities. By combining microautoradiography with a catalyzed reporter deposition-fluorescence in situ hybridization (MAR-CARD-FISH) technique, Alonso-Sáez *et al.* (2006) found that UVR effects on bacterial single-cell activities differed among taxonomic groups in the coastal NW Mediterranean. Flow cytometric analysis of the same samples showed *Synechococcus* to be significantly more resistant to UVR than *Prochlorococcus* (Sommaruga *et al.* 2005). Very recently, Kataoka *et al.* (2009) reported for the first time on UVB-resistant or sensitive bacteria in an oceanic environment with the use of PCR-DGGE combined with inmunocapturing techniques. Among all these methods, microautoradiography can be a good choice when assessing the role of sunlight on the uptake of radiolabeled DOM compounds by individual organisms within natural communities (Meyer-Reil, 1978). This technique is based on the detection of the radioactivity retained by active cells through precipitation of silver grains of a photographic emulsion around them; after development of samples, silver grains are visible like black dots under transmission mode of the microscope (see Fig. 2). Additionally, if used in combination with identification tools such as CARD-FISH, microautoradiography gives us information about both the identity and the activity of target groups.

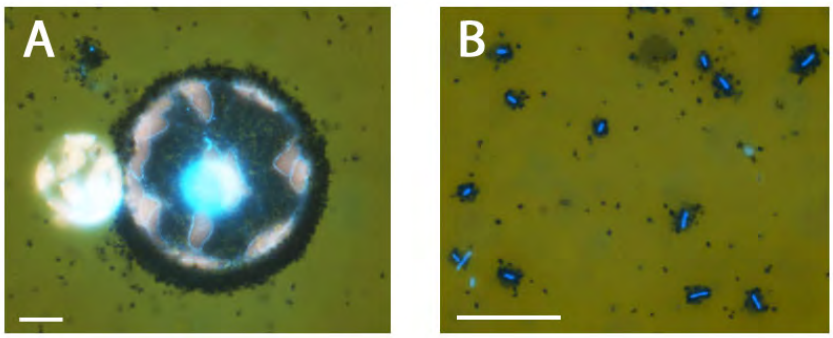

Fig. 2. Examples of microautoradiograms of marine algal (A) and bacterial (B) samples. Black dots surrounding cells indicate assimilation of the added radiolabeled substrate, and are formed when the radioactivity retained within the 'active' cells impacts onto a photographic emulsion precipitating silver grains that are visible upon development of the emulsion. The images are produced with UV excitation combined with transmitted light. Scale bars represent 10 μm (A) Two centric diatoms, one inactive at ^{3}H-leucine uptake (left) and one clearly active (right). On top of the emulsion we can observe red autofluorescence of the chlorophyll a and blue fluorescence from 4',6'-diamidino-2-phenylindole (DAPI) stained nucleus; (B) several bacteria labeled for ^{3}H-leucine uptake showing DAPI-stained nucleic acids.

Obviously, interspecific variability in metabolic activities and their responses to sunlight is not restricted to bacteria. Large differences in sensitivity to UVR and repair capabilities have been reported for a broad variety of organisms including phytoplankton, heterotrophic protists and even pelagic metazoans (Hessen, 2003; Karentz *et al.*, 1991; Llabrés and Agustí, 2006; Llabrés *et al.*, 2010; Sommaruga and Buma, 2000; Sommaruga *et al.*, 2005). Hence it seems that a good knowledge of the composition of the communities combined with analysis of the particular effects within species or groups of organisms may improve our understanding of the global impact of UVR on marine planktonic ecosystems.

Scales of variability in the exposure of marine microbes to sunlight

Coming on top of such a broad range of possible metabolic responses to sunlight, the inherent variability of the light conditions adds some more layers of complexity to the matter. The quality and intensity of solar radiation received by a single cell inhabiting the pelagic ocean will continuously fluctuate mainly following changes in the solar zenith angle, but also in the depth and intensity of the water mixing processes, the light attenuation of the water column, the cloud cover, or even the presence of ice or snow surfaces at the ocean's surface. All this translates into light conditions varying on both temporal scales (day/night cycles, seasons) and spatial scales (attachment to particles, depth, latitude). This large variability complicates the design of experimental settings and hampers the interpretation of experimental results, which will be greatly influenced by the differences between the experimental conditions and those encountered by the organisms in their natural environment.

Spatial variability

Latiudinal variations: from the tropics to the poles

The light regimes in our planet vary spatially as a direct consequence of solar elevation, UVR thus markedly decreasing as one moves from the tropics towards the poles. Nonetheless, although UVR irradiances are weaker in polar regions than in lower latitudes, much effort

has been devoted to studying the impact of UVR in the Arctic and Antarctica since these are the regions most affected by ozone depletion and global warming derived effects (e.g. loss of the ice cover and stronger stratification of the water column due to warmer temperatures (Johannessen *et al.*, 1999; Jones and Shanklin, 1995; Müller *et al.*, 1997; Rothrock *et al.*, 1999)). Besides, the organisms inhabiting these waters are exposed to continuous light during the long polar summer and are further believed to be more at risk because of the known repressed repair capabilities under low temperatures (Vincent et al 2006). On the other hand, tropical and subtropical waters experience strong surface heating, producing warm and salty upper layers that persist for long periods and isolate surface waters from the waters below, thus exposing organisms to high doses of UVR. And finally, falling in between, temperate regions go through seasons gradually changing from a deep mixed winter water column to a more stratified summer waters as temperature and irradiance increase or decrease throughout autumn and spring, respectively. Hence, depending their latitude, marine microbes will be exposed to very different sunlight conditions. However, the lack of comparable studies across different latitudes hampers any prediction of latitudinal trends in the physiological responses to light.

Vertical variations: moving up and down within the water column

Vertical mixing is another factor that determines the UVR exposure received by drifting organisms. Since shorter wavelengths (UVB) are absorbed faster than longer wavelengths (UVA and PAR) in the water column (Hargreaves et al 2003), DOM and microbes are continuously exposed to a changing light field as they move up and down within the upper mixing layer. Thus, depending on both the light penetration and mixing conditions, light exposures will be larger or smaller, and since the relative UVB:UVA and UVB:PAR ratios will decrease with increasing depth, the deeper the organisms move, the greater their chances for photoenzymatic or dark repair of any caused damage. Regardless of the difficulties of mimicking in situ mixing conditions, some studies attempting to address this issue have concluded that deep mixing prevents cells from being damaged during a long period of time because repair is allowed (Herndl *et al.*, 1997; Huot *et al.*, 2000; Jeffrey *et al.*, 1996b; Kaiser and Herndl, 1997; Neale *et al.*, 2003). All in all, there is a clear need for taking mixing into account when considering (and modeling) the exposure of planktonic microbes to sunlight.

Temporal variability

Diel versus seasonal variations

Variability in the light field is certainly not restricted to the spatial scale, but it also involves the temporal dimension. Throughout the day, marine microorganisms are exposed to changing conditions ranging from strong irradiances to darkness, except for those inhabiting very high latitudes during the solstices. Many biological parameters in the ocean vary daily as a direct consequence of the relationship between sunlight and marine biota, with many cycles being synchronized with the diel changes in light availability. As the most obvious example, diel peaks in primary production usually occur around noon, and this cycle in diel supply may thus drive some cyclic patterns in grazers or DOM consumers (Atkinson *et al.*, 1992a; Atkinson *et al.*, 1992b; Gasol *et al.*, 1998; Hernández-León *et al.*, 2001; Shiah, 1999). Similarly, UVR has been shown to directly affect organisms along a diel cycle: for example, Jeffrey *et al.*, (1996b) observed UVR-driven inhibition of ^{3}H-thymidine incorporation and a rapid increase in DNA damage in daytime samples from the Gulf of Mexico, whereas after sunset damage was quickly removed and bacterial activity started to increase again. In accordance with these results, Booth *et al.* (2001) showed that the expression of the DNA repair gene recA also peaked by the end of the day.

Superimposed to this diel scale of variability, the irradiance levels reaching the ocean vary trough seasons: they increase towards the summer, often leading to a shallower stratification of the water column due to warmer temperatures and hence, an increased exposure to strong sunlight levels due to confinement of organisms in this shallow layer. Very few studies have addressed the seasonal variability in the responses of natural microbial communities to the changing light conditions. Some studies with phytoplankton assemblages indicate that there is less inhibition by UVR during the summer months, probably because of seasonal shifts in UVR sensitivity (Gala and Giesy, 1991; Hobson and Hartley, 1983). On the other hand, Furgal and Smith (1997) found that midsummer phytoplankton assemblages were as sensitive to sunlight as spring or autumn communities. Other studies have reported different sensitivities of phytoplankton throughout the year suggesting that cell size, taxonomic composition, temperature, light or nutrient availability might be influencing the observed responses (Banaszak and Neale, 2001; Villafañe *et al.*, 2004). No study has hitherto addressed the seasonality of bacterial responses to radiation conditions. Only Alonso-Sáez *et al.* (2006), who carried out light incubation experiments both in spring and summer, suggested that selection for more photoresistant taxa might occur towards the summer in

Blanes Bay (NW Mediterranean). Also in this area the seasonal dynamics of *Prochlorococcus* and *Synechococcus* may be partly explained by their specific sensitivities to sunlight, since the former, more UVR-sensitive, dominates in fall whereas the abundances of *Synechococcus,* more resistant, increase towards the summer period (Sommaruga *et al.* 2005). It is thus possible that sunlight modifies to some extent the seasonality and succession of different heterotrophic prokaryotes.

Can bacteria adapt to changing light conditions?

There is still no clear evidence about whether bacterioplankton are able to adapt to UVR and, despite the reported interspecific variability in the sensitivity to UVR and the repair capabilities among marine bacterial isolates (Agogué *et al.*, 2005; Alonso-Sáez *et al.*, 2006; Arrieta *et al.*, 2000; Joux *et al.*, 1999; Kataoka *et al.*, 2009), as well as the existence of PRs with different absorption maxima (Béjà *et al.*, 2001), controversial results obscure the answer. Many studies have revealed no differences in light sensitivity between bacteria from high-light and low-light environments, suggesting the lack of adaptative strategies driven by differences in the light fields (Agogué *et al.*, 2005; Bailey *et al.*, 1983; Hernández *et al.,* 2007; Herndl *et al.*, 1993; Xenopoulos and Schindler, 2003). Other authors, however, have found evidences that suggest that photoadaptation might occur either through physiological acclimation or community change in favour of UV-resistant species. For example, Buma *et al.* (2003) compared different ecosystems and found the lowest mean damage ratio (i.e. damage normalized to the level of incident biologically effective irradiance) in samples from three high altitude lakes compared to marine areas. Similarly, observations of increased pigmented bacteria upon UV exposure (Thomson *et al.*, 1980), higher recovery of bacterial activities after the first day of exposure (Pakulski *et al.*, 1998), more sensitive bacteria in spring than in summer (Alonso-Sáez *et al.*, 2006), UVB responses of bacterial isolates in accordance with the irradiation levels of their native environments (Fernández-Zenoff *et al.*, 2006), more sensitivity to UVR in deeper than in surface bacterial communities (Joux *et al.*, 2009), or lower reduction in bacterial diversity and enhanced dark recovery potential in bacterioneuston than in bacterioplankton (Santos *et al.*, 2010) also point to some kind of bacterial adjustment to high UVR doses. Further temporal or spatial large scale comparisons of UVR vulnerability are needed in order to solidly accept a potential for photoadaptation in bacteria.

Closing the loop

In spite of all the sunlight effects on plankton enumerated above, the interactions between these two components do not only occur through 'one way arrows', as illustrated in figure 1. Plankton organisms themselves may also modulate to some extent the levels of UVR that reach the oceans' surface. Among, other possibilities, this has been suggested to occur through the production and release of the biogenic compound dimethylsulfoniopropionate (DMSP) and its volatile degradation product, dimethylsulfide (DMS). Research on DMS was initially encouraged in 1972 when James Lovelock measured it for the first time in surface seawater, estimated its sea-to-atmosphere flux and suggested that the emissions of this gas from the sea surface might close the budget of sulfur at the global scale. Later on, DMS research gained interest when Charslon *et al.* hypothesized in 1987 that this compound might play a role in climate regulation by the biosphere. These authors suggested that oxidation products of DMS in the atmosphere could act as cloud condensation nuclei and hence favor cloud formation, thus decreasing the radiative budget over the oceans.

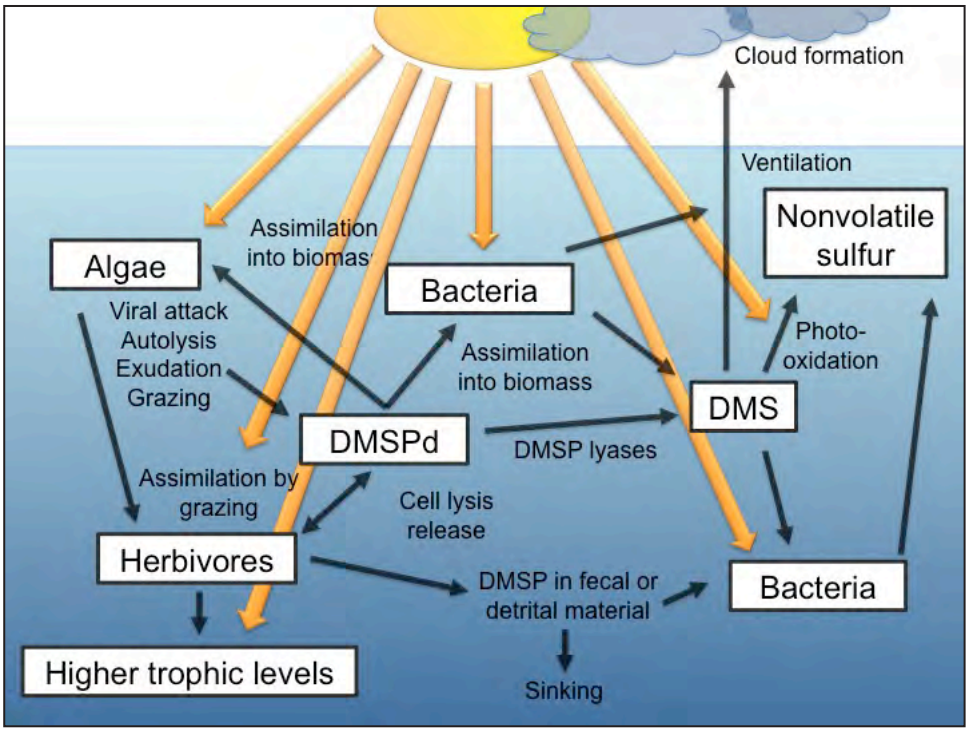

Fig. 3. Scheme of the DMSP/DMS cycle in the oceans. Only a small fraction of DMS escapes this tight cycling and vents to the atmosphere, potentially favouring cloud formation and thus light attenuation (adapted from Simó 2001).

The processes driving the synthesis, fluxes and transformations of DMSP and DMS are not yet fully understood (Simó, 2001; Stefels *et al.*, 2007). Briefly, the cycle of DMSP and DMS in the sea, sketched in Fig. 3, could be summarized as follows: DMSP is produced by many phytoplankton taxa mainly as an intracellular osmoregulator (Dickson and Kirst, 1987) although also cryoprotectant or antioxidant functions have been suggested (Malin and Kirst, 1997; Welsh, 2000). DMSP can either be degraded to DMS inside the algal cells or released into the dissolved pool through algal autolysis, viral attack, grazing or algal exudation released into the dissolved pool (Hill *et al.*, 1998; Laroche *et al.*, 1999; Wolfe *et al.*, 1994). Once released, it can be degraded by microbial communities, yet production of DMS (through the so-called 'cleavage' pathway, Ansede *et al.* (2001)) is not the primary fate of dissolved DMSP. Instead, most dissolved DMSP is transformed by bacteria through pathways initiated by demethylation that end up in nonvolatile sulfur compounds (Visscher *et al.*, 1992) and a fraction of the sulfur incorporated into bacterial biomass as protein (Kiene *et al.*, 1999). This latter process has been estimated to satisfy 1-15% of the total bacterial carbon demand and most of (sometimes virtually all) the bacterial S demand (Kiene and Linn, 2000; Simó *et al.*, 2002; Simó *et al.*, 2009; Zubkov *et al.*, 2001). Bacteria are thus thought to be the main consumers of this source of reduced sulfur that, although less abundant, is energetically cheaper to use than the ubiquitous sulfate (Kiene *et al.*, 1999). Incorporation of S from DMSP has been also recently reported for herbivore protozoans (Burkill *et al.*, 2002; Saló *et al.*, 2009; Simó, 2004; Simó *et al.*, 2002; Tang and Simó, 2003; Wolfe *et al.*, 1994) and even non or low DMSP-producing phytoplankton (Malmstrom *et al.*, 2005; Vila-Costa *et al.*, 2006b).

Additionally, grazing activity can also result in DMSP release and DMSP conversion to DMS if DMSP and DMSP-lyases are physically mixed following grazing (Kim *et al.*, 2010; Saló *et al.*, 2010; Stefels *et al.*, 2007; Wolfe and Steinke, 1996). DMS, in turn, can also be consumed by some bacteria (e.g. González *et al.*, 1999; Kiene and Bates, 1990; Vila-Costa *et al.*, 2006a), yet photolysis and ventilation (e.g. Toole *et al.*, 2003; Zemmelink *et al.*, 2004) are also significant sources of loss of DMS. Hence, depending on which pathway prevails and which organisms are involved, the flux of DMS to the atmosphere will be increased or decreased.

Once again, sunlight has a large potential as a modulator of the DMSP/DMS cycle, since most of the production, release, consumption and photolytic processes are directly or indirectly dependant on light levels (Kiene *et al.*, 2000; Stefels, 2000; Sunda *et al.*, 2002;

Vallina and Simó, 2007; Vila-Costa *et al.*, 2006b). In this regard, if this complex balance of synergistic and antagonistic light-driven effects ultimately results in an increased DMS flux to the atmosphere (Vallina and Simó, 2007), the loop might be closed through this negative plankton-irradiance feedback. Recent modelling estimates of the magnitude of this feedback suggest that it is not enough to significantly counteract the increased temperatures derived from global change (e.g., Vallina et al., 2007b), yet it may significantly act to attenuate sunlight fluctuations at shorter time scales, including seasonal, in the pristine ocean-atmosphere regions (Meskhidze and Nenes, 2006; Vallina et al. 2006, 2007a).

Elucidation of the biological players in the DMS cycle, their interplaying roles within planktonic food webs, and how they respond to environmental forcing (particularly solar radiation), are all necessary to better understand and predict this plankton/DMS/clouds/sunlight connection, which is a nice example of life impacting the physics of our planet.

AIMS AND OUTLINE OF THE THESIS

The main goal of this thesis is to provide a better understanding of the role of natural solar radiation upon the activity of microbial assemblages from surface marine waters, assessed from both a bulk and a single-cell point of view. We aim to address how sunlight influences the uptake of dissolved organic compounds by specific groups of bacteria and algae from different ecosystems (coastal Mediterranean, Arctic and Antarctic waters) with special emphasis on addressing to which extent the observed responses are explained by both the intensity and the spectral quality of the in situ light conditions. In order to address these and the more specific questions listed below, we carried out an array of light manipulation experiments in which the uptake of several radioactive organic compounds was quantified and analysed. For that purpose we combined bulk assimilation measurements with microautoradiographic approaches and flow cytometric sorting, and we further managed to contrast the observed responses with the measured PAR and UVR doses received by samples during experiments. This thesis is comprised of six separate studies structured in five main chapters that try to answer the specific issues listed below.

Chapter 1. Diel changes in bulk and single cell bacterial heterotrophic activity in winter surface waters of the NW Mediterranean Sea.

Diel variations in light levels are directly driving changes in many biological processes such as photosynthesis; marine bacteria may sometimes follow such diel patterns either due to UVR-damage or by responding to changes in DOM availability (e.g. photosynthate). However, it is not clear whether all the different groups of bacteria behave the same throughout diel cycles. In this chapter we wanted (1) to follow the diel activity of major bacterial groups from Blanes Bay and (2) to assess whether sunlight was directly responsible for any of the observed patterns in both thymidine and leucine incorporation.

Chapter 2. Seasonal variability in microbial responses to natural sunlight conditions:

2.1 Annual variability in light modulation of bacterial heterotrophic activity in surface NW Mediterranean waters.

Although there are many studies about UVR effects on bulk bacterial heterotrophic production, very few have addressed this subject throughout a whole seasonal period where the bacterial responses are expected to vary in accordance with seasonally changing light levels. Therefore we aimed (1) to assess the occurrence of seasonal patterns in the responses to in situ PAR and UVR of bacterial communities from the Blanes Bay Microbial Observatory sampling site, and (2) to compare them with the responses measured under invariant artificial light conditions. Further (3), we explored whether these patterns could be explained by the seasonality of other physical and biological variables to discern the main drivers affecting those responses at an annual scale.

2.2. Seasonal patterns in the sensitivity to sunlight of bacterioplankton from surface Mediterranean coastal waters

Different bacterial taxa from this area have shown different sensitivities to sunlight (Alonso-Sáez *et al.*, 2006); however, there is no information available about how these groups react to light levels typical of different seasons, and selection for photoresistant species towards the periods of higher irradiances has sometimes been suggested. The objective of this subchapter was to evaluate the seasonal variability in the sensitivity to sunlight of in situ dominating bacterial groups from the Blanes Bay Microbial Observatory sampling site.

Chapter 3. Sunlight effects on the DMSP and leucine assimilation activities of polar heterotrophic bacterioplankton

Marine organisms inhabiting polar waters are exposed to highly varying light conditions ranging from total darkness in winter to continuous light exposure in summer. We aimed to address the effect of sunlight (and specially UVB) on Arctic and Antarctic bacterial assemblages in terms of DMSP-sulfur assimilation. Since the highest concentrations of DMSP occur during the Arctic and Antarctic summers, we hypothesized that co-occurring bacterial

groups would be well adapted to use this source of reduced sulfur, and that continuous light exposure may affect the capabilities and rates of its incoporation. Therefore, the objective of this chapter was to test the group-specific sensitivities to sunlight among DMSP and leucine assimilating bacteria.

Chapter 4. Sunlight effects on the osmoheterotrophic behaviour of Arctic and Antarctic phytoplankton

Besides recognized for many years, very little is known about the potential ecological role of algal osmoheterotrophy within natural communities. Phytoplankton species from the polar regions have been suggested to switch to heterotrophic growth as a way to survive during the long aphotic polar winter, but so far, no study has addressed how UVR affects algal osmoheterotrophy. In this chapter, we aimed (1) to assess the occurrence and relevance of leucine and DMSP-sulfur utilization within summertime phytoplankton assemblages from the Arctic and Antarctica, and (2) to test the effect of natural solar radiation on the specific uptake of these compound by different algal groups.

Chapter 5. Solar radiation quality modulates the relative importance of bacteria and picophytoplankton in DMSP uptake

Even though there is ample evidence of the major role of heterotrophic bacteria in DMSP biogeochemistry, DMSP-sulfur uptake has been recently described for phototrophic microorganisms as well, and very little is known about their potential contributions to DMSP fluxes and how sunlight influences them. Previous studies have suggested that the heterotrophic uptake of DMSP-sulfur by picophytoplankton is stimulated by light; on this grounds, we hypothesized that sunlight exposure would lead to an enhanced competition of phytoplankton for DMSP compared to heterotrophic bacteria. Therefore, in this last chapter we aimed to address the role of light on the relative competition between Mediterranean prokaryotic picophytoplankton and heterotrophic bacteria for the use of DMSP-sulfur.

The six studies presented in this thesis have something in common: they focus on the role of sunlight as a modulator of the heterotrophic activities of marine bacteria or phytoplankton, and mainly from a species/group-specific perspective, yet they differ in some of the more specific topics they go through. Table 2 summarizes which of these subjects are addresses within each work.

		Paper 1	Paper 2	Paper 3	Paper 4	Paper 5	Paper 6
Temporal varibility	Diel cycles	■					
	Seasonal		■	■			
Geographic variability	Mediterranean	■	■	■			■
	Arctic				■	■	
	Antarctica						
Light quality	Artificial (PAR)	■	■				
	Sunlight PAR or UVR			■	■	■	■
Substrate considered	Leucine	■	■	■	■	■	■
	Thymidine	■					
	DMSP				■	■	■
Organisms considered	Het. bacteria	■	■	■	■	■	■
	Prok. phytoplankton	■	■	■			■
	Euk. phytoplankton					■	

Table 2. Schematic distribution of the six individual papers comprising this thesis with regard to the different topics that they address.

Chapter 1

Diel changes in bulk and single cell bacterial heterotrophic activity in winter surface waters of the NW Mediterranean Sea

Clara Ruiz-González, Thomas Lefort, Ramón Massana, Rafel Simó and Josep M. Gasol

ABSTRACT

Two diel cycle studies were conducted in order to assess the impact of day-night light changes on winter bacterial activity in the Blanes Bay (NW Mediterranean sea). Bacterial abundances, bacterial heterotrophic activity and grazing rates were determined at 4-h intervals during 3-day periods. Twice a day, the single-cell activity of major bacterial groups was further analysed by applying microautoradiography combined with catalyzed reporter deposition-fluorescence in situ hybridization (MAR-CARD-FISH). During the first cycle, all the measured parameters (including most group's number of active cells) presented a synchronized diel pattern with higher values at night and lower during the day. An episode of strong winds between the two studied periods seemed to disrupt this periodicity at the onset of the second cycle. The ^{3}H-leucine incorporation recovered the diel pattern after two days, mostly driven by the activity of *Gammaproteobacteria*. Occasional incubations under the light showed enhanced activity suggesting that bacteria were able to respond fast to light-driven algal release of dissolved organic matter (DOM); however, the observed night-time increase in the grazing activity of heterotrophic nanoflagellates suggests that predation-derived release of DOM was the main driver of the activity of most bacterial taxa.

INTRODUCTION

Day-night variations of biological parameters in the ocean are mainly a direct consequence of the relation between sunlight and marine organisms. Since the different components of the microbial food web are continuously interacting, any synchronization of life cycles and behaviours will ultimately be reflected in marine bacterial activity, having implications for carbon cycling at hourly scales.

Either the photosynthetic release or the excretion through grazing activities are thought to be major sources of dissolved organic matter (DOM) for marine bacteria in oceanic environments (see refs. in Nagata 2000). Since photosynthetic organisms have to deal with diurnal variations in light availability, and grazing activities are often synchronized with circadian cycles (Atkinson *et al.* 1992; Jakobsen and Strom 2004; Wikner *et al.* 1990), the rates of DOM supply for marine bacteria may also follow diel patterns. Thus, depending on the origin (and quality) of the DOM that bacteria use, and how fast they respond to substrate supplies, different diel bacterial activity trends will be detected. If bacteria are closely coupled to photosynthetic production of DOM, we should expect activity cycles showing peaks around noon and afternoon (Fuhrman *et al.* 1985; Gasol *et al.* 1998; Herndl and Malacic 1987). On the contrary, if bacterioplankton depend on DOM released by grazers (see refs. in Nagata 2000) or on allochthonous organic carbon, they will be synchronized to the rhythms of these supplies, if any. The diel pattern of bacterioplankton activity in the upper layers of the ocean, however, might also be influenced by other factors, such as ultraviolet radiation (UVR, Jeffrey *et al.* 1996*b*; VanWambeke *et al.* 2009), bacterivory (Christaki *et al.* 2002; Wikner *et al.* 1990) or viral lysis (Bratbak *et al.* 1992; Winter *et al.* 2004). Such short term variations seem to occur more intensively in oligotrophic environments, where substrate supplies are low and we may expect tightly coupled variations among bacteria and DOM production (Gasol *et al.* 1998; Shiah 1999). In coastal areas or more eutrophic waters, though, where DOM supply may be independent from circadian cycles and/or many different substrates are available, we might find inconsistent or unclear diel cycles (Gasol *et al.* 1998; Riemann and Sondergaard 1984; Santos *et al.* 2009).

Thus far, such diurnal variability of bacterial activity has been addressed mainly at the community level, while very few studies have considered this issue from a single-cell or group-specific point of view. Different phylogenetic groups are well known to show distinct seasonal patterns in their activities (Alonso-Sáez and Gasol 2007; Vila-Costa *et al.* 2007), yet

very little is known about how the different bacterial groups behave throughout a diel cycle. Among them, *Cyanobacteria* have been more carefully studied showing obvious diurnal rhythms in the uptake of organic substrates (Chen *et al.* 1991; Mary *et al.* 2008*a*). On the contrary, Pernthaler and Pernthaler (2005) did not find clear diel cycles in cell proliferation when focusing on three bacterial taxa. Considering that the different phylogenetic groups may differ in their preferences for organic substrates (Alonso and Pernthaler 2006; Alonso-Sáez and Gasol 2007; Vila-Costa *et al.* 2007) or for particular phytoplankton species (Pinhassi *et al.* 2004; Schäfer *et al.* 2002), and given that there are bacteria with differential sensitivities to sunlight (Alonso-Sáez *et al.* 2006; Arrieta *et al.* 2000; Sommaruga *et al.* 2005) or with potential photoheterotrophic capabilities (Béjà *et al.* 2000; Kolber *et al.* 2000), we expect that some populations within the bacterial assemblages exhibit higher diurnal fluctuations or different rhythms than others.

For that purpose, the short-term variability of bacterial activity was studied in seven bacterial taxa from coastal Northwestern Mediterranean waters in two consecutive, 72 h cycles, along with bulk bacterial heterotrophic activity measured as ^{3}H-leucine and ^{3}H-thymidine incorporation. Microautoradiography combined with catalyzed reported deposition-fluorescence in situ hybridization (MAR-CARD-FISH) allowed the analysis of diurnal variations at the single-cell level. In order to correctly interpret the observed patterns, several light incubations for bacterial activity and microscopic observations of grazing activities were performed. We expected to find variable behaviors of different bacterial groups depending on the light levels or DOM supplies, thus providing insight into the daily rhythms of particular bacterial taxa.

MATERIALS AND METHODS

Sampling and basic parameters. The study was carried out in the Blanes Bay Microbial Observatory, a shallow (20 m depth) oligotrophic coastal station in the NW Mediterranean Sea, located 800 m offshore of Blanes, Catalonia, Spain (41°39.90'N, 2°48.03E). Experiments were performed in two blocks, on February 20 to 23 (first cycle) and on February 26 to March 1 (second cycle), 2007. Surface water samples (0.5 m depth) were collected with polycarbonate carboys every 4 hours during these two 72 h periods. The sample

of the third day at noon could not be collected due to rough sea conditions. The temperature and salinity of the sampled waters were obtained with a SAIV A/S 204 CTD probe at every sampling time. Irradiance measurements during the sampling period were obtained from the nearby station of Malgrat de Mar (Catalan Meteorological Service, www.meteo.cat), located 5 km from the sampling station and at 4 m above sea level. The station recorded arithmetically averaged hourly air temperature and relative humidity at 1.5 m above ground, vector-averaged hourly wind speed and direction and global irradiance at 2 m, and accumulated rainfall at 1 m. Wave height data were collected from a scalar buoy (DATAWELL, Waverider) placed at 41º 38´49´´N, 02º 48' 56" E over a depth of 74 m (XIOM Network, www.boiescat.org). Chlorophyll *a* concentration was determined from 150 mL of seawater filtered through GF/F filters (Whatman) extracted in acetone (90% v/v), and fluorescence was measured with a Turner Designs fluorometer.

Abundance of prokaryotes. *Prochlorococcus*, *Synechococcus* and photosynthetic picoeukaryotes (Ppeuk) abundances were enumerated by flow cytometry in unstained samples and distinguished by their size and pigment properties following common procedures (Olson et al 1993). Heterotrophic prokaryotes were also quantified by flow cytometry after staining with SybrGreen I (Gasol and Del Giorgio 2000).

Bacterial heterotrophic activity. Bacterial activity was estimated every 4 hours from both radioactive ^{3}H-leucine and ^{3}H-thymidine incorporation. For leucine we used the ^{3}H-leucine assimilation method described by Kirchman *et al.* (1985) with the modifications of Smith and Azam (1992). Briefly, 4 aliquots (1.2 mL) and 2 TCA-killed controls were incubated with radiolabeled leucine (40 nmol L^{-1}, final conc., 160 Ci $mmol^{-1}$) for about 2 hours in the dark at in situ temperature. The incorporation was then stopped by adding 120 µl of cold TCA 50% to the samples, which were stored at –20ºC until processing by the centrifugation method of Smith and Azam (1992). Bacterial activity was also measured as ^{3}H-thymidine incorporation following Fuhrman and Azam (1980) with the modifications of Smith and Azam (1992). Samples were incubated with 10 nmol L^{-1} ^{3}H-thymidine (final concentration) and processed like the ^{3}H-leucine samples.

Furthermore, every day at 13:00 h six additional aliquots amended with ^{3}H-leucine were incubated under an artificial light source (~ 100 μmol photons m^{-2} s^{-1} of visible light only) parallel to standard dark incubations for comparison of both light and dark conditions.

Grazing activity of heterotrophic nanoflagellates. Samples of 30 mL fixed with glutaraldehyde (2% final conc.) and stained with 4,6- diamidino-2-phenylindole (DAPI) to a final concentration of 5 mg mL^{-1} were filtered through a 0.6 μm polycarbonate black filter (Poretics). Only samples from the first cycle were analyzed. Colorless flagellates < 5 μm in size (heterotrophic nanoflagellates, HNF) were enumerated with an Olympus BX61 epifluorescence microscope by UV excitation (to detect the protists) and blue light excitation (to check for the absence of chloroplasts). At this excitation, the presence of ingested pigmented prey (small PPeuk) was easily observed inside them. The HNF with ingested algae were distinguished from phototrophic forms by either the lack of conspicuous plastids and/ or the general morphology of both prey and predator.

Microautoradiography combined with Catalyzed Reporter Deposition-Fluorescence In Situ Hybridization (MAR-CARD-FISH). Twice a day (GMT 13:00 h and 01:00 h) 30 mL water samples were incubated with added radioactive ^{3}H-leucine (160 Ci $mmol^{-1}$, 0.5 nmol L^{-1} final conc.) for 4 hours in the dark at in situ temperature. Controls killed with paraformaldehide (PFA) were also run simultaneously with all live incubations. After 4 h the live samples were fixed overnight with PFA (1% final conc.) at 4°C in the dark.

For the analysis of the single-cell bacterial activity, we followed the protocol described by Alonso and Pernthaler (2005) with the modifications of Vila-Costa *et al.* (2007) and Alonso-Sáez and Gasol (2007). Aliquots of 10 mL were gently filtered through 0.2 μm polycarbonate filters (GTTP, Millipore), rinsed with milli Q, air dried and stored at –20°C until processing. Hybridization of the filters was done following the CARD-FISH protocol. Several horseradish peroxidase (HRP)-probes were used to characterize the composition of the bacterial community in the water samples: Eub338-II-III for most *Eubacteria* (Amann *et al.* 1990; Daims *et al.* 1999), Gam42a for most *Gammaproteobacteria* (Manz *et al.* 1992), CF319 for many clades belonging to the *Bacteroidetes* group (Manz *et al.* 1996), Ros537 for the *Roseobacter* clade (Eilers *et al.* 2001), SAR11-441R for the SAR11 cluster (Morris *et al.* 2002) and Syn405 for the cyanobacterial genus *Synechococcus* (West *et al.* 2001). The Eub antisense probe Non338 (Wallner *et al.* 1993) was used as a negative control. All probes were purchased from biomers.net (Ulm, Germany).

Filters were first permeabilized with lysozyme (10 mg mL^{-1}, 37°C, 1h) and achromopeptidase (60 U mL^{-1}, 37°C, 0.5 h) before the hybridization. Hybridizations were carried out on sections of the filters at 35°C overnight, and specific hybridization conditions

were established by addition of formamide to the hybridization buffers (45% formamide SAR11 probe, 20% for Non338, 60% for Syn405 and 55% for the rest of probes). Smaller pieces from each hybridized section were cut and stained (DAPI, 1μg mL^{-1}) to estimate the relative abundance of each group before applying the microautoradiography. Between 500 and 800 DAPI-positive-cells were counted manually within a minimum of 10 fields.

For microautoradiography, the filter sections were glued onto slides and embedded in 46ºC melted photographic emulsion (KODAK NTB-2) containing 0.1% agarose in a dark room. The slides were placed face-up on an ice-cold metal bar for about 5 min for the emulsion to solidify, and then stored inside black boxes at 4ºC until development. The optimal exposure time was determined for all the samples and resulted in an average of 8 days. Slides were developed by placing them into the developer (KODAK D19) for 3 min followed by fixation with KODAK Tmax fixer for 3 min and 5 min of washing with tap water. Slides were then dried in a dessicator overnight, stained with DAPI (1 μg mL^{-1}) and counted manually by epifluorescence microscopy.

RESULTS

Background information. Sampling was done at the coldest period of the year (average temperature ~ 13.4ºC), and temperature did not vary significantly different between both weeks. Chlorophyll *a* concentration was only estimated for the first sampling time of each cycle, and it almost doubled (from 0.47 to 0.89 μg L^{-1}) between the first and the second cycle (Table 1). Whereas the average number of heterotrophic bacteria did not significantly change between both cycles, averaged *Synechococcus*, *Prochlorococcus* and PPeuk abundances strongly increased from the first to the second week (Table 1), in concordance with the higher chlorophyll *a* concentrations found in the second week. During the unsampled weekend between the two cycles, wind direction changed (shifting from S/SW to E) causing increased sea turbulence and wave action (Fig. 1B), with simultaneous rainfall and low-light conditions (Fig. 1A).

	Temp. (ºC)	Salinity (psu)	Chl *a* (μg L^{-1})	Het. bact. (10^5 mL^{-1})	Syn. (10^4 mL^{-1})	Prochl. (10^4 mL^{-1})	PPeuk (10^4 mL^{-1})
1st. cycle	13.43 ± 0.04	38.27 ± 0.03	0.47 ± 0.02*	7.8 ± 1.1	0.6 ± 0.1*	0.5 ± 0.1*	1.1 ± 0.4*
2nd. cycle	13.36 ± 0.01	38.30 ± 0.01	0.89 ± 0.03*	8.3 ± 0.5	1.6 ± 0.3*	1.2 ± 0.2*	1.4 ± 0.3*

Table 1. Averaged measurements of temperature, salinity and cell concentration heterotrophic bacteria (Het. bact.), *Synechococcus* (Syn), *Prochlorococcus* (Prochl.) and picoeukaryotes (PPeuk) measured at every sampling time. Total chlorophyll *a* concentration, instead, was only analysed at the beginning of each week. Values represent means ± standard deviations. Asterisks (*) indicate significant differences between both cycles (ANOVA test $p < 0.05$).

Diel variations in bulk bacterial activity. Both ^{3}H-leucine and ^{3}H -thymidine incorporation rates were measured every 4 hours during two periods of 72 h in order to assess the impact of daily light changes on bacterial heterotrophic activity. During the first cycle (February 20 to 23) bacterial activity showed a marked diel pattern with higher ^{3}H-leucine and ^{3}H-thymidine incorporation values at night and lower values during the day (Fig. 2A). Throughout the 72 h there were extremely large variations in bacterial activity among sampling times. ^{3}H-leucine incorporation increased from 53.8 to 314.2 pmol L^{-1} h^{-1} between 13:00 h February 20 and 05:00 h February 21, meaning a ~ 6-fold increase in less than 24 h. On 22 and 23 February the peaks reached 269.8 and 243.9 pmol L^{-1} h^{-1}, respectively, and they appeared to be displaced in time with respect to the first day, because they both were reached at 21:00 h instead of at 05:00 h. Minimum values were around 50 and 75 pmol ^{3}H-leucine L^{-1} h^{-1}. ^{3}H–thymidine incorporation was also higher at night than during the day and it very closely paralleled the ^{3}H-leucine incorporation pattern. Maximum ^{3}H–thymidine incorporation values ranged from 80.4 pmol L^{-1} h^{-1} on February 20 to 59.4 pmol L^{-1} h^{-1} on February 22. Minimum values were obtained during daytime at around 5 and 20 pmol L^{-1} h^{-1} (Fig. 2A). During the second week (February 26 to March 1) the diel cycle of bacterial activity seemed to have been disrupted after the unsampled weekend (Fig. 2A). No diurnal pattern was found at the beginning of the week; however, in the course of the last two days, ^{3}H–leucine incorporation started to recover the pattern again, showing two maximum peaks at 01:00 h on February 27 and 28 (175.0 and 203.9 pmol L^{-1} h^{-1}, respectively). There was a general increasing trend in ^{3}H–leucine incorporation throughout the second 72 h (from 56.1 to 183.4 pmol L^{-1} h^{-1}).

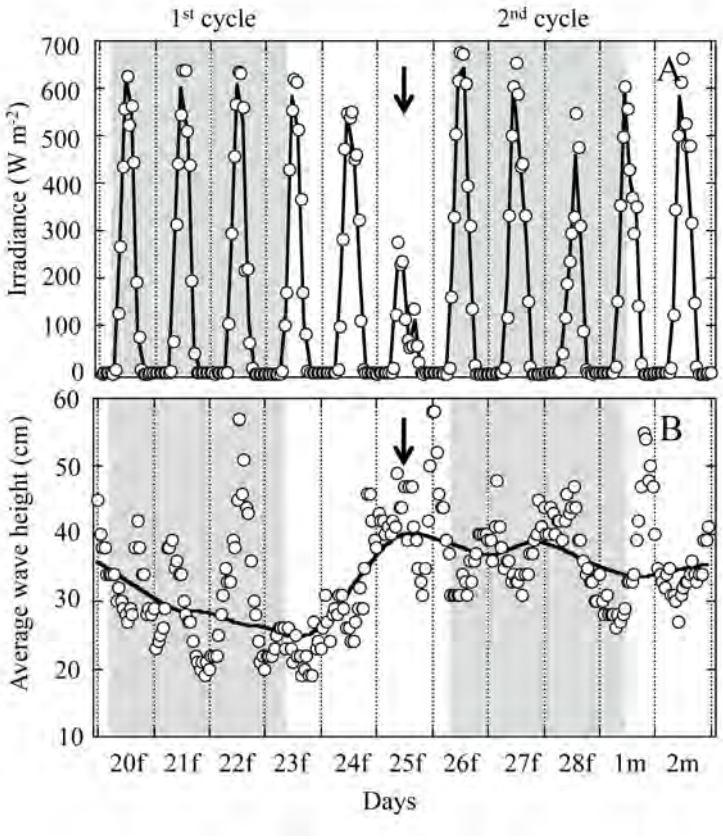

Fig. 1. (A) Irradiance measurements during the sampling period obtained from the station of Malgrat de Mar (Catalan Meteorological Service, SMC); (B) mean wave height measured by a scalar buoy throughout the sampling period (XIOM Network, www.boiescat.org). Grey areas behind show each 72 h cycle. Arrows indicate an episode of increased wave height and reduced irradiance just before the beginning of the second cycle. The line is a best fit smooth curve through the center of the data calculated using the locally weighted Least Squared error method (Kaleidagraph vs. 4.1.1., Synergy Software).

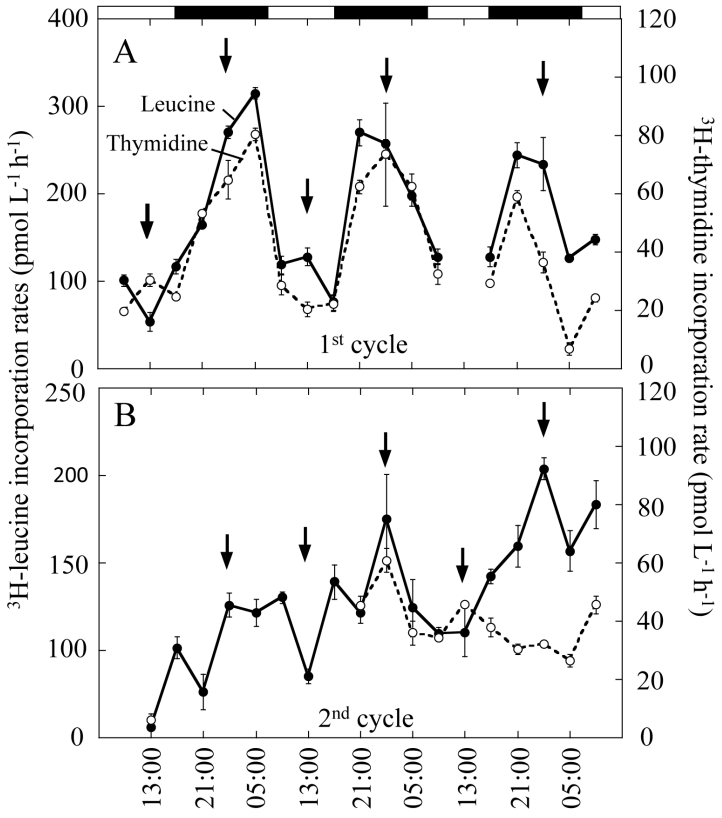

Fig. 2. Diel changes in ^{3}H–leucine and ^{3}H–thymidine bacterial incorporation rates during the first (A) and the second (B) cycles. The values are averages and standard errors of 4 replicated measurements. Arrows indicate sample incubations for MAR-CARD-FISH analysis. Solid bars on top axis represent dark periods.

During the second week we neither observed any consistent diel pattern for ^{3}H–thymidine incorporation. Values were variable and ranged from 6.1 pmol L^{-1} h^{-1} at the first sampling point to 60.8 pmol L^{-1} h^{-1} at 01:00 h on February 27. Unfortunately, we ran out of ^{3}H–thymidine at the beginning of this second week and missed 24 hours of incorporation before a new batch was obtained.

The cycle in bacterial abundances was not so pronounced (data not shown), and as a result, bacterial specific activities (Fig. 3) followed the same cycles as those of bulk bacterial activity. During the second week, however, bacterial specific activity did not present any clear cycle, although towards the end of the week we could detect two night peaks of ^{3}H-leucine specific incorporation rates, in a way similar as bulk incorporation did (Fig. 3B).

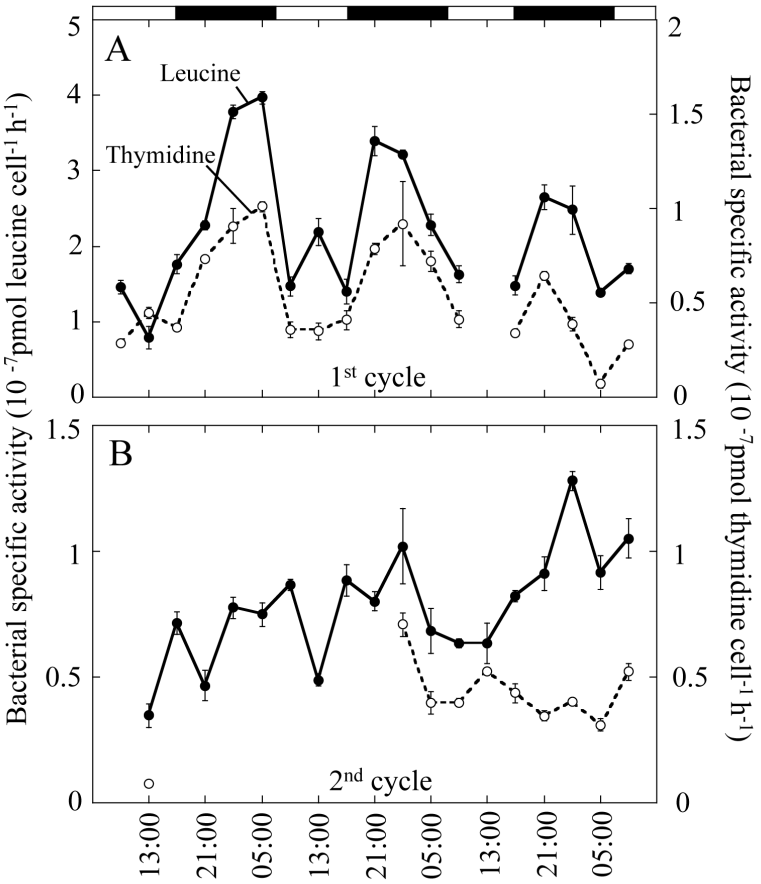

Fig. 3. Diel changes in ^{3}H–leucine and ^{3}H–thymidine bacterial specific activities during the first (A) and the second (B) cycles. The values were obtained by dividing bulk bacterial production by the concentration of bacteria at each sampling time. Solid bars on top axis represent dark periods.

On February 20, 21, 27 and 28 (days 1 to 4 as presented in Fig. 4) six subsamples from the noon sampling were amended with ^{3}H-leucine and ^{3}H-thymidine and incubated under an artificial visible-light source in parallel to standard dark incubations in order to explore the effects of light on bacterial activity measurements. ^{3}H-leucine incorporation was higher

by 33-57% (always significant at $p < 0.05$) when incubated in the light. The effect of light on ^{3}H-thymidine incorporation was more variable; it caused a significant increase (2 to 4-fold) on days 2 and 3 and no significant differences between treatments on days 1 and 4.

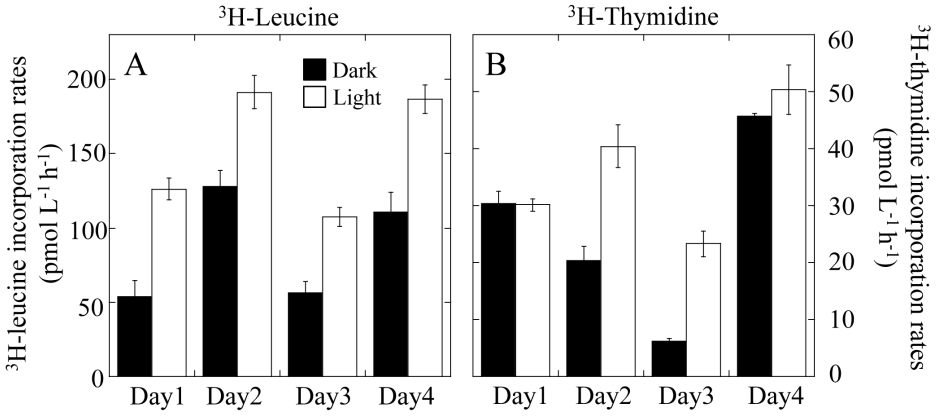

Fig 4. Bacterial activity measured as ^{3}H–leucine (A) and ^{3}H–thymidine (B) incorporation both in the dark and under an artificial light source at 13:00 h on February 20 and 21 (first cycle, days 1 and 2) and on February 27 and 28 (second cycle, days 3 and 4).

Diel variability of the grazing activity of heterotrophic nanoflagellates (HNF). During the first cycle, HNF and ingested photosynthetic picoeukaryotes (PPeuk) inside them were quantified every 4 h (Fig. 5). We found a clear pattern of higher grazing activity at night and nearly no ingestion during the day. This activity seemed to be explained by the availability of prey, as shown by the significant correlation between PPeuk abundances (T. Lefort, unpubl.) and ingested cells ($r = 0.73$, $p < 0.001$, $n = 18$). Interestingly, this relationship was more strongly correlated during the second day ($r = 0.99$, $p < 0.0001$, $n = 6$) compared to the other two days ($r = 0.88$ and $r = 0.82$ for the first and third day, respectively, $p < 0.05$, $n = 6$), and by the end of the sampling period it seemed that the HNF had caused a significant decrease in PPeuk abundances.

Diurnal variations in community composition and single-cell activities. The composition of the bacterial community during both cycles was analyzed by CARD-FISH for day (13:00 h) and night (01:00 h) samples (Table 2). The fraction of DAPI-stained cells hybridized with the probe for all bacteria (EUB338, -II, -III) ranged from 81% to 88% during the first week and from 78% to 88% during the second week. Hybridization with specific probes showed that the bacterial community was mainly dominated by the SAR11 clade of the *Alphaproteobacteria,* which accounted for 31% to 42% of the total DAPI counts. Also

Bacteroidetes comprised an important fraction of the bacterial community, with percentages ranging from 14% to 24%. The rest of the groups (*Gammaproteobacteria, Roseobacter,* and *Synechococcus)* were always below 11%. No significant differences in the average relative abundances of groups were found between the two cycles except for *Roseobacter,* which showed significantly lower numbers in cycle 2 than in cycle 1 (5 ± 1% and 8 ± 1% of DAPI counts, respectively, ANOVA test, $p < 0.05$).

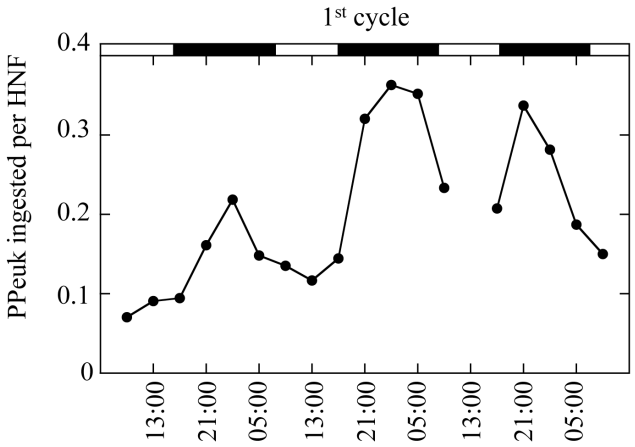

Fig. 5. Diel variations in the grazing activity of heterotrophic nanoflagellates (HNF) on photosynthetic picoeukaryotic (PPeuk) cells throughout the first cycle (February 20 to 23). Solid bars on top axis represent dark periods.

The relative abundances of the studied groups remained constant throughout the first week; only *Gammaproteobacteria* showed significantly lower numbers at 01:00 h on February 21 compared to the rest of the sampling times (Table 2A). On the contrary, the percentages of bacterial groups during the second week were more variable (Table 2B). Whereas *Roseobacter* or SAR11 did not show changes, total bacteria occurred at slightly lower percentages at 13:00 h on February 27 with respect to some of the sampling times. Conversely, *Gammaproteobacteria* and *Bacteroidetes* had significantly higher percentages at 13:00 h on February 28, but remained more or less constant during the rest of the cycle.

The diurnal variations in single-cell activity of the different phylogenetic groups of bacteria were investigated in the two cycles using the MAR-CARD-FISH technique. In the first week, the majority of the bacterial groups showed an obvious and similar diurnal pattern of activity, with greater percentages of active cells at night than during the day (Fig. 6). These changes between day and night were significant (Tukey's test $p < 0.05$) in most cases. Instead, no clear diurnal pattern was registered during the second cycle: just *Gammaproteobacteria* seemed to start to recover the same diel trend from the second night onwards (Fig. 7C).

B		Fraction (%) of total DAPI counts detected with CARD-FISH probe					
Date	Time	Eub338-II-III	CF319a	Gam42a	Ros537	Sar11-441R	Syn405
27-02	01:00	85 ± 1 [a]	16 ± 1 [a]	3 ± 1 [a]	4 ± 1 [a]	31 ± 2 [a]	2.0 ± 0.6 [a]
27-02	13:00	78 ± 1 [b]	15 ± 1 [a]	4 ± 1 [a]	5 ± 1 [a]	36 ± 2 [a]	1.1 ± 0.3 [a]
28-02	01:00	83 ± 2 [ab]	18 ± 2 [ab]	4 ± 0 [a]	6 ± 1 [a]	36 ± 2 [a]	1.5 ± 0.4 [a]
28-02	13:00	88 ± 1 [a]	22 ± 1 [b]	10 ± 1 [b]	4 ± 1 [a]	34 ± 2 [a]	1.6 ± 0.7 [a]
01-03	01:00	83 ± 2 [ab]	14 ± 1 [a]	6 ± 1 [a]	5 ± 0 [a]	37 ± 3 [a]	0.5 ± 0.3 [a]
	Avg ± se	83 ± 2	17 ± 1	5 ± 1	5 ± 0.4	35 ± 1	1.3 ± 0.2

A		Fraction (%) of total DAPI counts detected with CARD-FISH probe					
Date	Time	Eub338-II-III	CF319a	Gam42a	Ros537	Sar11-441R	Syn405
20-02	01:00	83 ± 1 [a]	17 ± 2 [a]	5 ± 1 [a]	6 ± 1 [a]	42 ± 2 [a]	0.9 ± 0.3 [a]
21-02	13:00	85 ± 2 [a]	24 ± 2 [a]	8 ± 1 [b]	10 ± 1 [a]	42 ± 2 [a]	1.0 ± 0.3 [a]
22-02	01:00	84 ± 1 [a]	21 ± 1 [a]	4 ± 1 [a]	7 ± 1 [a]	40 ± 2 [a]	0.5 ± 0.2 [a]
22-02	13:00	88 ± 2 [a]	21 ± 3 [a]	6 ± 1 [ab]	10 ± 1 [a]	38 ± 4 [a]	0.8 ± 0.3 [a]
23-02	01:00	81 ± 3 [a]	17 ± 2 [a]	5 ± 1 [a]	8 ± 1 [a]	34 ± 3 [a]	0.5 ± 0.3 [a]
	Avg ± se	84 ± 1	20 ± 1	6 ± 1	8 ± 1	39 ± 2	0.7 ± 0.1

Table 2. Bacterial assemblage structure described as percentage of hybridized cells with specific probes by CARD-FISH in five samples of the first cycle (A) and second cycle (B). Eub338-II-III (*Eubacteria*), CF319a (*Bacteroidetes)*, Gam42a (*Gammaproteobacteria*), Ros537 (*Roseobacter*), SAR11-441R (SAR11 clade) and Syn405 (*Synechococcus*). SAR11and *Roseobacter* are subgroups of *Alphaproteobacteria*. Values are expressed as percentage of the total DAPI counts (± standard error). Letters refers to results with a post hoc Tukey's test ($p < 0.05$). Different letters indicate significant differences among different sampling points.

On average, 28% (19-34%) of *Eubacteria* were labelled (active) in ^{3}H-leucine uptake during the first week, and 20% (14-27%) during the second week. In general, most of the studied groups showed no significant differences in averaged active cells between both cycles. Only members of *Gammaproteobacteria* presented on average much more active cells during the first cycle (39-87%, mean 65%) than during the second one (14-53%, mean 27%), despite the number of labelled cells tended to increase throughout the sampling period.

Considering the two weeks together, the average number of active *Bacteroidetes* was 6% (3-12%), members of the SAR11 cluster had 23% (13-41%) of active cells, *Roseobacter* were the most active group with 82% (68-93%) of labelled cells and active *Synechococcus* cells amounted 7% (5-11%) of the group abundance.

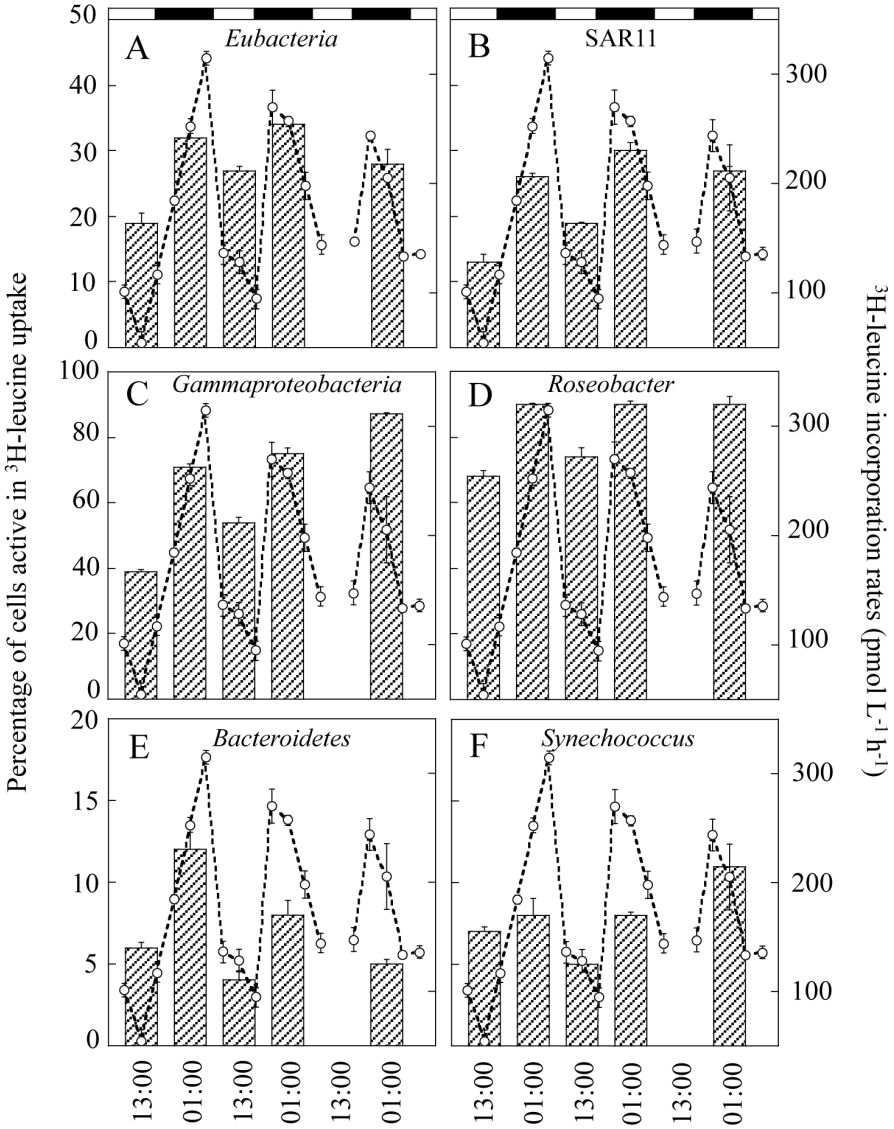

Fig. 6. Percentage of positively hybridized cells taking up ^{3}H-leucine (average ± standard error of duplicates) as measured by MAR-CARD-FISH during the first cycle (February 20 to 23) in both 13:00 h and 01:00 h samples. The dotted line behind shows bulk ^{3}H–leucine incorporation rates for comparison as displayed in Fig. 2a. Solid bars on top axis represent dark periods. 13:00h-sampling time is missing in the third day due to bad weather conditions.

With regard to variations in single-cell activity throughout the day in the first cycle (Fig. 6), we found that the number of labelled *Eubacteria* increased at nigh and decreased during the day by an average factor of ~ 1.5 (Fig. 6A). When we focused on bacterial groups, a similar response was generally found. Most groups showed a stronger increase in the number of active bacteria from 13:00 h on February 20 to 01:00 h on February 21 (the first night) than during

the second night. This was in agreement with the lower rates of ^{3}H-leucine incorporation registered at 01:00 h on February 22 compared to the first peak at 05:00 h on February 20 (Fig. 2A). *Gammaproteobacteria, Roseobacter* and SAR11 showed night:day increases of 45%, 24% and 50% during the first night, and 28%, 18% and 36% during the second night, respectively. This night stimulation of the number of active cells was the same during both nights for *Bacteroidetes* (50% increase), and *Synechococcus* did also show this nocturnal activation of their heterotrophic activities, although conversely to the rest of the groups, its stimulation was greater during the second night (38%) than during the first one (13%).

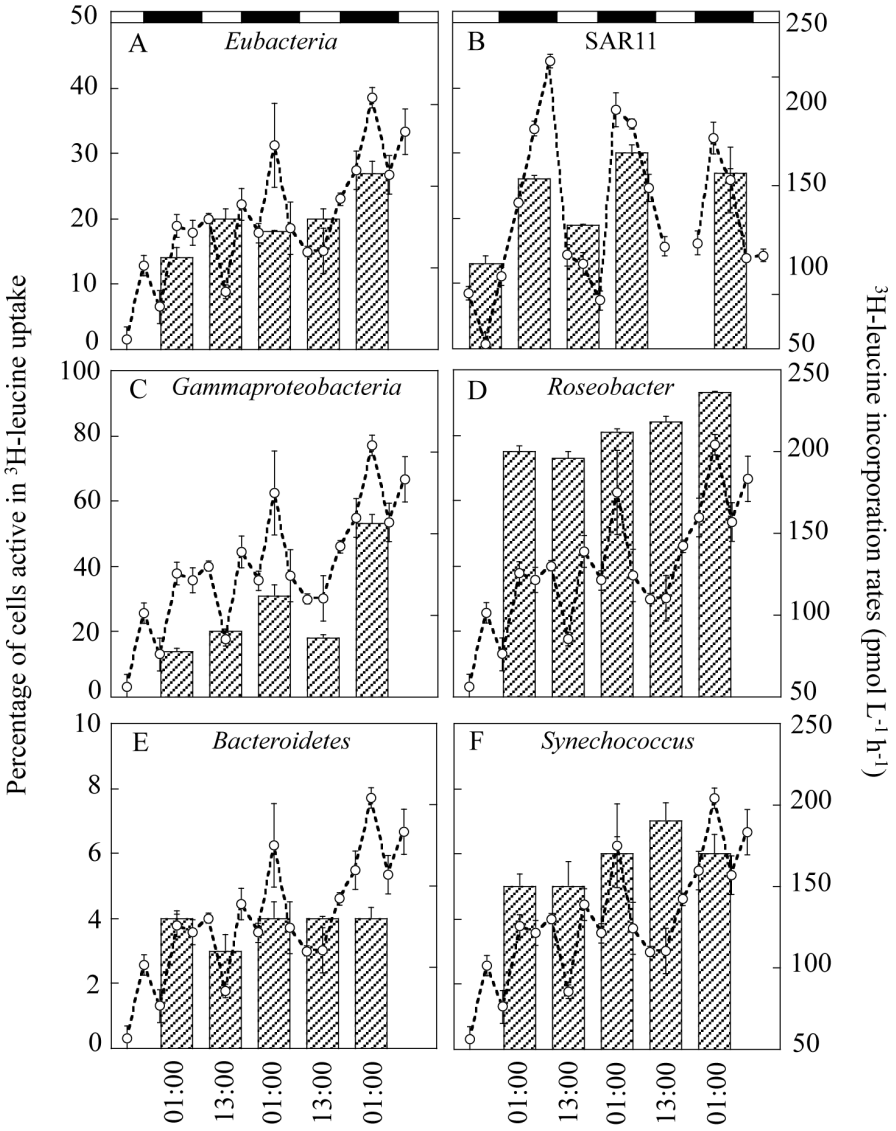

Fig. 7. Percentage of positively hybridized cells taking up ^{3}H-leucine (average ± standard error of duplicates) as measured by MAR-CARD-FISH during the second week (February 26 to March 1) in both, 13:00 h and 01:00 h samples. The dotted line behind shows bulk ^{3}H–leucine incorporation rates for comparison as displayed in Fig. 2a. Solid bars on top axis represent dark periods.

During the second cycle (February 26 to March 1) we could not find any consistent diel pattern but there was a general increase in the number of active cells towards the end of the sampling period (Fig. 7). Only *Bacteroidetes,* which remained constant during the whole cycle, did not show this significant increase in activity (Fig. 7E). Members of the *Gammaproteobacteria* cluster, instead, exhibited some diel pattern with significantly higher activities ($p < 0.05$) at night than during the last sampled day (from 18% at 13:00 h on February 28 to 53% at 01:00 h on March 1, Fig. 7C). Interestingly, whereas no significant correlation was found between bacterial activity and prokaryote abundances as measured by flow cytometry, it seemed to be nicely correlated with the number of active *Eubacteria* (Pearson's $r = 0.83$, $p < 0.005$, $n = 10$).

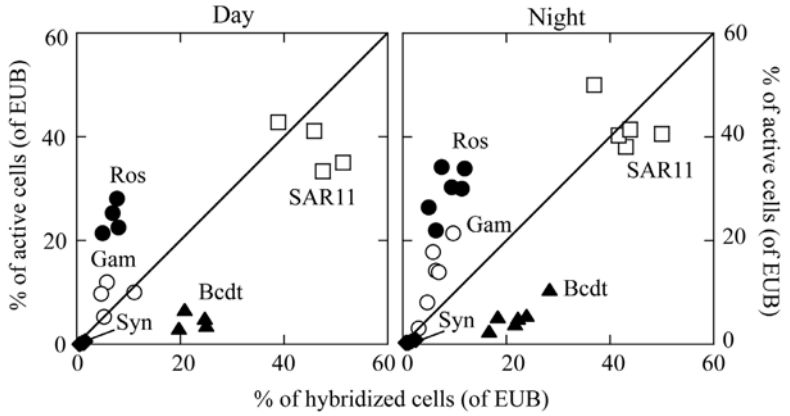

Fig. 8. Contributions of various phylogenetic groups (SAR11, *Roseobacter* [Ros], *Gammaproteobacteria* [Gam], *Bacteroidetes* [Bcdt] and *Synechococcus* [Syn]) to the number of cell active in ^{3}H-leucine uptake, presented against their contribution to the assemblage composition (relative abundance) in both day and night samples. Samples from the two cycles are plotted together. Percentages were calculated relative to eubacterial cells (probes EUB338, -II and -III).

Figure 8 shows the percentage contribution of each group to the total of cells active in ^{3}H-leucine uptake plotted against their relative contribution to total *Eubacteria* abundance in all the samples. Data points on, or near, the 1:1 line indicate groups that were participating in substrate uptake proportionally to their abundance share in situ. In both cycles, *Roseobacter* and to a less extent *Gammaproteobacteria* were overrepresented in ^{3}H-leucine uptake compared to their abundance. On the contrary, SAR11 were closer to the 1:1 line and members of the *Bacteroidetes* cluster were always underrepresented in the uptake. In general, the upwards displacement observed in the right panel was due to the significant nocturnal increases in the numbers of most groups during the first cycle.

DISCUSSION

The heterotrophic activity of marine bacteria in surface waters is driven by a complex framework of biological and physicochemical processes that are expected to undergo day-night variations as a consequence of the relation between sunlight and marine biota. In the course of the two cycles studied in February 2007, we first found that most of the analyzed variables showed clear diurnal patterns with maximum values at night and lower values during the day, but this trend seemed to be disrupted after the unsampled weekend between both cycles. During those two days, there was a rainfall episode and a change in the wind direction from S/SW to E, which seemed to alter the phased trend found during the first week. In this area, E winds are typically accompanied by relatively high waves and promote sediment resuspension (Ferré *et al.* 2005; Guillén *et al.* 2002) that might modify the quality of DOM and inorganic nutrients available for marine organisms and thus might alter diel patterns. Episodic resuspension events have been shown to affect planktonic activities in both laboratory and field studies (Cotner 2000; Garstecki *et al.* 2002), although no specific information is available on how this may affect the bacterial diel periodicity. In our study we found that towards the end of the second cycle, after relaxation of the E wind episode, some parameters appeared to recover the day-night pattern, suggesting that these microbial populations are resilient to this kind of physical perturbations.

Water mass characteristics during both cycles were usual for that time of the year in the NW Mediterranean, with cold temperatures, totally mixed water column and late winter pre-bloom conditions (Estrada *et al.* 1985). Our physical data showed no great changes in temperature or salinity over time, suggesting that we were sampling a reasonably stable and coherent patch of water during each week (details not shown).

Diel changes in bulk and specific bacterial activity. Marked diel cycles in bulk bacterial activity were detected during the first cycle but not during the second one. From February 19 to 23 bulk bacterial activity showed much higher ^{3}H-leucine and ^{3}H-thymidine incorporation rates at night than during the day. Likewise, the specific bacterial activity was also greater at night, meaning that the nocturnal increase in total ^{3}H-leucine and ^{3}H-thymidine incorporation was not just because there were more bacteria; on average, each cell grew faster throughout the night. Such diel fluctuations were surprisingly higher than the range reported for seasonal variation in bacterial activity within that year: the greatest change found during the first 24 h in ^{3}H-leucine incorporation rate was 260.8 pmol L^{-1} h^{-1} (from 13:00 h February 19 to

05:00 h February 20) whereas the maximum variation recorded for the whole 2007 (daytime sampling only) was found to be 188.6 pmol L^{-1} h^{-1} between March and October (unpublished data). Some authors (e.g. Stramska *et al.* 1995) had also found that the diel variability of marine parameters can be often more important that the variability at the weekly scale, or even at the annual scale. This scale of variability is often neglected in sampling strategies and thus the interpretation of seasonal data should be carefully considered.

The bacterial diel pattern found in this study is opposite to that reported by Gasol *et al.* (1998) in an offshore station near this area in June 1993 and 1995, although they sampled in summer instead of winter, which probably led to temporal differences in DOM quality or bacterial community composition, in addition to the spatial divergence between coastal and offshore communities. Indeed, these authors found that noon estimates of bacterial activity were more than twice the daily average in one oceanic station (~100 km offshore the Blanes Bay) and no clear trends near the coast, supporting the idea that under more oligotrophic conditions, the coupling of phytoplankton and bacterial activities should be more discernible. Most studies of diel variations in bacteria have suggested this link between primary production and bacterial use of the released DOM, usually leading to increased bacterial abundances or activity during daytime with maxima values in late afternoon and minima at night (Fuhrman *et al.* 1985; Herndl and Malacic 1987; Mével *et al.* 2008). Other authors have reported only weak or inconsistent diel patterns (Riemann and Sondergaard 1984; Torreton and Dufour 1996) but very few have observed this nocturnal stimulation of bacterial activity (Jeffrey *et al.* 1996*a*; Kuipers *et al.* 2000; Shiah 1999). However, the latter studies always measured activity in terms of DNA instead of protein synthesis and suggested that it might be a mechanism to avoid diurnal UVR-driven damage to DNA. During our first cycle, however, the uptake of both substrates was completely phased in time; therefore, the opposite patterns of ^{3}H-leucine incorporation and primary production may suggest that causes other than photosynthetic DOM are driving the changes in bacterial activity. Photoinhibition of bacterial heterotrophic production due to UVR has been widely shown (Aas *et al.* 1996; Herndl *et al.* 1993; Sommaruga *et al.* 1997) and some authors have concluded that direct or indirect photoinhibition of bacterial activities could be a significant factor in the diel cycling of organic matter in the euphotic zone, reporting on sunlight induced growth delay of surface bacteria (Sieracki and Sieburth 1986) or explaining why the algal release of DOM is not immediately taken up by bacteria (Burney 1986). Similarly, Jeffrey *et al.* (1996*b*) observed this diurnal UVR inhibition in samples collected from the northern Gulf

of Mexico, where they found a period of rapid DNA damage increase during daytime. After sunset, DNA damage was rapidly removed and ^{3}H-thymidine incorporation started to rise. This nocturnal recovery of DNA damage has been further supported by observations that the expression of the DNA repair gene recA also peaks just after sunset (Booth *et al.* 2001). More recently, VanWambeke *et al.* (2009) observed that increases in the daily UVB to UVA ratios were negatively correlated with bacterial activity measured in the South Pacific clear waters. On the contrary, Shiah (1999), who also found higher ^{3}H-thymidine incorporation rates at night, stated that UVR was not involved since it was excluded in his experimental set-up. Moreover, he could undoubtedly associate this increase in bacterial activity to DOM release by phytoplankton, since it occurred only in transparent carboys and not in the opaque ones. Interestingly, when we incubated noon samples both in the dark and under an artificial visible-light source (Fig. 4), we found that bacterial heterotrophic activity was clearly and rapidly responding to light exposure, indicating that in the absence of UVR, bacteria could readily use the products of photosynthesis, and further discarding a potentially retarded response of bacteria to photosyntate due to low temperatures. However, although the penetration of UVR can be important in these surface oligotrophic waters, it seems unlikely, regarding the low doses of UVR registered during winter in the study area, that UVR alone would be the main parameter influencing bacterial heterotrophic activity patterns.

Other possible reasons for the observed photostimulation of bacteria in our light-incubated noon samples could be that cyanobacteria or some eukaryotic microalgae were taking up more substrates under the light, as observed elsewhere (Mary *et al.* 2008*b*; Michelou *et al.* 2007; Rivkin and Putt 1987), but this hypothesis could not be tested since all our MAR-CARD-FISH incubations were performed in the dark. Further, since also all the bacterial activity measurements were carried out in the absence of light, we were probably underestimating the real diurnal rates of ^{3}H-leucine and ^{3}H-thymidine incorporation, and thus overestimating the magnitude of changes between day and night. In any case, even if including this light stimulated noon values in the cycle, the activity levels at night would still be much greater than during the day.

A more plausible explanation for the nocturnal enhancement of bacterial activity might be found in the greater grazing activity at night, as reported by the marked increase in the number of ingested PPeuk cells by HNF. Herbivore activity is a major source of labile DOM in oligotrophic waters (Nagata 2000). PPeuk divided at the beginning of the night, as seen by the increase in their numbers and the reduction in their specific fluorescence (T. Lefort, unpubl.),

thus leading to higher availability of prey for the HNF. Indeed, it is known that protozoa can feed selectively, and they have demonstrated the ability to graze on larger, actively growing and dividing cells rather than on smaller ones (Sherr *et al.* 1992). However, although the increase in PPeuk abundances itself seemed to trigger the activity of grazers, by the end of the sampling period this enhanced predation appeared to cause a significant reduction in prey abundance, therefore suggesting that this 3-day pattern was probably not maintained over time and that, subsequently, the derived release of DOM and bacterial activity would also be disrupted.

The release of DOM by heterotrophic protists appears to be fully accounted for by egestion (see refs. in Nagata 2000). This author proposed one model where grazers were the dominant source of DOM, greater than phytoplankton production: according to Nagata, up to 65% of total DOM production could be accounted by the release of DOM by grazers. Among grazers, protozoa, which dominate oligotrophic environments such as the Blanes Bay and graze on small phytoplankton or bacteria, play a major role in DOM production releasing large amounts of DOM rich in different nutrients (Nagata and Kirchman 1991; Nagata and Kirchman 1992; Strom *et al.* 1997). It could thus be that, because of the limiting elements or the nutritional requirements of bacteria within the studied period, the DOM released by grazers was the most suitable type of DOM for all bacteria, explaining the strong synchronization of their activities during the first week. In addition, whereas during the first cycle the picoplanktonic community was dominated by PPeuk, after the stormy weekend *Synechococcus* became the most abundant group and heterotrophic nanoflagellates were relatively less numerous (T. Lefort, unpubl.), pointing to a change in the composition of the community that might have altered the amount and quality of DOM released by grazing.

Bacterial competition for inorganic nutrients with phytoplankton during the day might also offers an alternative explanation to the nocturnal stimulation of bacterial activity (Kuipers *et al.* 2000). However, this hypothesis seems unlikely since the concentration of most inorganic nutrients was maximal during that time of the year (data not shown). Finally, since the abundances of some studied groups (PPeuk, *Synechococcus*) increased at night and decreased during the day (T. Lefort, unpubl.), it is also possible that some DOM was released as a result of the processes of cell growth and division, yet little information is still available on this topic (but see Kawasaki and Benner (2006) and references therein).

Overall, it seems that the most likely mechanisms leading to this opposite coupling between phyto- and bacterioplankton activities might be the higher release of DOM at night by microzooplankton grazing on top of the possible damage caused by UVR on bacterial assemblages during the day or a strong competition for inorganic nutrients.

Diurnal variations in single-cell activities of dominant bacterial groups. Many studies have tested the variations in bulk bacterial activity at daily scales, but very few have analyzed the changes within specific phylogenetic groups. Pernthaler and Pernthaler (2005) studied the cell proliferation of three bacterial taxa (*Roseobacter,* SAR86 and NOR5) looking for diurnal patterns of DNA synthesis within them, but they did not find any clear diel trends. Using flow cytometric cell sorting, Mary *et al.* (2008*a*) found obvious diurnal rhythms in ^{3}H-leucine and ^{35}S-methionine uptake by *Prochlorococcus* cells in the tropical Atlantic, with maximum values at dusk and minimum at midday, and Chen *et al.* (1991) described a circadian clock that regulates amino acid uptake in freshwater *Synechococcus,* which presented the highest uptake rates during the light period. To our knowledge, however, this is the first report showing clear diel patterns in the activities of in situ dominant bacterial groups.

The composition of the bacterial community, as assessed with CARD-FISH probes, was within the compositional variability previously reported for this area (Alonso-Sáez *et al.* 2007). Whereas the relative abundances of the studied groups did not change on a daily scale, greater numbers of active cells were observed at night for all groups during the first cycle, in accordance with the pattern of bulk activity. During the second cycle, instead, just *Gammaproteobacteria* seemed to start recovering the trend again from the second night onwards, and interestingly, this appeared to drive bulk ^{3}H-leucine incorporation rates, despite being one of the less abundant groups. In fact, although *Gammaproteobacteria* presented on average much less active cells during the second week than during the first one, a progressive increase in the number of labeled *Gammaproteobacteria* cells was recorded towards the end of the week, coinciding with the recovery of the bulk activity cycle. Cells belonging to *Gammaproteobacteria* clade have shown preference for amino acids rather than other compounds as a carbon source, with varying proportions of active cells depending on nutrient availability (Alonso-Sáez and Gasol 2007; Cottrell and Kirchman 2000; Elifantz *et al.* 2005). It is possible that changes in the quality of DOM derived from the resuspension episode had negatively affected the activity of *Gammaproteobacteria,* although they seemed to be the first in responding to the restoration of the DOM-supply cycle.

Remarkably, besides the fact that the studied groups may harbour a number of subgroups with rather different metabolic properties, all of them were responding in the same way showing a strong general synchronization with DOM supply, suggesting that the DOM released by grazers was suitable for all groups. Instead, if bacteria had been more tightly dependant on phytoplankton DOM, we might have found some favoured taxa, such as some *Roseobacter* closely related to blooms of particular phytoplankton species and thus to short-term fluctuations of primary production (Pinhassi *et al.* 2004; Schäfer *et al.* 2002). Similarly, if solar radiation had played a significant direct role, we could have found some differential activation or inhibition of bacteria throughout the daylight hours depending on each group's sensitivity to sunlight (Alonso-Sáez *et al.* 2006; Arrieta *et al.* 2000; Sommaruga *et al.* 2005) or their potential capabilities to derive energy from light (Béjà *et al.* 2000; Béjà *et al.* 2002).

When we compared the number of active cells within each group with their relative abundances, *Gammaproteobacteria* and specially *Roseobacter* were found to be overrepresented in terms of ^{3}H-leucine uptake, meaning that they contributed more to the total ^{3}H-leucine uptake than to the composition of the community, whereas *Bacteroidetes* was underrepresented, possibly explained by their lack of affinity for leucine and their preference for high molecular weight compounds. Cells belonging to the SAR11 clade participated in ^{3}H-leucine incorporation in proportion to their contribution to bulk abundance. This pattern, commonly found for these groups from this and other regions (e.g. Vila-Costa *et al.* 2007; Alonso-Sáez *et al.* 2008), did not change between day and night, except for a general displacement towards higher numbers of active cells in night samples. The figure also illustrates that the day-night cycle affected the activity but not the composition of the bacterial community. *Synechococcus* remained basically the same in both day and night samples, showing negligible contributions to both activity and community composition.

Dark standard measurements of bulk and single-cell bacterial activities may underestimate bacterial production during the day or neglect any potential UVR-driven effect, so future experiments under real light conditions will be needed in order to assess the actual magnitude of these diel variations. Moreover, since the relative activities of bacterial groups from the Blanes Bay vary between winter and summer (Alonso-Sáez and Gasol 2007), as does the availability of DOM (Alonso-Sáez *et al.* 2008), we cannot discard that completely different bacterial cycles occur at different times of the year, as shown by Ghiglione *et al.* (2008) between spring and summer samples. Further research on the relative contribution of the key players in DOM supply and the diel activities of different bacterial taxa will be required for better comprehension of their contribution to daily variations in carbon fluxes.

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Chapter 2.1

Annual variability in light modulation of bacterial heterotrophic activity in surface NW Mediterranean waters

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ABSTRACT

The effect of photosynthetically available radiation (PAR, 400-700 nm) and ultraviolet radiation (UVR; 280-400 nm) upon marine bacterial heterotrophic activity was assessed throughout a seasonal cycle in Blanes Bay (Northwestern Mediterranean Sea). We performed monthly incubations of seawater samples amended with ^{3}H-leucine directly exposed to in situ solar radiation under three radiation treatments: PAR + UVR (280-700 nm), PAR + UVA (320-700 nm) and PAR only. Parallel incubations in the dark and under a fixed artificial light source (PAR only) were also performed to be used as reference measurements obtained under invariant irradiance conditions. UVR exposure caused stronger inhibition when UVR doses were higher, whereas solar PAR incubations showed no significant effects. Within UVR, UVA radiation accounted for most of the reduction in ^{3}H-leucine incorporation, although its relative contribution to total inhibition was affected by the previous light exposure history of the samples, suggesting that overexposure in static incubations may affect the measured UVR effects. Constant (artificial) PAR-only exposure led to a general but seasonally variable increase in bacterial heterotrophic production compared to dark control, showing higher increases in spring and lower (and even negative) changes during summer. This light effect seemed to be caused by the stimulation of the bacterial group *Gammaproteobacteria*, which showed higher numbers of cells active in ^{3}H-leucine uptake after light exposure coincident with increases in bulk bacterial heterotrophic activity. Again, the previous light history of the samples seemed to partly explain such effects, and both the potential photoheterotrophy and an indirect effect of algal release on this bacterial group are discussed. Overall, our results show variable responses of bacterial activities with seasonally changing light conditions and communities, and stress the importance of realistic simulation of exposure conditions for accurate interpretation of the observed effects.

INTRODUCTION

Abundance and activity of marine heterotrophic bacteria are under the influence of several parameters that may fluctuate at different time scales. Temperature, nutrient concentration, dissolved organic matter (DOM) availability and the composition of the microbial community have been regarded among the main factors controlling DOM consumption by bacteria (Cotner *et al.*, 2000; Cottrell and Kirchman, 2003; Reinthaler and Herndl, 2005; White *et al.*, 1991). However, the relative importance of solar radiation as a modulator of bacterial production across spatial or seasonal patterns has received little attention. In surface waters, bacteria are exposed to damaging solar ultraviolet radiation (UVR, 280-400 nm) that can cause inhibition of metabolic activities such as protein and DNA synthesis (Herndl *et al.*, 1993; Sommaruga *et al.*, 1997), oxygen consumption (Pakulski *et al.*, 1998), and amino acid and ATP uptake (Alonso-Sáez *et al.*, 2006; Bailey *et al.*, 1983). Nevertheless, there is also evidence of a positive effect of UVA (320-400 nm) and photosynthetically available radiation (PAR, 400-700 nm) on bacterial activity due to photoenzymatic repair (Kaiser and Herndl, 1997), to the ability of some bacteria to derive energy from light using bacteriochlorophyll-*a* or proteorhodopsin (Béjà *et al.*, 2000; Gómez-Consarnau *et al.*, 2007; Kolber *et al.*, 2000), or even to the reported light-stimulation of cyanobacterial uptake of amino acids and related compounds (Church *et al.*, 2004; Mary *et al.*, 2008; Michelou *et al.*, 2007; Zubkov *et al.*, 2004). In addition, UVR can photolyze some recalcitrant DOM into more readily utilizable forms, making it more available to heterotrophs and thus enhancing their activity or, by contrast, initially labile DOM can be rendered more recalcitrant upon UVR exposure (Abboudi *et al.*, 2008; Benner and Biddanda, 1998; Herndl *et al.*, 1997; Obernosterer *et al.*, 1999; Obernosterer *et al.*, 2001; Tedetti *et al.*, 2009). And finally, heterotrophic bacteria might react to increased or decreased release of photosynthate from light-affected phytoplankton, all indicating that the interactions between heterotrophic bacteria and light are far from simple.

The Mediterranean Sea is characterized by relatively high solar radiation levels due to its weak cloud cover (Seckmeyer *et al.*, 2008; Vasilkov *et al.*, 2001) and a high penetration of solar radiation in the water column (Brunet *et al.*, 2007; Moutin and Raimbault, 2002) owing to its oligotrophic nature and highly transparent waters; however,

although some UVB (280-320 nm) and UVA underwater measurements are available for the Mediterranean (Joux *et al.*, 2009; Llabrés *et al.*, 2010; Sommaruga *et al.*, 2005; see refs. in Tedetti and Sempéré, 2006), there is still a remarkable dearth of data on UVR attenuation varying at different scales. A current temporal series in the Blanes Bay Microbial Observatory (NW Mediterranean), a shallow (20 m depth) oligotrophic area, shows maximum 10% attenuation depths of 7.6 and 32.4 m at the nominal wavelengths of 305 nm within the UVB range and 380 within the UVA region, respectively (M. Galí, unpubl.), indicating that a large % of UVA reaches the bottom waters, and UVB can reach half the maximum depth of this site, yet a great seasonal variability in underwater UV profiles is apparent. However, most of the UVR effects on prokaryotes reported for the Mediterranean are derived from experiments performed during short periods of time (hours), and mostly during spring or summer (Abboudi *et al.*, 2008; Alonso-Sáez *et al.*, 2006; Llabrés *et al.*, 2010; Sommaruga *et al.*, 2005; Tedetti *et al.*, 2009). Since both seasonal bacterial taxonomic succession (Schauer *et al.* 2003, Alonso-Sáez *et al.* 2007) and differential sensitivities to UVR of different bacterial groups have been described for this coastal region (Alonso-Sáez *et al.* 2006) seasonal variability in the bacterial responses to UVR might be expected in the area.

In this study we incubated natural bacterioplankton with radioactive leucine in UV-transparent 4 ml vials to (1) characterize the effects of natural radiation levels on bacterial heterotrophic activity, specifically on leucine incorporation rates (LIR), measured under in situ light conditions as compared to those measured with a constant light source, all throughout a seasonal cycle, and (2) analyze the main causes of such variability by comparison with changes in physical (temperature, irradiance, light history, mixing layer depth) or biological (chlorophyll *a,* primary productivity, bacterial abundance and community composition) parameters. Since both light levels and bacterial taxonomic composition change over a seasonal gradient, the differential responses of marine bacterial communities to natural sunlight seem essential to understand the role of sunlight as a modulator of organic matter fluxes in marine ecosystems. Finally, the results obtained may have implications for discussing the suitability of dark standard protocols for activity estimates of bacteria naturally exposed to varying PAR and UVR doses.

MATERIALS AND METHODS

Sampling and basic parameters. A monthly study was carried out in a shallow coastal station (the Blanes Bay Microbial Observatory, NW Mediterranean Sea, http://www.icm.csic.es/bio/projects/icmicrobis/bbmo) from January 2008 to April 2010. Surface waters (0.5 m depth) were sampled at about 800 m offshore (41º 40'N, 2º 48'E), filtered through a 200 μm mesh net and transported within an hour to the lab under dim light in 25 L polycarbonate carboys. Seawater temperature was measured in situ with a CTD-profiler and underwater PAR and UVR profiles were obtained with a PUV 2500 radiometer (Biospherical Instruments). Chlorophyll *a* concentration was determined by filtering 150 ml of seawater on GF/F filters (Whatman), extracting the pigment in acetone (90% v/v) in the dark at 4ºC for 24 h, measuring fluorescence with a Turner Designs fluorometer.

Different experiments were performed throughout the studied period. During the whole period (January 2008 to April 2010), parallel dark and artificial light (PAR-only) incubations for bacterial heterotrophic activity measurements (from now on ^{3}H-leucine incorporation rates, LIR) were carried out inside an indoor incubator at in situ temperature, in order to avoid natural variability in light levels and to allow comparison among responses of seasonally changing communities to invariable irradiance conditions (ca. 1500 μmol photons m^{-2} s^{-1}). From 15 January 2008 to 14 September 2009 additional LIR measurements were conducted under natural radiation conditions using different UVR filters inside a tank placed outside the laboratory in Barcelona, ca. 100 km south of the sampling site in the same coastline, and the PAR and UVR doses received by samples during experiments were recorded using a PUV 2500 radiometer placed underwater next to the samples. From 17 March 2009 to 13 April 2010 parallel dark and artificial PAR incubations were carried out for MAR-CARD-FISH analyses (see below) inside the same indoor incubator used for LIR measurements, in order to explain some of the patterns found. The rest of the variables (primary production, bacterial and picophytoplankton abundances, composition of the bacterial assemblages) were continuously monitored throughout the whole period.

Primary production. For the measurement of particulate primary production (pPP), fourteen 70 ml-bottles (Iwaki) and one dark control (bottle wrapped with

aluminium foil) were filled with seawater and inoculated with (10 μCi) $NaH^{14}CO_3$. The incubation was carried out in a water bath at in situ temperature for 2 hours in a gradient of light irradiance (ca. 10-1500 μmol photons m-2 s-1). Circulating water connected to a water bath maintained the temperature. Light was measured with a small size spherical light meter (Illuminova AB, Sweden). After the incubation, the samples were filtered at low vacuum pressure through cellulose ester filters (Millipore 0.22 μm), and the filters were subsequently exposed overnight to concentrated HCl fumes. Scintillation cocktail (4 ml Optiphase Hisafe 2) was then added to each filter, and the radioactivity was measured in a Beckton-Dickinson LS6000 scintillation counter. Average in situ pPP was estimated from the P-E curve and the hourly in situ PAR irradiance within the 'actively mixing layer' of the 24 h prior to sampling (see below).

Abundance of bacteria and photosynthetic phytoplankton. Aliquots for bacterial abundance were preserved with 1% paraformaldehyde and 0.05% glutaraldehyde (final conc.), frozen immediately in liquid nitrogen, and stored at -80°C until quantification with a FACSCalibur flow cytometer (Becton Dickinson) of cells stained with SybrGreen I (Molecular Probes, Eugene, Oreg.). *Synechoccocus* cells were enumerated by flow cytometry from unstained samples. Both groups were quantified and distinguished by their different size and fluorescence properties following common procedures (Gasol and Del Giorgio, 2000).

Leucine incorporation rates (LIR). LIR was estimated monthly using the ^{3}H-leucine incorporation method described by Kirchman et al. (1985). Four 1.2 ml aliquots and two TCA killed controls (5% final conc.) of each sample were incubated with 40 nmol l-1 ^{3}H-leucine for 2 h. The incubations were carried out in a water bath at in situ temperature both under in situ light or under fixed light irradiance (ca. 1500 μmol photons m-2 s-1, approximately the surface PAR irradiance of a summer day in this area). The incorporation was stopped by adding cold TCA (5% final conc.) to the vials and samples were kept at -20°C until processing as described by Smith and Azam (1992). Radioactivity was then counted on a Beckman scintillation counter.

From 15 January 2008 to 14 September 2009 LIR was also measured under exposure to natural sunlight. For that purpose, four UV-transparent cuvettes (4 ml, Plastibrand) and two formaldehyde-killed controls (4% final conc.) were amended with ^{3}H-leucine (40

nM final conc.) and incubated for 2 to 3 hours under different radiation conditions: full sunlight spectrum (PAR+UVR), the full spectrum minus UVB (PAR+UVA, covered with the plastic foil Mylar-D, which excludes UVB radiation), PAR only (wrapped with two layers of Ultraphan URUV farblos which removes all UVR) or darkness (wrapped with aluminium foil inside a black plastic bag to avoid reflection). Cuvettes were incubated at about 5 cm under the surface inside a black tank (200 litres) with running seawater to maintain in situ temperature. After incubation, 1.2 ml were transferred from each cuvette to centrifuge tubes, then killed with 120 µl cold TCA (5% final conc.) and processed as described above.

Microautoradiography combined with Catalysed Reporter Deposition-Fluorescence In Situ Hybridization (MAR-CARD-FISH). In the experiments between 17 March 2009 and 13 April 2010, 30 ml samples were amended with trace amount of ^{3}H-leucine (0.5 nM final conc., 160 Ci mmol^{-1}) and incubated parallel in the dark or in the light (PAR-only, ca. 1500 µmol photons m^{-2} s^{-1}) for 2 to 3 hours. After exposure, samples were fixed overnight with paraformaldehyde (PFA, 1% final conc.) at 4ºC in the dark and gently filtered on 0.2 µm polycarbonate filters (GTTP, Millipore). Sections of the filters were then hybridized following the CARD-FISH protocol (Pernthaler et al., 2002). We used a few horseradish peroxidase (HRP)-probes were used in order to search for potentially photostimulable groups: Gam42a that targets most *Gammaproteobacteria* (Manz et al., 1992), NOR5-730 for the NOR5 clade, which belongs to *Gammaproteobacteria* (Eilers et al., 2000b); Ros537 targeting the alphaproteobacterial *Roseobacter* clade (Eilers et al., 2001), CYA339 for *Cyanobacteria* (Nübel et al., 1997) and Eub338-II-III for inclusion of most *Eubacteria* (Amann et al., 1990; Daims et al., 1999). The relative abundance of each group was checked by cutting smaller pieces from each filter and staining them with 4,6-diamidino-2-phenylindole (DAPI, 1 µg ml^{-1}). Between 500-800 DAPI-positive-cells were counted manually within a minimum of 10 fields under an Olympus BX61 epifluorescence microscope.

For microautoradiography, we followed the protocols described in Alonso and Pernthaler (2005) but modified as in Alonso-Sáez and Gasol (2007) and Vila-Costa *et al.* (2007). The optimal exposure times were determined for each sampling point and ranged from 2 to 19 days. After development, slides were dried overnight, stained with DAPI

(1 μg ml^{-1}) and 500 to 700 hybridized cells were counted manually by epifluorescence microscopy within a minimum of 10 fields.

Measurement and calculation of PAR and UVR doses. A radiometer (Biospherical PUV 2500) was used in the field and also placed inside the incubation tanks, with the sensor covered by ca. 5 cm of water, and the downwelling cosine irradiance reaching the samples was recorded at a frequency of 5 s^{-1}. The wavelengths measured included 6 bands in the UVR (305, 313, 320, 340, 380 and 395 nm, in units of mW cm^{-2} nm^{-1}) and one integrated band in the visible (PAR, in mmol photons cm^{-2} s^{-1}). The mean spectral irradiance in the 6 UVR bands was converted to mean UVB and UVA irradiance (mW cm^{-2}) by integrating over the spectrum (sum of trapezoids), between 305 – 320 nm and 320 – 395 nm respectively. Finally, the mean UVB, UVA and PAR irradiance was multiplied by the duration of the experiment in order to obtain the radiation dose (in W m^{-2} for UVB and UVA, and mol photons m^{-2} for PAR).

The 'light' history of microbial communities, i.e. their previous UVR and PAR exposure, was calculated as a function of spectral irradiance at the water subsurface, vertical mixing depth and underwater attenuation of solar radiation (Vallina and Simó, 2007). For this purpose, two distinct exposure regimes were considered: 'seasonal exposure' and 'maximum daily exposure'. Seasonal exposure was calculated by combining the mean irradiance of the three days prior to sampling with the seasonal mixed layer depth (MLD), whereas maximum daily exposure was calculated as the combination of 'actively mixing layer' depth (mLD) with average irradiance at noon ± 2 hours. Total solar irradiance (with hourly resolution) was obtained from a meteorological station located 5 km SW from the BBMO sampling station (Malgrat de Mar, Catalan Meteorological Service, SCM). MLD and mLD were calculated from temperature profiles obtained from CTD casts, binned at 1 m intervals. MLD was defined as the depth where a jump in temperature larger than 0.15 ºC was encountered relative to 1 m depth while mLD was defined as the depth showing a 0.03 ºC departure from the 1 m reference. These criteria were optimized for our particular dataset, and yielded mLD or MLD estimates that were consistent with the vertical profiles of other variables (further details in Galí *et al.*, in prep.).

RESULTS

Background values. The seasonal changes of different parameters in the study area are shown in Fig. 1. The summer period was characterized by high surface temperatures (20-25 ºC) and low Chl *a* concentrations (< 0.4 µg l^{-1}) and the opposite trend was found in winter when the lowest temperatures (~ 12 ºC) and Chl *a* peaks (up to 2 µg l^{-1}) were recorded. In situ phytoplankton particulate primary production (pPP) also varied seasonally, reaching higher values in late winter (> 4 mg C m^{-3} h^{-1} in 2008) and variable peaks during spring and summer accordingly to higher irradiances and longer photoperiod (Fig. 1B). No winter pPP peak was apparent in 2009, although since the March data point is missing, it is possible that we missed that peak. No photoinhibition of pPP was detected at any measured irradiance.

Values of ^{3}H-leucine incorporation rates showed high variability among sampling dates (range 2-135 pmol ^{3}H-leucine L^{-1} h^{-1}) although the maximum rates tended to be measured after Chl *a* or pPP peaks, and in general higher values were found in summer and spring compared to autumn and winter, as reflected by a positive correlation between LIR and temperature (Pearson's R = 0.65, n= 36, P < 0.0001).

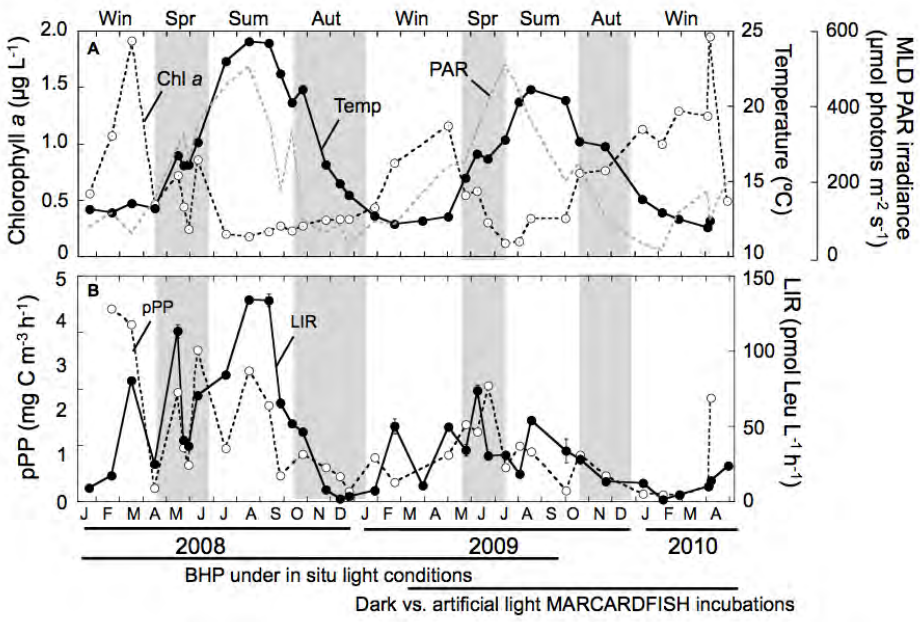

Fig. 1. (A) Temporal dynamics of seawater temperature (solid circles), chlorophyll *a* (open circles) and 'seasonal' PAR irradiance (i.e. mean irradiance of the 3 days prior to the sampling within the MLD, see text, grey line). (B) In situ primary production (PP, open circles) and leucine incorporation rates (LIR, solid circles) over the period January 2008-April 2010 in Blanes Bay, including a distinction of winter, spring, summer and autumn based on the average values of these parameters (see text).

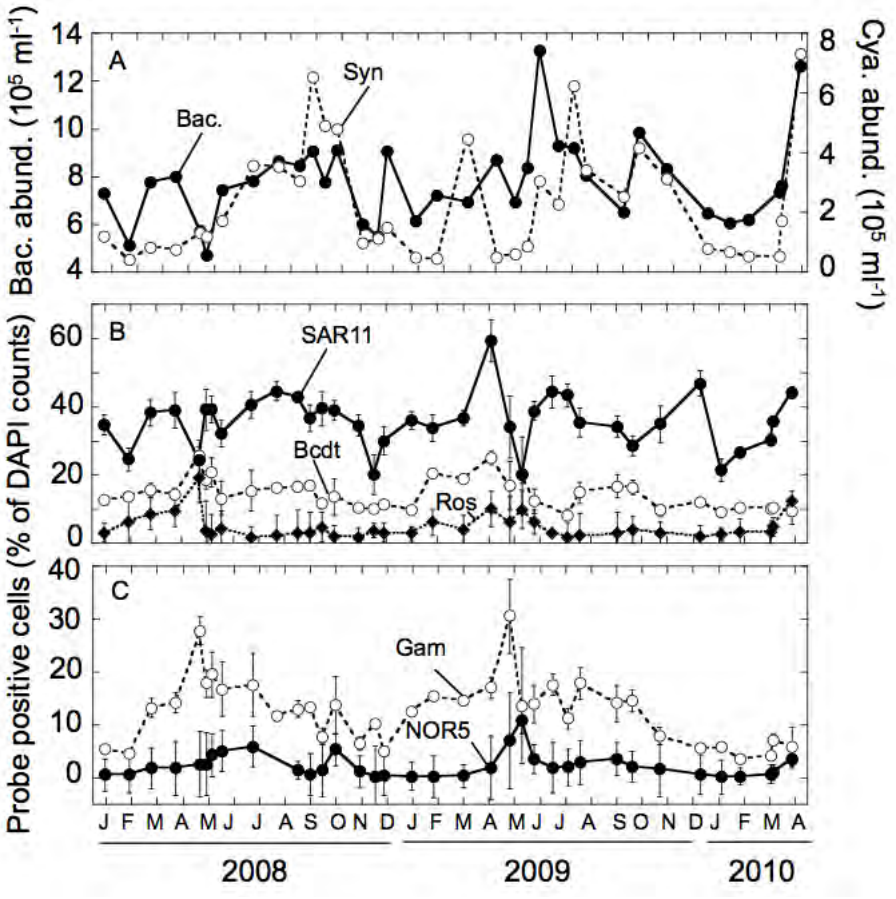

Fig. 2. (A) Seasonal variability in the abundance of bacteria (Het. Bac) and *Synechococcus* (Syn). Percentages of bacterial groups detected by CARD-FISH with HRP probes specific for: (B) *Bacteroidetes* (Bcdt), SAR11 and *Roseobacter* (Ros); (C) *Gammaproteobacteria* (Gam) and the NOR5 clade.

Bacterial abundances ranged from 0.4 to 1.3 x 10^6 cells ml^{-1} and tended to be higher during the summer. *Synechococcus* abundances also varied seasonally (Fig. 2 A) showing the highest numbers during summer (see also Sommaruga et al. 2005).

The bacterial community composition assessed by CARD-FISH was always dominated by the SAR11 group (average of 35% of cell counts) followed by similar proportions of both *Bacteroidetes* (15%) and *Gammaproteobacteria* (13%). Whereas the later ones showed a strong seasonality, increasing their numbers during winter and peaking in spring in both years closely following the Chl *a* peaks (up to 25% or 30% of cell counts, respectively), the SAR11 group was less clear and variable percentages were found ranging from 20% to 60% of total DAPI counts. *Roseobacter* and the gammaproteobacterial NOR5 group presented much lower numbers (average 5% and 2% of total DAPI counts, respectively) with higher peaks generally coincident with maximal Chl *a* concentrations.

Natural sunlight experiments. Exposure of samples to natural sunlight radiation caused a general but variable inhibition of LIR compared to the dark control (Fig. 3a). The lowest LIR rates were recorded in full sunlight exposure (up to 50% inhibition, average 17%) whereas PAR alone caused a significant decrease of LIR compared to the dark control in only five out of the twenty five experiments (range 20% - 33% decrease) and exceptionally, significant increases (range 25% - 50%) in PAR-measured LIR compared to dark could be observed (ANOVA test, $P < 0.05$). The degrees of inhibition due to both UVA and UVR, as compared to PAR values, were significantly correlated with increasing UVR doses measured during incubations (Figs. 3c and d, Pearson's $R = 0.61$, $P < 0.002$, n = 25 and $R = 0.43$, $P < 0.04$, n = 25, respectively). Conversely, no significant correlation was apparent between LIR measured under PAR radiation and its doses (Fig. 3b).

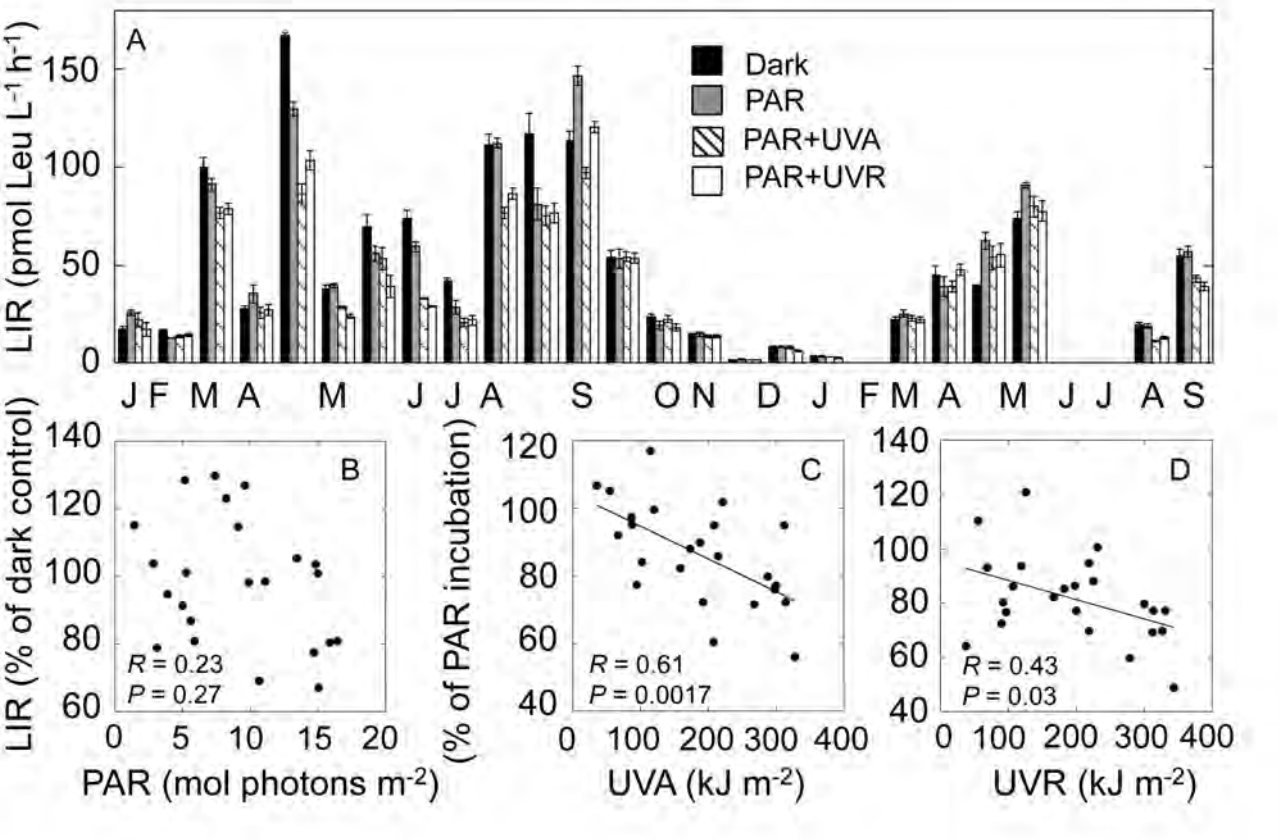

Fig. 3. (A) Bacterial heterotrophic production measured in the dark (black bars) and after exposure to PAR (grey bars), PAR+UVA (dashed bars) and PAR+UVR (open bars). (B) Relationship between inhibition of bacterial production measured under different light treatments (PAR, B; UVA, C and total UVR, D) and the PAR and UVR doses received by the samples during each experiment.

	In situ light conditions			Dark vs art. light
	Average PAR LIR (% of dark control)	Average UVA LIR (% of PAR value)	Average UVR LIR (% of PAR value)	Average light LIR (% of dark control)
Winter	103 ± 10^{a}	93 ± 3^{a}	92 ± 8^{a}	120 ± 10^{ab}
Spring	107 ± 11^{a}	76 ± 5^{b}	73 ± 5^{b}	153 ± 15^{a}
Summer	95 ± 10^{a}	73 ± 5^{b}	78 ± 4^{ab}	96 ± 13^{b}
Autumn	105 ± 7^{a}	99 ± 5^{a}	83 ± 6^{ab}	124 ± 15^{ab}
Annual average	103 ± 5	85 ± 3	81 ± 3	122 ± 7

Table 1. Seasonally averaged LIR measured under different light conditions and presented as percentages of the dark controls (PAR LIR) or percentage of the PAR values (UVA and UVR LIR), respectively. Last column: seasonally averaged LIR measured under artificial PAR light ('art. light') as percentage of the dark control. Values are average ± standard errors of sampling dates. Different letters indicate significant differences among seasonal averages measured under different light conditions (ANOVA test, $P < 0.05$).

Such sensitivity of LIR to UVR was not correlated with any other measured parameter including temperature, Chl *a*, nutrient concentration, mixing layer depth, nor the bacterial community composition and the abundance of the different bacterial groups as described by CARD-FISH (details not shown).

Seasonally averaged changes in LIR measured under in situ light conditions are summarized in Table 1 as percentages of the dark control (for PAR-LIR) or percentages of the PAR value (for UVA-LIR and UVR-LIR). Whereas no significant differences were found among seasons for LIR measured under PAR radiation, significant inhibition due to UVA was found in both the spring and the summer periods. Conversely, for the case of UVR, a significant inhibition could only be found in spring (Tukey's test, $P < 0.05$). When annually averaged, LIR measured under PAR conditions was significantly higher than LIR exposed to both UVA or UVR radiation (Tukey's test $P < 0.05$).

The relative contribution of UVA and UVB to the total photosynthetic inhibition (Fig. 4a) was calculated as follows:

$$\text{Inhibition due to UVR}_x = (\text{LIR}_{PAR}\text{-LIR}_{UVRx})*100/\text{LIR}_{PAR}$$

where LIR_{PAR} represents the ^{3}H-leucine incorporation rates under PAR-only incubation treatment, and LIR_{UVRx} means the LIR measured under each UVR treatment. Inhibition

due to UVB was further calculated as the difference between the relative inhibition due to UVA and UVR. The contribution of each type of UVR varied throughout the year, although in most cases, UVA was responsible for most of the observed inhibition (Fig. 4a) and no seasonality was apparent for the changes between the contributions of each UVR fraction, No correlation was found between the measured UVA to UVB ratio during incubations and the inhibition due to each fraction (Fig. 4b), whereas UVA and UVB relative inhibitions were significantly correlated with the UVA to UVB ratio measured in situ (calculated from the in situ UV irradiance measured during the 4 hours of maximum radiation of the previous day, see Methods) and a higher inhibition of UVB was found with increasing in situ UVA/UVB ratios (R = 0.66, P < 0.0001, n = 25, Fig 4c). In other words, the lower the UVB doses relative to UVA received by samples in situ, the higher the UVB-driven inhibition during incubations. This was partially explained by the difference between in situ UVA/UVB ratio and the one received by samples during our incubations, since the contribution to inhibition of each UV fraction varied accordingly with how much did these two ratios differed (data not shown). Thus, it seems that if the ratio measured during our incubations was lower than that found in situ, a more important effect due to UVB would be observed due to the artifactually higher UVB doses received by samples inside the incubator.

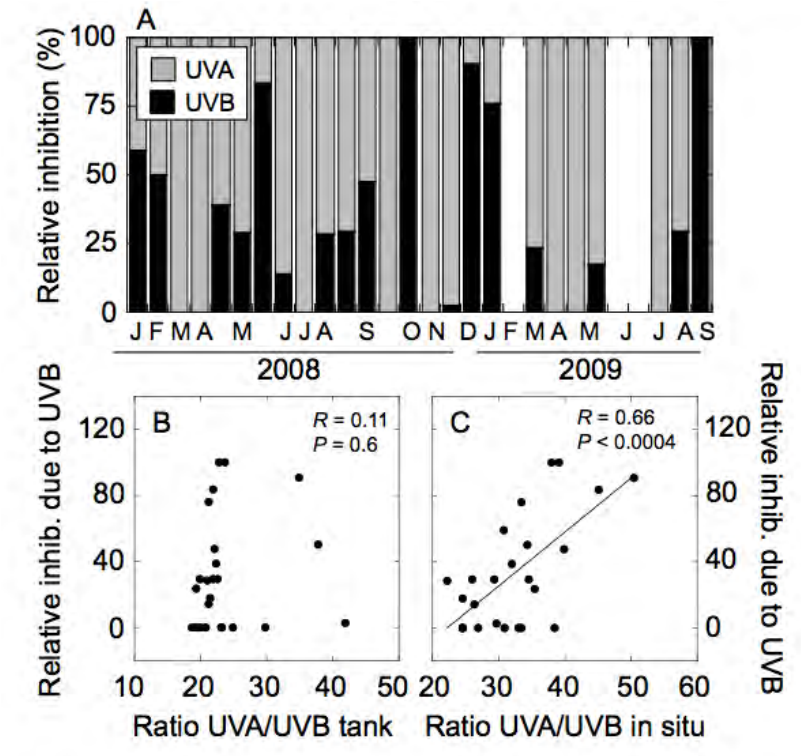

Fig. 4. (A) Relative contribution of UVA (grey bars) and UVB (black bars) to total inhibition of bacterial production with respect to the PAR treatment. Variation of the relative contribution of UVB to total UVR-inhibition with respect to (B) the ratio UVA/UVB experienced by samples during incubations or (C) to the in situ UVA/UVB ratio (calculated from the in situ UV irradiance measured during the 4 hours of maximum radiation of the previous day, see Methods).

Constant light experiments. An artificial light source (PAR only, ~ ca. 1500 µmol photons m^{-2} s^{-1}) was further used for comparison between the LIR measured in the dark and under constant light conditions in order to check if the responses of the bacterial communities varied independently of the light intensity, as well as to avoid the possible variability in the light regime experienced by the samples during the incubations, i.e. due to passing clouds and changing weather. In general, LIR measured in the light was stimulated with respect to the dark control (range 20% to 151% increase), although also inhibition (range -20% to 65% decrease) or no effects at all were sometimes observed (Fig. 5a). Such variability could not be explained by other parameters such as temperature, Chl *a*, nutrient concentration, primary productivity or the composition of the bacterial community. The only apparent relationship was found between the increase in the light-measured LIR compared to dark and the maximum daily PAR irradiance (mean irradiance at noon ± 2h of the previous day within mLD, see Methods) shown in Fig. 5b.

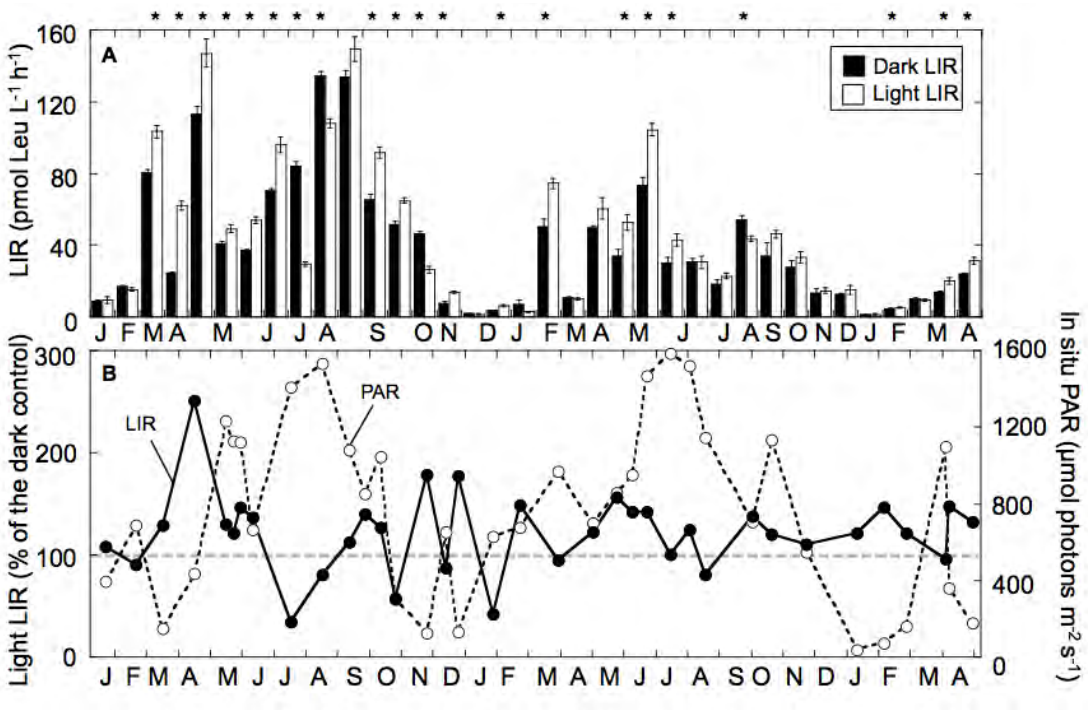

Fig. 5. (A) Seasonal variability in bacterial heterotrophic production measured in the dark and under a fixed light source (ca. 1500 µmol photons m^{-2} s^{-1}). Asterisks (*) indicate significant differences between dark and light incubations (ANOVA test, n = 4, $P < 0.05$) Arrows indicate dates when MARCARDFISH incubations were performed. (B) Comparison between the variation in LIR measured in the light (scaled to the dark control value) with the irradiance history of the samples, expressed as the in situ PAR irradiance measured during the 4 hours of maximum radiation of the previous day (see Methods). Grey dashed line behind represents LIR 100% of the dark control, meaning no effect between dark and light incubations.

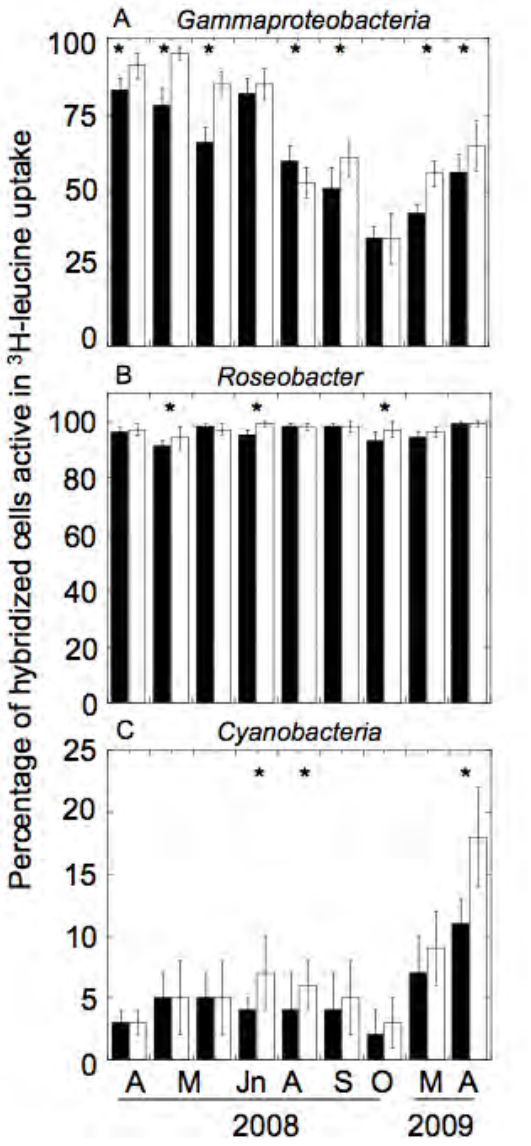

Fig. 6. Percentage of positively hybridized cells with probes for *Gammaproteobacteria* (a), *Roseobacter* (b), and *Cyanobacteria* (c) taking up ^{3}H-leucine (average ± standard deviation of fields) as measured by MAR-CARD-FISH after exposure to a fixed light (ca. 1500 µmol photons m^{-2} s^{-1}, open bars) or kept in the dark (black bars). Asterisks (*) indicate significant differences between light and dark treatments (ANOVA, $P < 0.05$). Only samples where a clear light-driven response in LIR was apparent were processed and quantified.

In general, the LIR measured in the light was higher than the dark values when lower irradiances were present in situ, and instead, inhibition or no effects were found when the natural irradiances approached the artificial levels. In terms of seasons, the average photostimulation was maximal in spring whereas photoinhibition generally occurred in summer where similar light levels were experienced by samples in both the incubator and the sea (Table 1).

To further investigate the reasons of such light-driven variability in LIR, several MAR-CARD-FISH incubations were performed to search for potentially photostimulable bacterial groups. *Gammaproteobacteria*, *Roseobacter*, NOR5 and *Cyanobacteria* were selected for that purpose, and only results of the dates when differences between dark and light LIR were more apparent are presented (Fig. 6). Given the low abundances of the NOR5 group it was not possible to quantify their number of active cells and they were thus rejected as potential drivers of the observed light-stimulation patterns.

Variable numbers of *Gammaproteobacteria* active in the uptake of ^{3}H-leucine were found all through the year (range 40%-90% of active cells), showing higher percentages

in summer and spring compared to autumn samples (Fig. 6a). This group showed a repeated pattern of significant stimulation due to light, except in August 2009 when they were slightly inhibited with respect to the dark control, coincident with the observed decrease in bulk light-LIR. A good positive correlation was found between the light-driven increases in the number of active *Gammaproteobacteria* and the increase in bulk LIR caused by light in those samples where significant changes were found between both treatments (n = 7, $R = 0.94$, $P < 0.0001$) and moreover, significant correlations were found between the number of active *Gammaproteobacteria* and bulk LIR rates (n = 17 $R = 0.59$, $P < 0.02$) and between active *Gammaproteobacteria* and active *Eubacteria* (n = 17 $R = 0.66$, $P < 0.003$) measured in the dark. Both correlations adjusted better under the light (n = 17 $R = 0.67$, $P < 0.004$ and n = 17, $R = 0.82$, $P < 0.0001$, respectively). Remarkably, *Gammaproteobacteria* abundances seemed to be explained in part by the underwater ambient PAR levels (n = 29, $R = 0.61$, $P < 0.0007$), showing greater abundances in more illuminated waters, whereas no correlation was found with temperature, Chl *a* or primary production data. Conversely, neither *Roseobacter* nor *Cyanobacteria* explained the observed light-driven differences due to light. Very high percentages of *Roseobacter* were active in ^{3}H-leucine uptake throughout the year (> 95% of labelled cells) but no significant differences were usually found between dark and light treatments. The fraction of active *Cyanobacteria* rarely exceeded 10%, and although some differences were detected between treatments (up to two fold increase), their low activity and different stimulation patterns excluded them as candidates responsible for the light enhancement of LIR.

DISCUSSION

Very few seasonal studies have considered the responses of marine microbial communities to temporally variable natural radiation levels, UVR being often omitted as a significant driver of microbial activities. UV radiation has been shown to impact on phytoplankton and bacterioplankton production (Herndl *et al.*, 1993; Villafañe *et al.*, 2003), DOM lability and release (Herndl *et al.*, 1997; Obernosterer *et al.*, 1999; Pausz and Herndl, 1999; Tedetti *et al.*, 2009), grazer pressure (Ochs, 1997) and viral infectivity

(Suttle and Cheng, 1992), all of which might ultimately affect the cycling of carbon through the ecosystem. Heterotrophic marine bacteria are main players in the ocean carbon cycle (Azam *et al.*, 1983) and, due to their small size, they are considered among the groups of plankton more susceptible to sunlight damage (García-Pichel, 1994). Given the high transparency of most oceanic waters, particularly those of Blanes Bay, to UV radiation, and the reported specific UVR sensitivity of some bacterial groups from this area (Alonso-Sáez *et al.*, 2006), seasonal variations in light intensity and penetration into the water column might differentially affect the year-round bulk incorporation of DOM by bacteria, thus buffering the effects of other environmental variables that are more easily and often measured.

Our approach consisted of incubations in full light and with the radioisotope tracer already added, which was possible by using UVR-transparent vials and filters to distinguish the effects of different wavebands (PAR, UVA and UVB). This approach combines the potential positive or negative effects of light on the microbes with any possible effects on the lability of DOM, yet we believe it is the closest possible to natural circumstances. Any further UVR impacts on the added leucine tracer were discarded since no significant reduction in LIR was found when samples were amended with previously exposed ^{3}H-leucine to both natural or artificial UVR (data not shown). With this approach, we found an average 3% increase of LIR under PAR (with respect to darkness), and a 19% inhibition (with respect to PAR values) under full UVR, these values being variable with the seasons (see Table 1).

Exposure of samples amended with ^{3}H-leucine to in situ radiation levels caused, as reported by others (Aas *et al.*, 1996; Herndl *et al.*, 1993; Sommaruga *et al.*, 1997), a significant decrease in LIR rates with respect to those in dark incubations. In general, we found low or no inhibition when irradiance values were low (winter and autumn) whereas in spring and summer, when higher radiation levels occurred, we observed higher UVR inhibition of LIR (up to 50%).

In contrast, the PAR-only treatment did not generally affect the measured LIR regardless of the irradiance, except for a few cases where a slight decrease or an occasional increase in bacterial production were observed. Other studies have also observed different degrees of inhibition (Aas *et al.*, 1996; Morán *et al.*, 2001; Sommaruga *et al.*,

1997) or stimulation (Aas *et al.*, 1996; Pakulski *et al.*, 2007) when measuring LIR under in situ PAR irradiance. Morán *et a*l. (2001) suggested that apparent PAR inhibition in samples from the same area was a result of stimulation of bacterial growth in the dark, although the exact mechanisms were not identified. Besides radiation levels received, the response of bacteria to solar radiation has been shown to depend on many other environmental or biological factors, such as temperature (Rae and Vincent, 1998; Roos and Vincent, 1998), nutrient status (Pausz and Herndl, 2002), mixing (Huot *et al.*, 2000; Neale *et al.*, 2003), or the specific sensitivities or recovery capabilities of different bacteria (Agogué *et al.*, 2005; Alonso-Sáez *et al.*, 2006; Arrieta *et al.*, 2000; Joux *et al.*, 1999; Kataoka *et al.*, 2009). During our annual cycle, neither temperature, Chl *a*, pPP or nutrient concentration seemed to influence the light-driven responses of LIR to natural sunlight. Also, no significant correlations were found between the LIR response to light and bacterial community composition in terms of group relative abundances, but since different phylotypes from this area have been shown to present distinct seasonal patterns in their activities (Alonso-Sáez and Gasol, 2007; Vila-Costa *et al.*, 2007), it is possible that we could have only found such a relationship if comparing with the relative contribution of each group to bulk activity instead of with their abundances alone.

Another interesting aspect of the UVR effects refers to the relative contribution of UVA and UVB to the total LIR inhibition. The contribution of UVA in our experiments was generally higher than that of UVB (Fig. 4), as also seen by other authors (Sommaruga *et al.*, 1997; Tedetti *et al.*, 2009), which might be attributed to the fact that besides UVB contains more energy than UVA, the amount of UVA energy that reaches the sea surface is much higher than that of the UVB region. However, in some occasions, a higher inhibition due to UVB was also detected. In those cases, the increase in the relative inhibition due to UVB was associated with increasing in situ mixing layer depths ($n = 25$, $R = 0.59$, $P < 0.002$, data not shown). Vertical mixing controls the residence time of marine bacteria in surface waters (Neale et al. 2003) and it has been shown to be an important factor regulating the impacts of UVR (Huot *et al.*, 2000; Van Wambeke *et al.*, 2009). Since the attenuation of UVB in the water column is much higher than that of UVA or PAR (Smith and Baker, 1979), bacterioplankton transported within a deeper mixing layer (e.g. in winter) will be exposed to higher UVA/UVB ratios than cells retained in a strongly stratified and shallower layer (e.g. in summer), where they will have less chances for UVA-

or PAR-driven photorepair of DNA damage (Friedberg et al 1995). Thus, it seems that our static incubations led to an overexposure to UVB of organisms naturally inhabiting a well-mixed water column and thus acclimated to lower UVB doses, resulting in an enhanced UVB inhibition compared to UVA. This was supported by the fact that higher inhibition due to UVB was found with increasing in situ UVA/UVB ratios (data not shown, Fig. 4c) that is, when less UVB was present in the natural environment with respect to UVA and, moreover, when the larger was the difference between our incubation conditions and in the situ light regime (data not shown). UVB has often been regarded as the main contributor to bacterial damage (Aas *et al.*, 1996; Herndl *et al.*, 1993) but, in view of our results, it is possible that depending on the environmental characteristics of the samples, the use of artificial UV lamps or long surface incubations that neglect natural mixing effects might cause an artificial enrichment of such UVR waveband thus leading to unreal effects. All this points out the relevance of taking into account vertical mixing and the past light-exposure history of samples for an accurate interpretation of results, and further highlights the difficulty of mimicking natural light conditions.

Although in situ PAR levels did not generally increase bacterial activity rates, incubation of samples under an artificial PAR light (equivalent to the annual mean surface irradiance in this area) led to a general stimulation of activity in comparison to the dark control. However, such an effect was more pronounced during spring than in summer, when no changes or even inhibition occurred instead. These variable effects among different samples receiving exactly the same amount of light discarded an exclusive dependence of bacterial responses on light intensity and pointed to a major role of community structure in explaining the observed light-driven variability. Such a light stimulation of bulk LIR has been also reported by several studies where the results were usually consistent with light-enhanced amino acid uptake by cyanobacteria (Church *et al.*, 2004; Michelou *et al.*, 2007; Zubkov *et al.*, 2004). Conversely, our MAR-CARD-FISH data (Fig. 6c) discarded this group as the main drivers of the observed responses due to the low numbers of active cells and the lack of significant light-enhancement in these numbers concomitant with LIR increases. Bacterial groups other than cyanobacteria have also been shown to augment their activity under the light (Alonso-Sáez *et al.*, 2006; Mary *et al.*, 2008) and such an enhancement has been related to the photoheterotrophic capabilities of some phylotypes containing light-harvesting proteorhodopsins or bacteriochlorophyll *a* (Béjà,

2002; Béjà *et al.*, 2000; Cottrell *et al.*, 2006; Gómez-Consarnau *et al.*, 2007; Kolber *et al.*, 2000). However, still no direct evidence is available of this kind of photoheterotrophic enhancement of amino acid uptake by bacteria. Therefore, to check whether any non-photosynthetic bacterial group was responsible for the observed increases in LIR, probes for *Gammaproteobacteria*, *Roseobacter* and the NOR5 group were selected on the basis of previously published data from this area that indicated that these groups occasionally presented light-enhanced activity (Alonso-Sáez *et al.*, 2006, **Chapter 2.2**). SAR11 and *Bacteroidetes* were not considered due to the reported negative sensitivity to light of the former and the latter's preference for high molecular weight DOM and low numbers of cells active in ^{3}H-leucine uptake (Alonso-Sáez *et al.*, 2006, **Chapter 2.2**)

While the great majority of *Roseobacter* cells were active in both light and dark treatments throughout the year, increases in the number of active cells due to light were often negligible, so it is unlikely that they account for the observed light-stimulated LIR. Conversely, the numbers of active *Gammaproteobacteria* cells significantly increased in the light during most of the study period, showing a stimulation pattern very similar to that of bulk LIR (o community LIR). Even the observed LIR decrease in August was mirrored by a decrease in active *Gammaproteobacteria*, thus pointing to a major role of this group in the community response to light. This role, remarkable because *Gammaproteobacteria* were not the most abundant prokaryotes, was further supported by the good positive correlations observed between the number of active cells within this group and both the number of active *Eubacteria* and the bulk LIR rates. Some members of the *Gammaproteobacteria* apparently maintain high levels of ribosomes during extended periods of non-growth, which would allow these bacteria to rapidly initiate growth at changing environmental conditions (Eilers *et al.*, 2000a; Pernthaler *et al.*, 2001). Thus, they might also take fast advantage of changes in light conditions and react faster than other groups.

Interestingly, the *Gammaproteobacteria* showed a preference for highly illuminated environments as seen by the good correlation between the seasonal irradiance and cell abundances, not seen for other variables. This is in accordance with their apparent ability to benefit from light and it is further supported by the relatively high resistance to UVR reported for *Gammaproteobacteria* from the NW Mediterranean Sea (Agogué *et al.*, 2005; Alonso-Sáez *et al.*, 2006, **Chapter 2.2**)

Within *Gammaproteobacteria*, the NOR5 clade was analysed in detail because one sequenced member of the group has shown capability for aerobic anoxygenic photosynthesis (Fuchs *et al.*, 2007). Unfortunately, their very low abundances throughout most of the year prevented an accurate activity quantification. However, in a parallel study in the Blanes Bay we found that their ^{3}H-leucine uptake was occasionally stimulated by natural PAR radiation (**Chapter 2.1**). On May 26th 2009, when they comprised up to 90% of all *Gammaproteobacteria*, they might indeed have driven the light response of the whole group.

Despite these observations, we could not unequivocally determine whether the observed light increase in bulk ^{3}H-leucine uptake was solely the result of bacterial photoheterotrophy. A rapid response of bacteria to photosynthate leaks from phytoplankton upon light exposure could also account for such an increase of activity. *Gammaproteobacteria* abundances seemed to follow the peaks in Chl *a*, and this group has sometimes been found associated to phytoplankton blooms (Buck and Pierce, 1989; Fandino *et al.*, 2001; Wichels *et al.*, 2004) as if rapidly responding to short-term variations in PP. However, no significant correlation was obtained between increases in bulk LIR or active *Gammaproteobacteria* numbers and Chl *a* concentrations or primary productivity rates. We did not estimate the rates of photosynthetically extracellular release (PER), which has been sometimes shown to increase due to abrupt changes in irradiance (Mague *et al.*, 1980; Wood *et al.*, 1992) and in this area may vary throughout the year independently from particulate PP (Alonso-Sáez *et al.*, 2008). Thus, a potential role of light-induced PER in light LIR increase could not be ruled out.

Only the history of previous light exposure seemed to partially explain the observed light effects on LIR. Interestingly, the higher the in situ maximal daily irradiance bacteria had been exposed to, the smaller was the increase caused by our artificial light source. In contrast, smaller or even negative effects of light were observed when the natural maximal irradiance was more similar to the experimental irradiance. In other words, the bigger the difference between the natural and the experimental light conditions, the greater the effects that we should expect. It is thus possible that overexposure of algae with respect to their previous in situ light conditions would have resulted in enhanced release of DOM, thus stimulating the activity of *Gammaproteobacteria*.

However, the excess of pPP due to the difference between the in situ and the artificial light conditions did not seem to explain the observed variability, although pPP and PER do not necessarily react equally to light-stress (Mague *et al.*, 1980). As an alternative hypothesis we can consider a photoheterotrophic response of *Gammaproteobacteria* by use of proteorhodopsins or bacteriochlorophyll *a* in high light conditions, specially in oligotrophic waters such as those of Blanes Bay, where strong year-round phosphorous limitation of LIR is known to occur (Pinhassi *et al.*, 2006). Such a strategy has been reported by Gómez-Consarnau *et al.* (2007) for a cultured proteorhodopsin-containing *Bacteroidetes* isolated from Blanes Bay, which indeed showed higher stimulation of growth upon low DOM concentrations; however, to date no field measurement has consistently supported this hypothesis. Alternatively, differences in DOM quality might also explain the differences in the bacterial responses. In the NW Mediterranean, Tedetti *et al.* (2009) reported a significant PAR enhancement of DOM bioavailability and bacterial activity in spring, whereas light exposure of DOM in summer caused inhibition of LIR (mainly due to UVA). We cannot test if this was the case in our study because we had no data of DOM phototransformations and bioavailability; nonetheless, the facts that the largest light-driven increases in LIR were observed in spring and that UVA was the main inhibitor of bacterial activity support this argumentation.

Finally, the heterotrophic uptake of DOM reported for many algae (Amblard, 1991; Neilson and Lewin, 1974) further complicates the picture. Significant numbers of the diatom *Chaetoceros* spp. labelled for ^{3}H-leucine were found in March 2009 microautoradiography filters (> 20 cells ml^{-1}, up to 80 cells ml^{-1}, details not shown), yet no differences were visually apparent between the numbers of active diatoms in light and dark bottles. Similarly, radiolabeled *Pseudonitzschia* spp. cells were often found, but again no obvious differences were apparent between the two treatments. Using a microautoradiographic approach like ours, Paerl (1991) unveiled that large phytoplankton (mainly diatoms) from different oceanic regions occasionally showed active incorporation of organic substrates, mainly during bloom events, which seemed to be the case in March 2009. Hence, it is possible that during some months eukaryotic algae contributed to the measured uptake of ^{3}H-leucine, although apparently not to the differences between both dark and light treatments.

In summary, the response of bacterial heterotrophic activity to light is certainly not only a function of irradiance levels but also depends on a number of other environmental (light-exposure history, mixing, nutrient status) or biological (e.g. community taxonomic composition, specific sensitivity of groups, recovery capacity, photoheterotrophic potential) factors. Given the importance of bacteria for carbon and energy fluxes and nutrient cycling in the pelagic ocean, the observed effects of light on bacterial activity may have ecosystem implications and should not be ignored. Although measurements for bacterial heterotrophic production are often performed in the dark, which clearly avoids algal stimulation and circumvents the problem of reproducing ambient light levels, marine planktonic communities are naturally exposed to varying radiation conditions and parallel light (including the full spectra) and dark incubations are recommended for realistic interpretations of results.

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Chapter 2.2

Seasonal patterns in the sensitivity to sunlight of bacterioplankton from Mediterranean surface coastal waters

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ABSTRACT

The sensitivity of coastal marine bacterioplankton (Blanes Bay Microbial Observatory, NW Mediterranean Sea) to natural sunlight was evaluated in five experiments done over a seasonal cycle. The short-term effect of natural sunlight quality on bacterial bulk and single-cell activity was assessed by exposing surface samples to different treatments: darkness, photosynthetically active radiation (PAR, 400-700 nm), PAR + ultraviolet A radiation (UVA), 320-700 nm, and full spectrum (280-700 nm). Exposure to sunlight generally inhibited bulk activities or damaged membrane integrity when PAR or UV doses were high (i.e., spring and summer experiments) and, in general, UVB (280-320 nm) accounted for most of the inhibition. Winter communities seemed the most sensitive per unit of radiation, but this was apparently caused by overexposure of samples due to our experimental approach. When assessing activity (^{3}H-leucine uptake) at the single-cell level by microautoradiography combined with catalyzed reporter deposition-fluorescence in situ hybridization of RNA (MAR-CARD-FISH), some groups such as *Roseobacter*, SAR11 and *Gammaproteobacteria* were more responsive in summer (e.g., PAR-stimulation in *Roseobacter* and UVR inhibition in SAR11 and *Gammaproteobacteria*). When assessed on a per photon basis, though, the inhibition per unit of radiation was generally higher in autumn or spring than in summer, pointing to a certain degree of photoadaptation of bacteria towards higher irradiances. Light exposure greatly enhanced *Synechococcus* heterotrophic uptake of ^{3}H-leucine and, independently of the radiation levels received, *Bacteroidetes* appeared to be highly resistant to UV radiation. Springtime photostimulation of *Gammaproteobacteria* could be explained by the occasional dominance of the NOR5 subgroup capable of enhanced activity upon light exposure, stressing the importance of the changes in community composition and the relative contribution to total activity of each group in the observed global responses to UVR. We conclude that radiation levels, previous light history, and taxonomic composition of the community are essential for understanding UVR effects on prokaryotic picoplankton.

INTRODUCTION

Marine plankton communities are naturally exposed to fluctuating radiation regimes in their environment. The photosynthetically active radiation (PAR, 400-700 nm) and ultraviolet radiation (UVR, 280-400 nm) levels reaching planktonic organisms vary throughout the year, mainly because of changes in solar zenith angle (Madronich, 1993), but also in cloud cover, water transparency, and the depth of the surface mixing layer. Marine bacteria are a major components of aquatic ecosystems and play a key role in biogeochemical processes (Azam *et al.*, 1983; Cotner and Biddanda, 2002). They are specially sensitive to solar radiation since they are too small for efficient protection by pigments (García-Pichel, 1994). Although the effects of UVR, and mainly UV-B (280-320 nm), on bacterial communities as a whole have been studied in the past two decades, very few studies have addressed the impact of UVR on in situ bacterial community composition and group-specific activities (Winter *et al.,* 2001; Alonso-Sáez *et al.*, 2006; Kataoka *et al.*, 2009). Most of them, moreover, analysed these effects within a particular period of time and none considered the responses of changing communities throughout seasons.

Since marine bacterial communities are known to show gradual changes in their taxonomic composition throughout the year (Murray *et al.*, 1998; Pinhassi and Hagström, 2000; Schauer *et al.*, 2003), and given that different bacterial groups may display different sensitivities to sunlight (Joux *et al.*, 1999; Arrieta *et al.*, 2000; Agogué *et al.,* 2005; Alonso-Sáez *et al.*, 2006; Kataoka *et al.*, 2009; Matallana-Surget *et al.*, 2009), it seems reasonable to expect seasonal changes in the responses to sunlight of changing bacterial phylotypes. So far, only Alonso-Sáez *et al.* (2006) have addressed this issue with samples from Blanes Bay (NW Mediterranean Sea) in two different seasons, spring and summer. This coastal area is characterized by a marked seasonality of water temperature and solar radiation typical of temperate zones that causes a strong stratification in summer and deep mixing in winter. Among the studied bacterial groups, they found that *Gammaproteobacteria* appeared to be more resistant to UVR in summer than in spring, and they suggested that selection for photoresistant species might occur towards the periods of higher radiation intensity, yet it remained untested if autumn or wintertime communities are more sensitive to UVR because of the much lower radiation doses reaching the water column.

No clear evidence has yet been posed to support that bacterioplankton are able to adapt to UVR. Despite the aforementioned interspecific variability in the sensitivity to UVR and in

the repair capabilities among marine bacterial taxa, many studies have revealed no differences between the sensitivity of bacteria from high-light and low-light environments (e.g., the surface layer vs. deeper waters, Agogué *et al.*, 2005; Bailey *et al.*, 1983; Hernández *et al.*, 2007; Herndl *et al.*, 1993; Xenopoulos and Schindler, 2003), suggesting the absence of adaptative strategies driven by differences in the light conditions. By contrast, photoadaptation in marine bacteria has been inferred from circumstantial evidences, e.g., an increase in the percentage of pigmented cells during UV exposure of estuarine bacteria (Thomson *et al.*, 1980), a greater recovery of bacterial production and respiration during the second day of exposure compared to the first day (Pakulski *et al.*, 1998), different UVB responses and recovery potential of bacterial isolates according to the irradiation levels of their native environments (Fernández-Zenoff et al., 2006), more sensitivity to UVR in deeper than surface bacterial communities (Joux et al., 2009), or lower reduction in bacterial diversity and enhanced dark recovery potential in bacterioneuston than in bacterioplankton (Santos *et al.*, 2010). Altogether, hence, there is no consensus about whether bacteria can efficiently adapt to or protect from UVR, and which mechanisms they use for that purpose.

We present here the results of five experiments (conducted in different seasons) designed to evaluate the short-term responses to sunlight of different marine bacterioplankton assemblages from Blanes Bay. Flow cytometry and bulk measurements of bacterial heterotrophic activity were used together with microautoradiography combined with catalyzed reporter deposition-fluorescence in situ hybridization (MAR-CARD-FISH) to detect sunlight-driven changes in the whole community, as well as in the uptake of radiolabelled leucine at the single-cell level of dominant bacterial groups. Our results report for the first time seasonally varying sensitivities to UVR of in situ dominating bacterial groups.

MATERIALS Y METHODS

Study area and sample collection. The study was done in the Blanes Bay Microbial Observatory, a shallow (20 m depth) oligotrophic coastal station in the NW Mediterranean Sea, located 800 m off the shore of Blanes, Spain (41º39.90'N, 2º48.03E). Surface samples (0.5 m depth) were collected with polycarbonate carboys and taken to the lab under dim light. Water was collected at dawn to avoid exposure to sunlight before the experiments. Chlorophyll *a* concentration was determined by filtering 150 ml of seawater on GF/F filters

(Whatman). The pigment was then extracted in acetone (90% v/v) in the dark at 4ºC for 24 h and fluorescence measured with a Turner Designs fluorometer. Underwater PAR and UVR profiles of the day prior to sampling were obtained with a PUV 2500 radiometer (Biospherical Instruments).

Experimental design. Experiments were carried out in five occasions corresponding to different seasons: 9 July 2008 (summer experiment 1, Sm1), 30 September 2008 (autumn experiment, Aut), 11 December 2008 (winter experiment, Win), 26 May 2009 (spring experiment, Spr), and 21 July 2009 (summer experiment 2, Sm2).

Briefly, 50 and 100-ml water samples were incubated for 4 h in UV-transparent quartz glass bottles under different light conditions. Bottles were exposed to the full sunlight spectrum (PAR+UVR), the full spectrum minus UVB (PAR+UVA, covered with one layer of the plastic foil Mylar-D of 150 μm thickness, 50% transmission at 325 nm), the full spectrum minus UVR (PAR only, wrapped with two layers of Ultraphan URUV colourless, 0.1 mm thickness, 50% transmission at 380 nm) or kept in the dark (wrapped with aluminium foil). Bottles were incubated 5 cm under the surface inside a black tank (200 litres) with running seawater to maintain the in situ temperature. In the spring experiment, the samples were placed below an optically neutral mesh that reduced surface irradiances by 40%, trying to simulate the average reduction naturally experienced by spring samples due to their movement within the mixing layer that sets up at that time of the year, and to avoid excessive damage due to the high UVR doses commonly recorded in spring.

Five quartz bottles were used for each treatment: three 100 ml replicates were used for flow cytometric measurements, ^{3}H-leucine incorporation and ectoenzyme activity analysis, and two 50 ml replicates were amended with radioactive ^{3}H-leucine for MAR-CARD-FISH incubations. Only in the experiment Sm1, we incubated two replicates for general parameters and just one for MAR-CARD-FISH**.**

Measurement and calculation of PAR and UVR doses. UVR and PAR radiation were continuously monitored throughout the incubations. A radiometer (Biospherical PUV 2500) was placed inside the incubation tanks, with the sensor covered by ca. 5 cm of water, and the downwelling cosine irradiance reaching the samples was recorded at a frequency of 5 s^{-1}. The wavelengths measured included 6 bands in the UV (305, 313, 320, 340, 380, and 395 nm, in units of mW cm^{-2} nm^{-1}) and one integrated band in the visible (PAR, in mmol

photons cm^{-2} s^{-1}). The mean spectral irradiance in the 6 UV bands was converted to mean UV-B and UV-A irradiance (mW cm^{-2}) by integrating over the spectrum (sum of trapezoids), between 305 – 320 nm and 320 – 395 nm respectively. Finally, the mean UVB, UVA and PAR irradiances were multiplied by the duration of the experiment to obtain the radiation doses (in kJ m^{-2} for UVB and UV-A, and E $m^{-2}$for PAR).

The previous light exposure history of the sampled microbial communities was calculated for comparison with the doses measured during incubations as described in **Chapter 2.1**. For that purpose, we combined the maximun irradiance values (average irradiance at noon ± 2 h) collected at the meteorological station Malgrat de Mar (Catalan Meteorological Service, www.meteo.cat) of the three days prior to sampling, the mixing layer depth, and the underwater attenuation of solar radiation. Further details on these calculations will be provided in Galí *et al.,* in prep.

Abundance of prokaryotes. Samples for enumeration of bacteria were preserved with 1% paraformaldehyde and 0.05% glutaraldehyde (final concentrations) and kept frozen at -80ºC until analysis with a FACSCalibur flow cytometer (Becton-Dickinson). Bacteria were stained with SybrGreen I (Molecular Probes, Eugene, Oreg.) and counted by their signature on the SSC versus FL1 (green fluorescence) plot (Gasol and del Giorgio, 2000). *Synechococcus* abundances were estimated from unstained samples, and abundances were quantified by their signature when plotting side scatter (SSC) vs. red fluorescence (FL3), and that one versus orange fluorescence (FL2)

CTC labelling. Aliquots of 0.5 ml were spiked with CTC (5-cyano-2,3-ditolyl tetrazolium chloride, 5 mM final conc., Polysciences) and incubated for 1.5 h in the dark at in situ temperature. When actively respiring prokaryotes reduce CTC, it turns into a red fluorescent formazan that is detectable by flow cytometry (Sieracki *et al*., 1999). The samples were immediately counted with the FACSCalibur flow cytometer. An additional aliquot fixed with paraformaldehyde (PFA) was used as a background control of CTC fluorescence on dead samples. CTC particles were identified by their signature when plotting light scatter versus FL3 (see Gasol and Arístegui, 2007). We used the FL2 (orange fluorescence) versus FL3 plot to differentiate the populations of photosynthetic microbes (*Synechococcus, Prochlorococcus,* and picoeukaryotes) from the CTC particles.

Nucleic acid double staining (NADS). We used SYBR green I (Molecular Probes, Eugene, Oreg) and propidium iodide (PI; Sigma Chemical Co.) for the double staining of nucleic acids as described by Gregori *et al.* (2001) and Falcioni *et al.* (2008). Samples were stained with 1:10,000 (vol/vol) SYBR green I and 10 g ml^{-1} PI commercial solutions and analysed by flow cytometry after 20 min of incubation in the dark. Plotting red (PI) versus green fluorescence (SYBR green I) allowed differentiation of 'live' cells (i.e., with undamaged membranes) from those considered 'dead' (with damaged or compromised membranes).

^{3}H-leucine incorporation rates (LIR). Bacterial heterotrophic activity was estimated before and after exposure to sunlight using the ^{3}H-leucine incorporation method described by Kirchman *et al.* (1985) modified as in Smith and Azam (1992). From each quartz bottle, three aliquots (1.2 ml) and one trichloroacetic acid-killed control were incubated with ^{3}H-leucine (40 nM final conc., 160 Ci $mmol^{-1}$) for 2 h in the dark at in situ temperature.

Ectoenzyme activity. For the determination of the activities of ectoenzymes (beta-glucosidase [βglu], aminopeptidase [AMA], and alkaline phosphatase [APA]), we used fluorogenic substrates and followed the method described by Hoppe (1983) modified as in Sala *et al.* (2010). In brief, each ectoenzyme activity was assayed by observing the release of fluorescence after the addition of the fluorogenic substrates: 4-MUF-beta-glucoside for βglu, 4-MUF-P-phosphate for APA and L-leucine-7-amido-4-methyl-coumarin for AMA. Substrates were added at saturating concentrations (100 μM final concentration) to 0.9 ml replicate subsamples and fluorescence was measured immediately after addition, and after a 1-3 h incubation. Fluorescence was read on a Shimadzu spectrofluorometer RF-540 at 365 nm excitation and 446 nm emission wavelengths. Increase of fluorescence units during the incubation time was converted into activity by preparing a standard curve with the end products of the reactions.

Microautoradiography combined with Catalyzed Reporter Deposition-Fluorescence In Situ Hybridization (MAR-CARD-FISH). For the analysis of single-cell bacterial activity, 50 ml of seawater were incubated under the different light treatments with added trace ^{3}H-leucine (0.5 nM final conc., 160 Ci $mmol^{-1}$) for 4 h. Controls killed with paraformaldehyde (PFA, 1% final conc.) were also run simultaneously with all live incubations. After exposure, live samples were fixed overnight with PFA at 4°C in the dark, gently filtered on 0.2 μm polycarbonate filters (GTTP, 25 mm diameter, Millipore), and the filters then hybridized by CARD-FISH as described in Pernthaler *et al.* (2002). We used the following

horseradish peroxidase (HRP)-probes: Eub338-II-III for *Eubacteria* (Amann *et al.*, 1990; Daims *et al.*, 1999), Gam42a for *Gammaproteobacteria* (Manz *et al.*, 1992), CF319 for clades belonging to the *Bacteroidetes* group (Manz *et al.*, 1996), Ros537 for the *Roseobacter* clade (Eilers *et al.*, 2001), SAR11-441R for the SAR11 cluster (Morris *et al.*, 2002), NOR5-730 for the NOR5 clade (Eilers *et al.*, 2000), and Syn405 for the cyanobacterial genus *Synechococcus* (West *et al.*, 2001). Before subjecting samples to microautoradiography, smaller pieces from each hybridized section were cut and stained with 4,6-diamidino-2-phenylindole (DAPI, 1 μg ml^{-1}) to estimate the relative abundance of each group. Between 500 and 800 DAPI-positive-cells were counted manually in an Olympus BX61 epifluorescence microscope within a minimum of 10 fields. For microautoradiography, we followed the protocol described in Alonso and Pernthaler (2005) modified as in Alonso-Sáez and Gasol (2007). The optimal exposure time was determined for each experiment an resulted in 3 days for the summer experiment 1, 5 days for the autumn and summer experiment 2, 17 days for the winter experiment and 2 days for the spring experiment. Slides were developed as described previously (Alonso-Sáez and Gasol, 2007), dried in a dessicator overnight, stained with DAPI (1 μg ml^{-1}) and counted manually in an Olympus BX61 epifluorescence microscope. Between 500 and 700 hybridized cells were counted manually within a minimum of 10 fields.

RESULTS

Background information and irradiance measurements. The surface water (0.5 m) characteristics at the sampling time differed among experiments and were typical for each season at Blanes Bay (Fig. 1, Table 1), with minimum temperatures in winter (14 ºC) and maximal in summer (23 and 20ºC in experiments Sm1 and 2, respectively). Chlorophyll *a* concentrations ranged from 0.13 (Sm2) to 0.58 μg l^{-1} (Spr), and the in situ bulk ^{3}H-leucine incorporation activities varied between 4.0 pmol l^{-1} h^{-1} in winter and 73.7 pmol l^{-1} h^{-1} in spring. Remarkably, bulk bacterial heterotrophic activity measured at the beginning of the spring experiment was the highest recorded for that year, and it closely followed the spring phytoplankton bloom that peaked at the beginning of May, only a few days before the set up of the spring experiment (Fig. 1). Water transparency varied slightly between samplings (Table 1). The diffuse attenuation coefficient for 320 nm (K_{d320}) measured during the day

prior to sampling ranged from 0.26 to 0.35, which represented a variability in 1% irradiance depth (i. e. the depth where 1% of surface UVR at 320 nm remains) between 13 and 17 m.

During the experiments, a PAR-UV radiometer installed in the centre of the incubation tank recorded irradiances at PAR and 6 bands within the UV region. Total UVR and PAR doses varied among experiments mainly due to seasonal variations in the solar angle, since all experiments were set up on clear days. As an example, the highest doses were observed on July 2008 and 2009 (both summer experiments) with cumulative UV-B exposure reaching 22.0 and 23.9 kJ m^{-2}, respectively, whereas in December 2008 samples received only 4.1 kJ m^{-2} throughout the whole incubation (Table 1). Spring values are the result of a 40% reduction by a neutral mesh, meaning that in situ surface doses were almost as high as the summer ones.

Experiment	Date (day/mo/yr)	Temp (ºC)	Chl*a* (µg l^{-1})	LIR (pM h^{-1})	K_{d320} (m^{-1})	UV-B (kJ m^{-2})	UV-A (kJ m^{-2})	PAR (E m^{-2})
Sm1	09/07/08	23.0	0.20	84.8 ± 2.1	0.35	22.0	419.4	20.6
Au	30/09/08	20.3	0.23	51.5 ± 2.0	0.34	11.4	262.2	13.3
Win	11/12/08	14.1	0.33	4.0 ± 0.5	0.35	4.1	135.7	8.0
Spr	26/05/09	16.8	0.58	73.7 ± 8.2	0.34	13.7	278.1	13.5
Sm2	21/07/09	20.3	0.13	18.5 ± 2.1	0.26	23.9	455.7	22.2

Table 1. Temperature, chlorophyll *a* concentration, and bacterial heterotrophic activity (measured as ^{3}H-leucine incorporation rates, LIR) in the in situ starting samples of each experiment, downwelling diffuse attenuation coefficients for UVR at 320 nm (K_{d320}) measured for the day prior to sampling, and integrated doses of PAR, UV-A and UV-B received by the samples during the experiments. Sm1: Summer experiment #1, Sm2: Summer experiment #2, Spr: Spring, Aut: Autumn, Win: Winter.

Effects of sunlight on prokaryote abundances. The picophytoplankton community was dominated by *Synechococcus*, and its abundance varied seasonally (Table 2), showing maximum values in summer and autumn. Heterotrophic bacterial numbers, on the contrary, remained more or less constant throughout the year. Spring and summer *Synechoccocus* abundances did not seem to be affected by sunlight exposure (Table 3), whereas their numbers were significantly reduced upon UV-A exposure in Aut and to a less extent in the Win experiments (23% and 5% decrease, respectively, Tukey's test, $p < 0.05$). For heterotrophic bacteria, instead, we only found a significant light-driven decrease of ca. 20% in experiment Sm1, the one receiving the highest radiation dose (Table 3).

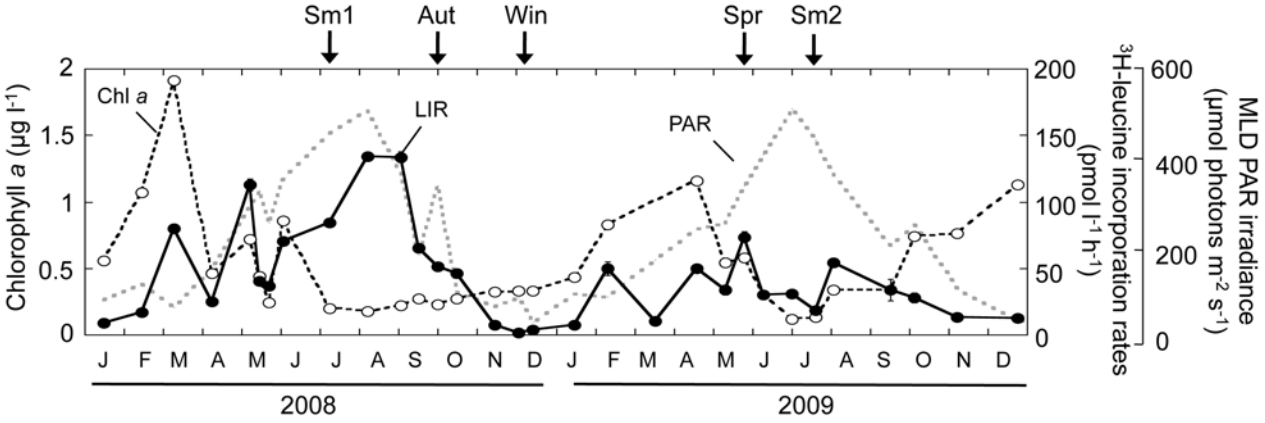

Fig. 1. Temporal dynamics of chlorophyll *a* (Chl *a,* open circles), ^{3}H-leucine incorporation rates (LIR, solid circles, average ± standard error) and mean PAR irradiance within the surface mixed layer depth (grey dashed line) during 2008 and 2009. Arrows indicate the timing of each of the experiments: Sm1 (summer exp. 1), Aut (autumn exp.), Win (winter exp.), Sp (spring exp.) and Sm2 (summer exp. 2).

Experiment	Date (day/mo/yr)	Bac (10^5 ml^{-1})	Syn (10^4 ml^{-1})	Fraction (%) of total DAPI counts						
				Eub	Gam	Bcdt	Sar11	Ros	NOR5	Syn
Sm1	09/07/08	7.8	3.6	88 ± 7	18 ± 4	15 ± 3	40 ± 1	2 ± 1	4.1 ± 1.7	1 ± 1
Aut	30/09/08	7.7	4.9	67 ± 7	8 ± 5	12 ± 5	40 ± 1	5 ± 3	2.4 ± 1.3	4 ± 4
Win	11/12/08	9.1	1.5	93 ± 4	5 ± 4	11 ± 3	30 ± 1	3 ± 4	0.3 ± 0.5	2 ± 2
Spr	26/05/09	8.4	0.9	82± 4	14 ± 3	20 ± 6	20 ± 1	10 ± 3	12.2 ± 2.6	2 ± 1
Sm2	21/07/09	9.2	6.2	87 ± 7	11 ± 4	8 ± 5	43 ± 6	2 ± 2	6.3 ± 3.2	7 ± 3

Table 2. Initial abundances of bacteria (Bac), *Synechococcus* (Syn), and bacterial assemblage structure described as percentages of hybridized cells with specific probes by CARD-FISH (over total DAPI-positive prokaryotes) measured at the beginning of each experiment. Eub, Eubacteria; Gam, *Gammaproteobacteria*; Bcdt, *Bacteroidetes*; SAR11; Ros, *Roseobacter*; NOR5; Syn, *Synechococcus*. CARD-FISH values represent means ± standard deviations.

The effect of sunlight on cell membrane integrity was further assessed by quantification of NADS green-positive cells (a surrogate for 'live' cells, see Falcioni et al. 2008). In all experiments, except for the autumn one, the number of cells with intact membranes was significantly reduced by UV exposure compared to dark controls (20% - 30% decline). Only in the spring experiment, PAR alone had a negative effect on membrane integrity accounting for half of the observed effect (ca. 15%, Table 3).

Experiment	Treatment	Bac abund. (10^5 ml^{-1})	Syn abund. (10^3 ml^{-1})	'Live' cells. (10^5 ml^{-1})	CTC$^+$ cells (10^4 ml^{-1})
Sm1	**DARK**	9.2 ± 0.7	11.9 ± 0.2	10.2 ± 0.1^a	12.0 ± 0.6
	PAR	8.7 ± 0.3	12.1 ± 0.1	10.9 ± 0.1^a	11.9 ± 0.02
	PAR+UVA	9.1 ± 0.3	12.8 ± 0.2	8.5 ± 0.3^a	10.7 ± 0.5
	PAR+UVR	8.2 ± 0.2	11.8 ± 0.4	8.1 ± 0.4^b	10.6 ± 0.04
Aut	**DARK**	7.5 ± 0.2	14.5 ± 0.2^a	8.1 ± 0.3	4.7 ± 0.0^a
	PAR	7.4 ± 0.3	14.4 ± 0.2^a	7.5 ± 0.2	3.0 ± 0.7^b
	PAR+UVA	7.1 ± 0.2	11.7 ± 0.1^b	7.6 ± 0.2	3.2 ± 1.4^b
	PAR+UVR	6.8 ± 0.2	11.1 ± 0.2^b	7.5 ± 0.2	2.8 ± 0.5^b
Win	**DARK**	8.8 ± 0.3	5.8 ± 0.1^a	7.3 ± 0.02^a	3.6 ± 0.8
	PAR	-	-	-	-
	PAR+UVA	9.0 ± 0.6	5.5 ± 0.01^b	6.4 ± 0.1^{ab}	4.0 ± 1.2
	PAR+UVR	10.0 ± 0.3	5.5 ± 0.02^b	5.8 ± 0.2^b	4.8 ± 0.4
Spr	**DARK**	14.3 ± 0.6	2.9 ± 0.01	20.6 ± 1.6^a	17.2 ± 11.0^{ab}
	PAR	12.7 ± 1.5	2.8 ± 0.1	17.6 ± 0.4^b	18.6 ± 1.0^a
	PAR+UVA	11.2 ± 1.4	2.7 ± 0.1	14.7 ± 0.2^c	15.4 ± 0.4^b
	PAR+UVR	11.4 ± 0.7	2.8 ± 0.1	14.6 ± 0.2^c	16.2 ± 0.9^{ab}
Sm2	**DARK**	12.1 ± 0.1^a	34.1 ± 0.2	5.1 ± 0.4^a	14.1 ± 4.2^a
	PAR	9.9 ± 0.1^b	33.4 ± 0.4	5.1 ± 0.02^a	8.2 ± 0.5^b
	PAR+UVA	9.9 ± 0.2^b	32.8 ± 0.5	4.7± 0.1^{ab}	7.2 ± 0.6^b
	PAR+UVR	10.1 ± 0.2^b	34.1 ± 0.1	4.3 ± 0.1^b	5.1 ± 0.1^b

Table 3. Cell abundances of bacteria (Bac), *Synechococcus* (Syn), cells with intact membranes ('live' cells, NADS green-positive cells) and actively respiring cells (CTC$^+$ cells) measured by flow cytometry after exposure to the different treatments. Values represent means ± standard errors. The PAR treatment is missing in the winter experiments. Letters refer to results of an ANOVA with a Tukey's post hoc test (p < 0.05). Different letters indicate significant differences among treatments.

Effect of solar radiation on bacterial metabolism. We did not observe any consistent effect of light on the number of actively respiring cells (CTC$^+$ cells, Table 3). Only in two experiments, Aut and Sm2, lower numbers of CTC$^+$ cells were found after light exposure compared to the dark control, and it seemed to be mainly caused by PAR. In contrast, exposure to full sunlight significantly inhibited bulk ^{3}H-leucine incorporation rates measured after the incubations (Tukey's test, p < 0.05, Fig. 2) compared to the dark treatments in autumn, winter, and in both summer experiments. Instead, the effect was not significant in spring, in spite of the substantial UV doses received by those samples. The response of bacteria to the different wavebands presented some variability. Thus, inhibition of bacterial activity due to PAR exposure was only significant in the summer experiments when PAR doses were highest. We did not detect significant differences between PAR and PAR+UV-A treatments in any of the experiments, indicating that UV-B alone was responsible for most of the observed

inhibition. However, no correlation was found between the degree of inhibition and the doses received during experiments and, although the highest UV levels were recorded in summer, a much stronger UVR-driven inhibition was found in winter (68% decrease compared to dark control) than in summer samples (36% and 45% decrease in Sm1 and Sm2, respectively).

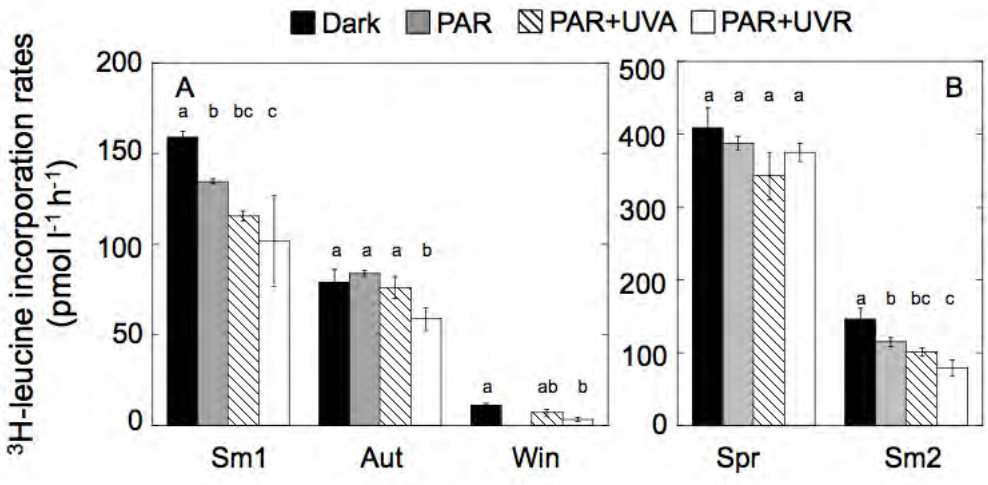

Fig. 2. Bulk bacterial activity measured as ^{3}H–leucine incorporation rates after 4 h exposure to different wavebands of natural sunlight or kept in the dark in the five experiments: summer (Sm1), autumn (Au) and winter (Win) of 2008 (A), and spring (Spr) and (Sm2) of 2009 (B). Bars represent means ± standard errors. Letters refer to results of an ANOVA followed by a post hoc Tukey's test ($p < 0.05$). Different letters indicate significant differences among treatments.

Activities of βglu, APA and AMA were analyzed immediately after 4 h of exposure to the different light conditions (Fig. 3). In general, exposure to the full sunlight spectrum caused the greatest inhibition of enzyme activities compared to PAR exposure, showing more intense effects in spring and summer. There seemed to be a tendency for higher activities after dark incubation except for βglu activity in experiment Sm1, where exposure to PAR caused a ca. 60% stimulation of this enzyme. A similar trend was found in the spring experiment, although these differences were not statistically significant at the level of $p < 0.05$. On the contrary, PAR radiation in the Sm2 experiment was the major contributor to βglu inhibition, and, whereas UVA was responsible for most of the inhibition of AMA activity in the spring experiment, the inclusion of UV-B caused an increase in its activity compared to the PAR+UV-A treatment (Fig. 3).

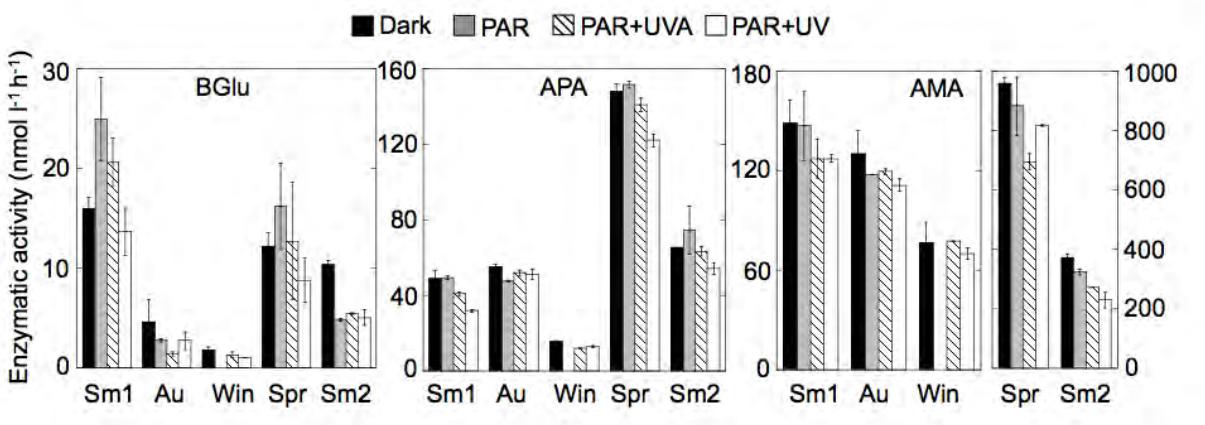

Fig. 3. Activities of the ectoenzyme β-glucosidase (βGlu), alkaline phosphatase (APA) and aminopeptidase measured after exposure to the different light treatments or kept in the dark (average ± standard deviation).

In spite of the low UVR levels in winter, the percentage of inhibition on a per photon basis of most of the measured parameters was higher in winter samples (Fig. 4). When we compared the UV-B doses received by winter samples with their in situ UVB levels (calculated as the mean UV-B irradiance measured within the mixing layer during the 4 h of maximal irradiance of the 3 days prior to the sampling, see Methods), we found that they had been overexposed (Fig. 4, dashed line). Only the actively respiring cells (CTC^+ cells) and the AMA activity showed no significant reduction in winter after full sunlight exposure compared to dark incubation. For the rest of the experiments, instead, the degree of inhibition per radiation unit seemed to be quite comparable among all the different parameters. For this comparison, we only considered the absolute inhibition values when significant differences were found between full sunlight and dark treatments; when differences were not significant, the percentage of change compared to the dark control was assigned a value of zero (following Pakulski et al. 2007).

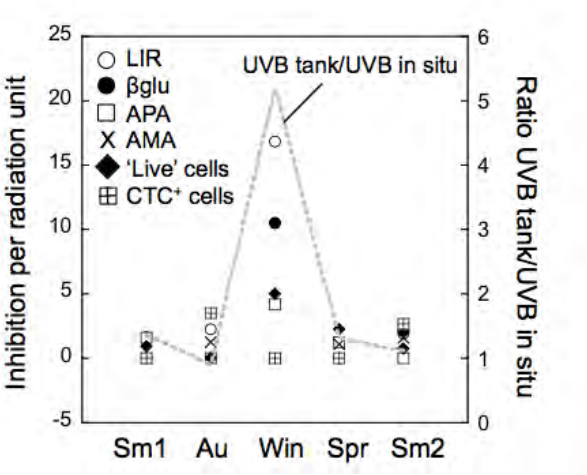

Fig. 4. Percentage of full sunlight-driven inhibition of different parameters relative to UVB doses. [LIR] ^{3}H-leucine incorporation rates; [βglu] β-glucosidase, [APA] alkaline phosphatase and [AMA] aminopeptidase activities. Dashed line behind shows the ratio between the UVB doses measured during incubation and the UVB doses naturally experienced by samples within the surface mixing layer depth during the 4 hours of maximal irradiance of the 3 days prior to the sampling (i.e. UVB in situ, see Methods).

Differential sensitivity to light of the dominant bacterial phylotypes. The seasonal differences in the sensitivity to light of distinct bacterial groups were assessed by applying the MAR-CARD-FISH technique. Hybridization with specific probes showed that the relative abundances of the studied groups varied among experiments (Table 2), although the alphaproteobacterial clade SAR11 was always the most abundant one, accounting for 20 - 54% of the total DAPI counts. *Bacteroidetes* and *Gammaproteobacteria* showed variable contributions depending on the season (range: 8% - 20 % and 5% - 18%, respectively) whereas *Roseobacter*, *Synechococcus* and the NOR5 clade always remained below 13%. The number of cells of each group active in the uptake of ^{3}H-leucine varied among treatments and depended on the studied season. In accordance to the lower levels of bacterial heterotrophic activity measured in winter samples, most of the groups were much less active in this experiment, with weakly labelled cells (i.e., much smaller silver grain areas).

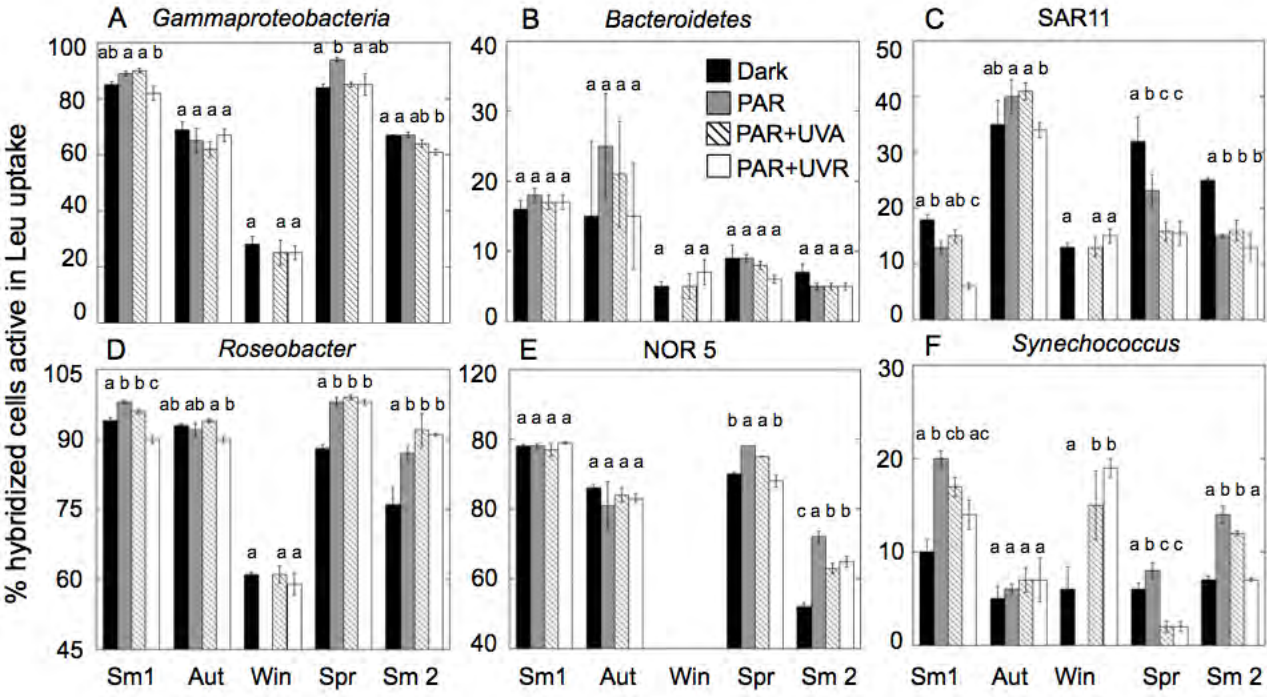

Fig. 5. Percentage of positively hybridized cells with probes for *Gammaproteobacteria* (a), *Bacteroidetes* (b), SAR11 (c), *Roseobacter* (d), NOR5 (e) and *Synechococcus* (f) taking up ^{3}H-leucine (average ± standard error of duplicates) as measured by MAR-CARD-FISH after exposure to each treatment in the five experiments. PAR treatment is missing in the winter experiment, and NOR5 cells from the winter experiment could not be counted due to low abundances. Note that the Y axes show different scales. Letters refer to results with a post hoc Tukey's test ($p < 0.05$). Different letters indicate significant differences among treatments.

Members of the *Gammaproteobacteria* appeared to be moderately resistant to solar radiation (Fig. 5a), showing no significant effects due to light except in the spring and both summer experiments, where inclusion of UV-B led to a reduction of 34%, 8% and 9% of the percentages of active cells compared to PAR treatment, respectively. Remarkably, *Gammaproteobacteria* from spring showed a significant 12% increase in the number of labelled cells after PAR exposure that was not observed in the rest of experiments.

No significant light effects were observed for *Bacteroidetes* in any of the experiments (Fig. 5b), although this group was never very active in the uptake of the amino acid. The great error bars in the autumn experiment were due to the presence of aggregates where members of this group were abundant and much more active. Except for that experiment, *Bacteroidetes* always presented numbers of labelled cells < 20%.

We also tested the sensitivity of four more specific groups: SAR11 and *Roseobacter* within *Alphaproteobacteria*, the gammaproteobacterial clade NOR5, and the photosynthetic cyanobacterium *Synechococcus*. Members of the SAR11 clade (Fig. 5c) showed a consistent strong inhibition after full sunlight exposure compared to dark controls in the experiments with the highest doses of UV-B (64%, 52% and 48% reduction in percentage of active cells in the uptake of ^{3}H-leucine in Sm1, Spr and Sm2, respectively) although the pattern was not the same for all: whereas in experiment Sm1 exposure to UVR was responsible for most of the inhibition, in experiments Spr and Sm2, it seemed to be mainly caused by UV-A and PAR treatments, respectively. *Roseobacter* showed no sensitivity to UVR except for a 8% reduction in experiment Sm1 caused by UV-B as compared to the PAR treatment, whereas cells from Spr and Sm2 samples appeared to be stimulated with all light incubations (Fig. 5d). However, this stimulation was never higher than 12% because the members of this group were already highly active. In autumn and winter, on the contrary, they did not show this light-driven stimulation.

Within *Gammaproteobacteria*, the NOR5 group (Fig. 5e) showed no responses to light in the experiments Sm1 and Aut, while a significant light-enhancement was apparent in spring and Sm2 experiments (9% and 38% increase with respect to the dark control, respectively). Interestingly, this group comprised up to 90% of all spring *Gammaproteobacteria,* which also showed such a PAR-driven stimulation. Active NOR5 cells from the winter samples could not be quantified due to their very low abundances.

The heterotrophic activity of *Synechococcus* was also assessed by MAR-CARD-FISH (Fig. 5f), and the number of active cells clearly increased after PAR exposure compared to dark treatments in all experiments except in autumn. No data on PAR response are available for winter samples, although it seems obvious to assume some kind of PAR-driven stimulation given that the rest of light treatments also induced such an increase. A significant reduction in activity was observed after full sunlight exposure compared to PAR treatment in the spring and both summer experiments, showing 80%, 70% and 53% inhibition in the number of active cells, respectively. However, in experiment Spr the decline seemed to be entirely caused by UV-A radiation, as we did not find significant differences between both UV treatments.

In general, none of the groups appeared to be directly responding to light levels, since no correlations were found between the degree of change in activity and the doses received. Only *Synechococcus* showed significant higher numbers of active cells with increasing PAR doses (Spearman's rho = 0.96, $p < 0.00001$), although since winter PAR samples are missing, this behaviour is not conclusive.

Although the absolute light-driven changes in the number of active cells were generally greater during the periods of higher radiation intensity (summer), the inhibition per unit of solar radiation was generally lower during this season compared to autumn and spring, pointing to a relatively lower resistance of these communities naturally adapted to less intense irradiances.

Contribution of each group to substrate-assimilating cells relative to their abundances. The contribution of the studied groups to the total number of cells assimilating ^{3}H-leucine was calculated from the fraction of active cells in the group and its abundance (with respect to total eubacteria) relative to the percentage of total eubacteria active in ^{3}H-leucine uptake. Figure 6 shows the comparison between the relative contribution of each group to the total number of active cells measured after dark and full sunlight treatments, to address the effect of UVR in the role of the different bacteria. In general, group contributions to total active cells varied among experiments, with SAR11 and *Gammaproteobacteria* generally being the main contributors. *Roseobacter* and NOR5 accounted for an important share of active cells especially in spring, whereas the contribution of *Bacteroidetes* and *Synechococcus* always remained below 8% and 4%, respectively. Interestingly, exposure to full sunlight reduced the contribution of SAR11 and, consequently, the large contribution of both *Roseobacter* and *Gammaproteobacteria* in spring was further increased after exposure

(46% and 31% increase, respectively, with NOR5 accounting for the very most of the increase due to *Gammaproteobacteria*).

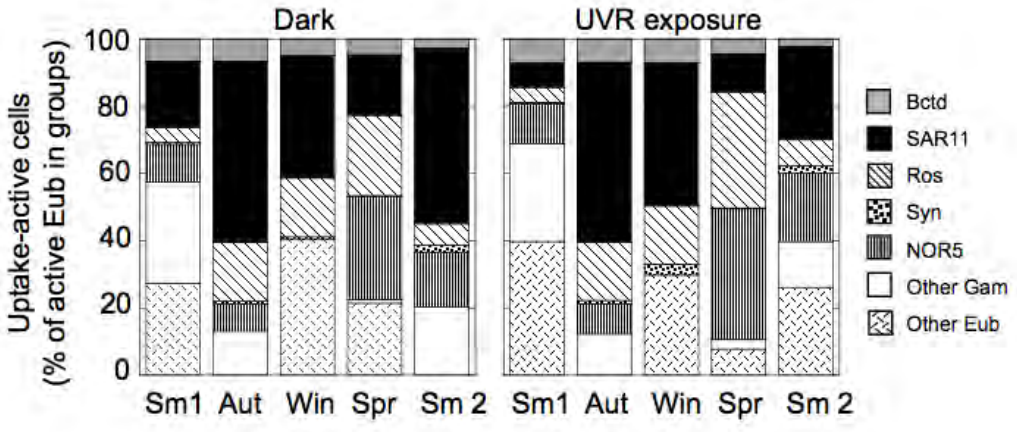

Fig. 6. Seasonal variation of the contribution of the different bacterial groups to total number of active *Eubacteria* [Eub] after dark incubation (A) or full sunlight exposure (B). *Gammaproteobacteria* [Gam], *Bacteroidetes* [Bcdt], SAR11, *Roseobacter* [Ros], NOR5 and [Syn] *Synechococcus*. Note that the relative contribution to total active cells after full sunlight exposure in spring was largely dominated by *Roseobacter* and NOR5, two groups that were highly resistant or even stimulated by light treatments.

DISCUSSION

The Bay of Blanes is a highly transparent shallow coastal area where UVR can easily reach the bottom at least during the spring and summer months (Sommaruga *et al.*, 2005). To evaluate the short-term responses of seasonally different communities to in situ radiation levels, we made five experiments from July 2008 to July 2009. The water characteristics varied among experiments (Fig. 1 and Table 1) according to the expected seasonal changes in the study area, changing from a totally mixed water column during winter and early spring to a summer shallow mixing layer due to warmer surface waters.

Exposure to 4 hours of natural sunlight resulted in a significant decrease in bacterial numbers only in the summer experiment Sm2; the one where the highest radiation doses and the lowest Chl *a* concentration were recorded. Short-term reductions in bacterial numbers have been reported elsewhere (Pakulski *et al.*, 1998; Alonso-Sáez *et al.*, 2006), although, in

general, longer incubations are needed to observe such effect (Müller-Niklas *et al.*, 1995; Pakulski *et al.*, 1998; Santos *et al.*, 2010). Despite the general lack of effects on bacterial abundances, we observed a consistent decline in the number of cells with intact membranes in most experiments caused mainly by UVR exposure, thus indicating that natural light levels were indeed damaging cells. This contrasts with the results of Alonso-Sáez *et al.* (2006) in experiments in summer and spring where they found most bacteria maintaining membrane integrity after light exposure; instead, Maranger *et al.* (2002), using a different exclusion stain (TOPRO-1) found significant increases in the number of damaged cells from lake samples after UVR exposure, yet they exposed samples for a few days.

Decreased *Synechococcus* abundances after UVR exposure were observed, but only in autumn and to a less extent in winter samples. Sommaruga *et al.* (2005) evidenced for the first time a high resistance of *Synechococcus* in this area, showing no decrease in either cell abundance or cell-specific fluorescence upon sunlight exposure. However, those experiments were done in summer and agree with our summer results; whereas the higher sensitivity observed in autumn and winter *Synechococcus* might imply a selection for spring and summertime phylotypes with increasing UVR-resistance. UVR has been shown to directly cause cell death among picophytoplankton communities from different ecosystems (Llabrés and Agustí, 2006; Llabrés *et al.*, 2010), and although in general, *Synechococcus* has shown higher resistance than *Prochlorococcus* or picoeukaryotes, their sensitivity to light seems to vary depending on the location of origin, depth and time of the year, maybe indicating changing phylotypes with different resistance capabilities. The *Synechococcus* from our experiments appeared to be all the same as checked by PCR-DGGE with cyanobacterial primers (B. Díez, unpubl. data), thus pointing to physiological acclimation rather than succession towards photoresistant strains.

Bacterial activity seemed to be more clearly affected by exposure to solar radiation, showing a general decrease in the bulk bacterial incorporation of ^{3}H-leucine and ectoenzyme activities (both indirect measurements of DOM processing). In contrast, the number of respiring cells (CTC^{+} cells) was only significantly reduced in the autumn and Sm2 experiments. Similar to the findings of other authors (Herndl *et al.*, 1993; Helbling *et al.*, 1995; Müller-Niklas *et al.*, 1995; Jeffrey *et al.*, 1996; Santos *et al.*, 2010; Sommaruga *et al.*, 1997; Wilhelm and Smith, 2000), inclusion of UV-B remarkably inhibited bulk ^{3}H-leucine incorporation compared to dark controls in all the experiments, but in spring, in spite of the substantial UVR doses received by spring samples. Remarkably, the highest Chl *a* concentration was

found in spring, and phytoplankton themselves and DOM released by algae can serve as sunscreens for bacteria due to absorption and scattering of UVR (Aas *et al.*, 1996; Joux *et al.* 2009; Sommaruga *et al.*, 1997). It is plausible, thus, that the higher productivity of the spring waters could have relieved bacterial UVR stress. However, Alonso-Sáez *et al.* (2006) tested whether the removal of phytoplankton was a relevant factor influencing bacterial sensitivity to UVR in Blanes Bay, and found no relevant effects.

UV-A did not seem to have any significant impact on LIR, and PAR alone was only responsible for some inhibition in summer, when PAR levels were highest. Other studies have reported an important (or even dominant) inhibitory effect of PAR and UVA on bacterial activity (Aas *et al.*, 1996; Sommaruga *et al.*, 1997; Pakulski *et al.*, 2007) that, instead of occurring through direct molecular damage as in the case of UVB, is believed to act indirectly through the formation of reactive oxygen species or free radicals that can interact with DNA or other cellular components (Harrison, 1967; Mitchell, 1995). In any case, current information on the effects of different portions of the solar spectrum on bacterial heterotrophic activity is not conclusive, and it is possible that distinct bacterial populations with different susceptibilities to UV such as those observed from our MAR-CARD-FISH data (see below), as well as sunlight effects on phytoplankton and DOM all interact to produce the observed effect.

Bacterial extracellular enzymatic activity is thought to be a major agent in processing and cleaving DOM (Chróst, 1991). Of the three studied enzymes, βGlu and AMA (Chróst, 1991, 1992) are considered mainly of bacterial origin, whereas APA can also be produced by algae and zooplankton (Cembella *et al.*, 1985; Myklestad and Sakshaug, 1983). Ectoenzyme activities fell within the range previously measured at the Blanes Bay Microbial Observatory sampling site (Alonso-Sáez *et al.*, 2008), and varied depending on the time of the year. Exposure to sunlight generally inhibited total ectoenzyme activities compared to dark incubations, but some variability was measured in the responses to the different wavebands among experiments. Similar to the results of other authors (Herndl *et al.,* 1993; Müller-Niklas *et al.*, 1995; Garde and Gustavson, 1999; Espeland and Wetzel, 2001; Santos *et al.,* 2010), exposure to UV-B significantly decreased total enzyme activities in some cases, while UV-A and PAR were responsible for significant inhibition in others. Moreover, in experiments Sm1 and Spr, exposure to PAR increased βGlu activity compared to the dark control. Extracellular enzymes can be inactivated in natural waters either directly (through direct damage by UVB absorption) or indirectly by a mechanism involving UV-A whereby photoreduced Fe binds with the enzyme and inactivates it upon oxidization by H_2O_2 (Scully *et al.*, 2003). Likewise,

the increased βGlu activity after PAR could be related to some evidence that suggests that the complexed Fe can be photoreduced by PAR (Emmenegger *et al.*, 2001) allowing Fe-bound enzymes to be reactivated in the absence of UVR. Additionally, light driven changes in DOM availability due to photosyntate release, photoalteration of DOM or even cell death (Herndl *et al.*, 1997; Benner and Biddanda, 1998; Obernosterer *et al.*, 1999; Pausz and Herndl, 1999; Marañón *et al.*, 2004; Llabrés and Agustí, 2006) might indirectly decrease or enhance enzyme activities. Although the observed decrease in the number of cells with intact membranes could have been associated with the release of cell metabolites into the dissolved pool, we could not find any correlation between the number of damaged cells and the ectoenzyme responses.

A less clear pattern was found for the number of actively respiring cells; only those from the experiments Aut and Sm2 seemed to be significantly inhibited by light exposure, with PAR accounting for most of the inhibition. Alonso-Sáez *et al.* (2006) found such a reduction in the number of CTC^+ cells due to sunlight (mainly UVB), but their results were only from summer experiments.

Although no significant correlation was found between sunlight doses and the degree of inhibition of the abovementioned activity measurements, in general, higher inhibition due to UVR occurred in the summer experiments, the ones with the highest irradiances. However, for the case of ^{3}H-leucine incorporation, the greatest decrease was found in winter. Indeed, when we considered the absolute values of bulk measurements inhibited by full sunlight exposure relative to the total doses of UV-B received during each experiment, we found higher inhibition per unit of radiation in winter in all the cases, but in the number of CTC^+ cells and the AMA activity (which were not significantly reduced upon UV-B exposure). In the rest of the experiments, despite the higher UVR doses, inhibition per unit of radiation was much lower. This might suggest that winter bacteria were more sensitive to in situ UVR levels than the rest of the assemblages. Joux *et al.* (1999) showed that several isolated bacteria accumulate DNA damage when exposed to UV-B doses < ~ 1 kJ m^{-2}, so the dose received by the winter sample (4 kJ m^{-2}) was high enough to potentially damage bacteria. In our case, however, a different reason could be invoked for the greater UVR-sensitivity found in winter. When we compared the doses measured inside the tank with the ones naturally occurring in the environment the bacteria were sampled from, it was evident that winter samples were exposed to up to 5-fold more UV-B than the in situ levels. Water column mixing in winter prevents cells from being damaged (Jeffrey *et al.*, 1996), and our 4 h incubation right under the surface caused sample overexposure to sunlight and, consequently, stronger negative effects than those expected

to occur naturally. This highlights the importance and the difficulty of mimicking real light conditions, and further stresses the relevance of the past light exposure history of samples for an accurate interpretation of the results.

Application of MAR-CARD-FISH revealed that the sunlight levels bacteria were exposed in autumn and winter experiments were in general too low to decrease the numbers of cells actively taking up ^{3}H-leucine, with the exception of SAR11 in experiment Aut. Nearly all groups exhibited inhibition of activity due to UV-B in the summer experiments and, unlike the bulk bacterial activity measurements, most of the phylotypes from the spring experiment clearly responded to sunlight exposure. Using the same experimental design, Alonso-Sáez et al. (2006) found some variability in the photosensitivity of *Gammaproteobacteria* from Blanes Bay between spring and summer, and suggested that selection for photoresistant species might occur in periods of higher solar radiation intensity, although no data from autumn or winter assemblages were available for comparison. Their observed pattern of sunlight stimulation of *Roseobacter* and strong inhibition of SAR11 members (Alonso-Sáez *et al.* 2006) was also found here, and appeared to be more or less constant throughout the year, but more pronounced during spring and summer. The low proportions of active *Bacteroidetes* cells throughout the year were accompanied by a lack of significant responses to light. Members of the *Gammaproteobacteria* appeared to be highly resistant to UVR, but, unlike Alonso-Sáez *et al.* (2006), who found that this group showed lower sensitivity to UVR in summer than in spring, we observed a PAR-induced increase in the number of active *Gammaproteobacteria* in spring and a slight but significant UV-B-driven inhibition in the two summer experiments. Therefore, when considering the absolute proportions of active cells, our results do not support the hypothesis of selection for photoresistant strains with increasing radiation levels, because most groups showed higher inhibition in spring and summer. However, when the same data were re-calculated as percentage of change from dark or PAR values with respect to the doses received, there was a tendency for greater changes in the percentages of active cells in autumn than in spring, and in spring than in summer. This hints to the existence of a radiative threshold. Thus, while winter levels are too low to cause a response in the activity of the different groups, once irradiance is strong enough, it seems that upon the same radiation intensity, summer assemblages were more resistant to UVR probably due to acclimation to higher irradiances.

These seasonal differences in the responses of bacteria might indicate the occurrence of different phylotypes within the probed broad phylogenetic groups all through the year, as reported for the same sampling site by Schauer *et al.* (2003). For instance, the PAR-driven increase in the number of active *Gammaproteobacteria* cells in spring might be explained by the appearance of different gammaproteobacterial taxa associated to the phytoplankton bloom that occurred just before the experiment. Indeed, that year's maximum number of *Gammaproteobacteria* was recorded together with the maximum in Chl *a* concentration, and when this group was exposed to an artificial PAR source (**Chapter 2.1**), they were repeatedly stimulated in their uptake of ^{3}H-leucine, probably indicating that they can rapidly use dissolved organic compounds produced by phytoplankton. In addition, some members of this group have been shown to be capable of aerobic anoxygenic photosynthesis (NOR5, Fuchs *et al.*, 2007) and several studies have associated this lineage with different phytoplankton populations (Eilers *et al.*, 2001; Alonso-Sáez *et al.*, 2007b). Spring samples hybridized with the NOR5 probe showed much higher numbers compared with the rest of the experiments, with this clade accounting for up to 90% of the total *Gammaproteobacteria*, and, remarkably, they also presented this pattern of photostimulation of activity in both Spr and Sm2 experiments. The cultured representative characterized by Fuchs *et al.* (2007) showed an enhanced cell yield with artificial PAR light, although these experiments were preliminary. Thus, it seems that the spring enhancement in the activity of *Gammaproteobacteria* after exposure to PAR was driven by the increase in the numbers of the NOR5 clade and their stimulation by light. Consistently, in experiment Sm2, the lower contribution of NOR5 to *Gammaproteobacteria* resulted in the absence of such stimulation.

Similarly, some members of the *Roseobacter* group are known to contain bacteriochlorophyll *a* (Shiba *et al.,* 1979; Shiba, 1991; Allgaier *et al.*, 2003) and this factor, or alternatively the presence of the widespread proteorhodopsin (Béjà *et al.*, 2000), could be the reason for the light-driven stimulation of the number of active cells. However, to date no study has demonstrated any increase in ^{3}H-leucine uptake caused by light exposure in any of the proteorhodopsin or bacteriochlorophyll *a*-containing isolates. Again, a rapid response of *Roseobacter* to the release of photosynthate in the presence of light could be an additional explanation for the observed increases in active cells. Actually, *Roseobacter* cells have been reported to occur in association with phytoplankton blooms in the North Atlantic (González *et al.*, 2000; Buchan *et al.*, 2005; Alonso-Sáez *et al.*, 2007a) and in Blanes Bay (Alonso-Sáez

et al., 2007b; **Chapter 2.1**). Therefore, if rates of photosynthetically extracellular release by phytoplankton increase with higher irradiances (Alonso-Sáez *et al.*, 2008), activity of bacteria such as *Roseobacter* or NOR5 might be stimulated. Further experiments with isolates are needed for understanding the relative roles of direct and indirect effects of light on specific activities.

The cyanobacteria *Synechococcus* showed a consistent stimulation of the number of active cells caused by sunlight exposure; only spring cells were strongly inhibited by UVR to percentages lower than those of the dark control. Contrary to previous reports on the relative resistance of *Synechococcus* to UVR in terms of cell counts, fluorescence, or mortality (Sommaruga *et al.*, 2005; Llabrés and Agustí, 2006; Llabrés *et al.*, 2010), our results show that their heterotrophic activity is largely stimulated by PAR, but generally inhibited by UV-A and UV-B radiation. Uptake of amino acids by cyanobacteria has been shown to be stimulated by light exposure, as was the case of *Prochlorococcus* in the North Pacific Ocean (Church *et al.*, 2006) and the central Atlantic Ocean (Mary *et al.*, 2008) and of a freshwater *Synechococcus* isolate (Chen *et al.*, 1991). To our knowledge, this is the first evidence for UVR effects on the heterotrophic activity of this widely distributed marine cyanobacteria.

Looking at the results altogether, decreases in the number of prokaryotic active cells were generally mostly attributable to UV-B, although in some cases UV-A or even PAR were responsible for most of the inhibition (i.e., SAR11 in Spr and Sm2, respectively). Besides its potential to cause damage, UVA radiation is also involved in photorepair mechanisms (Sancar and Sancar, 1988). Environmental characteristics such as vertical mixing or water transparency influence the relative exposures to UV-B versus longer wavelengths, and hence, the balance between damage and repair (Herndl *et al.*, 1997; Kaiser and Herndl, 1997). As stated above, different phylotypes differ not only in their sensitivities to UVR, but also in their recovery capabilities (Agogué *et al.*, 2005) and even show preferences for different types of repair (e.g., photo-enzymatic vs. dark repair, Arrieta *et al.*, 2000)><urls><related . Hence, changes in the quality of solar radiation might have caused some of the observed variability in the responses to the different wavelengths among and within bacterial groups.

It is remarkable the lack of effects in the spring bulk ^{3}H-leucine incorporation in spite of the clear responses found at the single-cell level for some of the studied groups. However, when we calculated the contribution of each phylogenetic group to the total number of ^{3}H-leucine assimilating cells, variations in these contributions did explain some of the bulk

responses. In general, SAR11 and *Gammaproteobacteria* were responsible for the largest share to the total numbers of active cells, with variable roles depending on the seasons. Only in the spring experiment, the lower abundances of SAR11, together with the increase in the numbers of *Roseobacter* and NOR5, resulted in a great contribution of these latter two clades that was even greater under full sunlight exposure. The lack of inhibition of bulk community ^{3}H-leucine incorporation in spring could be explained by a compensation of the strong inhibition of SAR11 cells with stimulation or higher resistance of NOR5 and *Roseobacter* cells. This is an example of patterns observed at the community level being driven by identifiable behaviors at the level of taxonomic groups.

One main difference exists between the methodology used in this study and that used by Alonso-Sáez *et al.* (2006). They exposed the samples for 4 h and measured the number of active cells afterwards by using dark incubations of 4 h, so they were measuring the result of the effects of radiation after exposure as they hold for a while in the dark. Kaiser and Herndl (1997) had shown that 3 h of darkness are enough for bacteria to recover the activity levels previous to UV-B exposure (fig 2a). We thus wanted to avoid this possibility by exposing the organisms together with the added ^{3}H-leucine. This approach allows a more realistic estimation of the in situ incorporation rates because irradiation and uptake processes are not separated in space and time. However, it also carries its own uncertainties. First, caution has been called for a potential photodegradation of leucine by UVR (Sommaruga *et al.*, 1997), as photolysis and photoalteration of recalcitrant organic molecules have been demonstrated (De Haan, 1993). We did some tests where we measured dark ^{3}H-leucine incorporation rates with radiotracer that had been irradiated under natural or artificial UVR, and found no differences from incubations performed with non-irradiated ^{3}H-leucine (data not shown), and in accordance with a recent study that discards a negative effect of UVR on the integrity of this compound (Vaughan *et al.,* 2010). Second, addition of the radiotracer at the very beginning of the light exposure allows microbes to start taking it up before their activity becomes progressively inhibited by the cumulative UVR dose. Should this 'early labeling' of cells occur, it will tend to reduce the magnitude of the detrimental effects of UVR. This may explain why the inhibitory effects observed by Alonso-Sáez *et al.* (2006) using post-exposure dark incubation were sometimes greater than the ones we measured.

In summary, our results confirm that sunlight, and particularly UVR, is an important driver of the seasonal variations in microbial heterotrophic carbon processing in natural waters. Its effects, however, are far from simple, and depend not only on the physics of

the water column and its optical characteristics, but also –and this is often overlooked- on the taxonomic composition of the community. In addition, the apparent high sensitivity to UVR of winter bacteria drew attention onto the importance of the previous light history of the plankton community for the outcome of the light-manipulation experiments. Studying the dynamical influence of sunlight on plankton with realistic manipulation experiments represents a formidable challenge.

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Chapter 3

Sunlight effects on the DMSP and leucine assimilation activities of polar heterotrophic bacterioplankton

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ABSTRACT

The influence of solar ultraviolet radiation (UVR) and photosynthetically active radiation (PAR) on summertime bacterial uptake and assimilation of sulfur from radiolabelled dimethlysulfoniopropionate (^{35}S-DMSP) was studied in Arctic and Antarctic surface waters. Parallel incubations with ^{3}H-leucine were also conducted for comparative purposes as a measurement of bacterial uptake for protein synthesis. Arctic waters were characterized by large numbers of colonial *Phaeocystis pouchetii* and higher DMSP concentrations than in the diatom-dominated Antarctic waters. Exposure to full sunlight radiation (280-700 nm), and to a lesser extent to PAR+UVA (320-700 nm), generally decreased the bacterial assimilation of ^{3}H-leucine with respect to darkness, and caused variable yet less inhibitory effects on ^{35}S-DMSP assimilation. We used a single-cell approach involving microautoradiography and RNA hybridization to identify the bacterial groups responsible for the ^{35}S-DMSP and ^{3}H-leucine uptake as well as their sensitivity to UVR. High percentages of sulfur-assimilating cells were recorded for all the studied phylogenetic groups (*Gammaproteobacteria, Bacteroidetes,* SAR11 and *Roseobacter*) despite the different DMSP concentrations between Arctic and Antarctic waters. The dominant SAR11 clade contributed 50-70% of the cells assimilating both substrates in the Arctic stations, whereas either *Gammaproteobacteria* or SAR11 were the largest contributors to active cells in the Antarctic stations. PAR+UVA repeatedly increased the number of SAR11 cells assimilating ^{3}H-leucine, a pattern that also occurred, yet more occasionally, with ^{35}S-DMSP-assimilating SAR11, *Bacteroidetes* and *Roseobacter* cells. Our results support a widespread capability of polar bacteria to assimilate DMSP-sulfur during the season of maximum DMSP concentrations, and for the first time show that all major taxa (including *Bacteroidetes*) can be highly active at this assimilation under the appropriate circumstances. Our data also indicate that the bacterial use of leucine is more sensitive to UVR than the use of DMSP-sulfur, yet this sensitivity occurs more through a decrease in assimilation rates than through a decrease in active cell numbers. This study suggests that incubations under realistic sunlight conditions are needed if we are to quantify actual substrate uptake and assimilation rates by natural (either bulk or taxon-resolved) bacterioplankton assemblages.

INTRODUCTION

Dimethylsulfoniopropionate (DMSP) is a ubiquitous sulfur compound produced by many phytoplankton taxa (Keller *et al.*, 1989) as an intracellular osmolyte (Kirst 1996), although other functions such as cryoprotectant or antioxidant have been also suggested (Karsten *et al.*, 1996; Sunda *et al.*, 2002). Once released into the dissolved pool mainly through grazing, viral lysis or phytoplankton autolysis (Simó, 2001), DMSP becomes a significant carbon source and a major source of reduced sulfur for marine bacteria (Howard *et al.*, 2006; Kiene *et al.*, 2000; Kiene *et al.*, 1999; Simó *et al.*, 2002; Tang and Simó, 2003; Vila-Costa *et al.*, 2007) herbivore protozoans (Burkill *et al.*, 2002; Saló *et al.*, 2009; Tang and Simó, 2003) and even low-DMSP producing phytoplankton (Vila-Costa *et al.*, 2006).

Algal DMSP production varies both among species (Keller *et al.*, 1989) and within species depending on environmental conditions (Stefels *et al.*, 2007). One of the strongest DMSP producers is the haptophyte *Phaeocystis* spp. (see refs. in Liss *et al.*, 1994) which is known to form massive blooms causing elevated concentrations of this compound either in the particulate or the dissolved forms. In polar waters, summer *Phaeocystis* blooms triggered by ice-melting stratification are accompanied with very high DMSP levels (Curran and Jones, 2000; Ditullio *et al.* 2003; Matrai and Vernet, 1997). Within marine bacteria, significant assimilation of DMSP-sulfur has been observed in all major taxonomic groups, although not all cells uniformly assimilate it (Malmstrom *et al.*, 2004a; Malmstrom *et al.*, 2004b; Vila *et al.*, 2004; Vila-Costa *et al.*, 2007). This widespread use of DMSP is consistent with its suggested role as a source of reduced sulfur for protein synthesis, energetically advantageous with respect to the assimilative sulfate reduction chain (Kiene et al. 1999). It has been suggested, though, that more than the taxonomic composition of the bacterial assemblage, it is the contribution of DMSP to available reduced sulfur in the ecosystem that controls its assimilation (Pinhassi *et al.*, 2005; Vila-Costa *et al.*, 2007). Therefore, given that large DMSP production rates occur in polar waters during summer, it is of interest to examine whether this is reflected in the sulfur-assimilation activity and taxonomic composition of the bacterial assemblages. To date, the only study focusing on the identity of polar bacteria taking up DMSP was carried out during the Arctic winter and early spring, where low numbers of active bacteria were found associated with very low DMSP concentrations (Vila-Costa *et al.*, 2008).

Among the environmental factors with a high potential to regulate the availability of DMSP to bacteria and its uptake and assimilation, there is ultraviolet radiation (UVR, 280-

400 nm). Exposure to UVR has been found to either increase (Slezak and Herndl, 2003; Sunda *et al.*, 2002) or reduce (Sakka *et al.*, 1997) the cellular DMSP content of marine algae. Whether UVR hampers or enhances the DMSP production machinery is, therefore, uncertain, but a recent study with cultures of the haptophyte *Emiliania huxleyi* reported the UVR-enhanced release of DMSP and its cleavage product dimethylsulfide (DMS) (Archer *et al.* 2010). Besides potentially influencing the release of DMSP by phytoplankton, high doses of solar radiation can moreover inhibit the growth and activity of marine bacteria (Aas *et al.*, 1996; Herndl *et al.*, 1993), and there is direct evidence that they can decrease DMSP consumption (Slezak *et al.*, 2001; Slezak *et al.*, 2007).

All this, together with the reported variable sensitivities to UVR within bacterial and phytoplankton species (Agogué *et al.*, 2005; Arrieta *et al.*, 2000; Hernández *et al.*, 2006; Joux *et al.*, 1999; Neale *et al.*, 1998), and the fact that polar summers are characterized by long days of almost continuous light, points to an important role of natural solar radiation in the assimilation of DMSP-sulfur by polar bacterial taxa.

UV radiation studies in the polar regions have attracted increasing attention since ozone depletion (Jones and Shanklin, 1995; Müller *et al.*, 1997) and the ongoing thinning of the ice covers in both the Arctic (Johannessen *et al.*, 1999; Rothrock *et al.*, 1999) and some regions of Antarctica (Rignot *et al.*, 2008) are leading to a higher UVR penetration in the water column. However, although some information is available about the impact of UVR on bacterial communities or isolates from the Arctic (Wickham and Carstens, 1998) and Antarctica (Booth *et al.*, 2001; Buma *et al.*, 2001a; Davidson and van der Heijden, 2000; Helbling *et al.*, 1995; Hernández *et al.*, 2006), to our knowledge no studies have examined the effects of natural solar radiation on the single-cell activities of different polar bacterial groups.

To that end we used an experimental single-cell approach combining CARD-FISH (catalyzed reporter deposition-fluorescence in situ hybridization of RNA) for bacterial identification with microautoradiography for quantification of the active cells. This was applied to natural bacterial assemblages in Arctic and Antarctic waters during summer cruises. With the aim at deciphering the bacterial taxa responsible for DMSP-sulfur, radiolabeled (^{35}S) DMSP was used as a substrate. ^{3}H-leucine was used in parallel for comparative purposes since it is considered one of the most universal substrates for marine bacterioplankton and its assimilation is used to estimate protein synthesis (Kirchman *et al.*, 1985). The effect of

natural sunlight (and specially UVB, the most harmful fraction within UVR and the one affected by changes in ozone concentration) on substrate assimilation was further assessed by exposing samples under different light conditions. Our starting hypotheses were that (a) the bacterial assemblages in summer polar waters would be adapted to an efficient use of highly available DMSP as a source of reduced sulfur, and (b) that uptake of labile dissolved organic compounds, sulfur assimilation and protein synthesis by bacteria would be dependent on the spectrum of solar radiation, with implications for bacterial activity in situ in response to changes in surface irradiance, mixing, and light penetration.

MATERIALS AND METHODS

Study area, sampling and basic parameters. The study was carried out on board RV Hespérides during the ATOS I (Arctic) and II (Antarctica) cruises. In July 2007, ATOS I visited the Atlantic sector of the Arctic with a transect from Iceland, parallel to the eastern Greenland current, up to the ice cap edge (ca. 81ºN) located north/northwest of Svalbard. In February 2009, ATOS II cruised around the Antarctic Peninsula, from the Weddell Sea (65ºS) through the Bransfield Strait and into the Bellinghausen Sea (ca. 69ºS). Samples were collected at 5 m depth (except for station AN2, which was sampled at 20 m depth) with a rosette of Niskin bottles mounted on a CTD profiler (Seabird SBE 911). Water characteristics of the sampled stations together with the irradiance measurements and time of incubation during experiments are shown in Table 1.

Arctic	Stn	Date (day/mo/yr)	Longitude	Latitude	Sampling depth (m)	SW temp (ºC)	DMSPt:Chl *a* (nmol μg^{-1})	Incub. time(h)	UVB (kJ m^{-2})
	AR3	05/07/07	1º 39.82'W	77º 23.23'N	5	3.22	31	12	9.2
	AR4	07/07/07	2º58.49'W	78º43.72'N	5	2.15	42	11.5	4.6
	AR5	12/07/07	10º 11.44'E	80º 13.99'N	5	0.15	167	11.8	7.4
	AR7	19/07/07	13º 14.22' E	80º 49.57'N	5	0.21	21	9.5	4.5
Antarctica									
	AN1	03/02/09	55º 45.43' W	65º 01.17'S	5	-0.17	2	7.6	9.1
	AN2	06/02/09	57º 14.42' W	62º 10.63'S	20	1.67	17	8	1.2

Table 1. Characteristics of the sampling stations, total DMSP to chlorophyll *a* ratio, time of exposure and integrated UVB irradiances during deck board incubations.

DMSP analysis. We could not reliably measure dissolved DMSP concentrations because even low volume drip filtration (Kiene and Slezak 2006) seemed to break *Phaeocystis* sp. colonies and enrich the dissolved fraction with intracellular DMSP. Only total DMSP concentrations (DMSPt, Table 1) were measured instead. Water samples were collected directly from the Niskin bottles into glass vials (120 ml) avoiding bubbling. Subsamples of 3-5 ml were syringe-filtered through GF/F into glass vials, and analyzed for DMS by purging, cryotrapping, and sulfur-specific gas chromatography followed by flame photometry as described (Simó *et al.*, 1996). Aliquots of 40 ml of the original sample were stored in crimp glass vials with two added pellets of NaOH (45 mg each), which hydrolyzed all DMSP into DMS. DMSPt was determined in 0.2-1 ml subsamples the following day as the evolved minus the pre-existing DMS.

Bacterial abundance. In situ bacterial abundances were analyzed by flow cytometry. Samples of 1.2 ml were preserved with 1% paraformaldehyde and 0.05% glutaraldehyde (final concentrations) and kept frozen at -80ºC until analysis with a Becton-Dickinson FACSCalibur flow cytometer after staining with SybrGreen I (Molecular Probes, Eugene, Oreg.) at 10x. Regions were stablished on the SSC versus FL1 (green fluorescence) plot to discriminate cells with high nucleic acid content (HNA) from cells with low nucleic acid content (LNA), and cell abundance was determined for each subgroup (Gasol and Del Giorgio, 2000).

Experimental design. Six experiments were performed to assess the impact of natural sunlight on the heterotrophic activity of polar bacterial assemblages. The substrates used were chemically synthesized and purified ^{35}S-DMSP, which was kindly donated by Ronald P. Kiene (University of South Alabama, Dauphin Island Sea Laboratory, USA), and ^{3}H-leucine (Amersham).

Briefly, water samples were incubated in 50 ml UV-transparent quartz bottles with the addition of trace concentrations of ^{35}S-DMSP or ^{3}H-leucine under different light conditions. The bottles were exposed either to the full sunlight spectrum (PAR+UVR), the full spectrum without UVB (i.e., PAR+UVA, covered with the plastic foil Mylar-D that excludes only UVB radiation) or kept in the dark (wrapped with aluminum foil and a black plastic bag). The samples were incubated on deck inside a black tank with running seawater to maintain the in situ temperature. To simulate the irradiance level of 5 m, samples were placed 5 cm under the surface below an optically neutral mesh that reduced surface irradiances by 40%, which was approximately the reduction naturally occurring at 5 m. Samples from station AN2 (20 m depth) were covered with a double neutral mesh.

Radiation measurements. UVR and PAR irradiances were continuously monitored throughout the incubations by using a Biospherical PUV-radiometer 2500 installed inside the incubation tank. Downwelling cosine irradiance was measured at a frequency of 5 s^{-1} in six bands in the UV region (305, 315, 320, 340, 380, 395 nm). The mean spectral irradiance during the incubation was calculated for each UV band, and the total energy received in the UVB region was computed as the integral of mean spectral irradiance over a given spectral interval and time. UVB measured irradiances during experiments are shown in Table 1.

^{3}H-leucine incorporation rates (LIR). Bacterial heterotrophic activities was estimated before and after exposure to sunlight using the ^{3}H-leucine method described by Kirchman *et al.* (1985) modified as in Smith and Azam (1992). From each quartz bottle, six aliquots (1.2 ml) plus two trichloroacetic acid-killed controls were incubated with ^{3}H-leucine (40 nM final conc., 160 Ci $mmol^{-1}$) for 2 to 3 h in the dark at in situ temperature.

Trace isotope assimilation during incubations. Samples of 50 ml were incubated for 7 to 12 h in quartz bottles with added trace concentrations of ^{35}S-DMSP (845 Ci $mmol^{-1}$, 0.8 pM final conc. for Arctic samples and 120-145 Ci $mmol^{-1}$, 2.5-3 pM final conc. for Antarctic samples) or ^{3}H-leucine (161 Ci $mmol^{-1}$, 0.5 nM final conc.). Killed controls were prepared in 30 ml Teflon flasks by adding paraformaldehide (PFA, 1% final conc.) before the addition of the radioisotope. After exposure, the incorporation of substrate was stopped by fixing samples overnight with PFA (1% final conc.) at 4°C in the dark. Triplicate aliquots (previously filtered through 5 μm pore-sized filters to exclude the larger organisms) were filtered through 0.2 μm pore-sized filters (GNWP, Millipore) and rinsed with 15 ml of 0.2 μm-filtered seawater. Macromolecules were precipitated by treating filters with 5 ml of cold TCA 5% for 5 min. The filters were then rinsed three times with milliQ water and their radioactivity determined by placing them into 5 ml of scintillation cocktail (Optimal HiSafe) and counting them with a Beckman scintillation counter. Incorporation of ^{35}S-DMSP and ^{3}H-leucine in PFA-killed controls was always < 1.5 % of that in live samples.

MAR-CARD-FISH (microautoradiography combined with catalyzed reporter deposition-fluorescence in situ hybridization). Samples of 50 ml were incubated under the different light treatments with added radioactive ^{35}S-DMSP (845 Ci $mmol^{-1}$, 0.04 nM final conc. for Arctic samples and 145 Ci $mmol^{-1}$, 0.03 nM final conc. for Antarctic samples) or ^{3}H-leucine (161 Ci $mmol^{-1}$, 0.5 nM final conc.) for 7 to 12 hours. PFA-killed controls were run simultaneously with all live incubations. Microautoradiography of

^{35}S-DMSP samples from station AN2 could not be performed due to insufficient amount of the isotope left for a visible signal.

After sunlight exposure, live samples were fixed overnight with PFA (1% final conc.) at 4°C in the dark. Aliquots of 10-15 ml were first filtered through 5 μm polycarbonate filters (Osmonics, inc.) for identification of the particle-attached bacteria and subsequently filtered through 0.22 μm polycarbonate filters (GTTP, 25 mm diameter, Millipore), rinsed with milliQ, air dried and stored at -20°C until processing. Hybridizations were made following the CARD-FISH protocol (Pernthaler *et al.* 2002). We used a suite of six horseradish peroxidase (HRP)-probes to characterize the composition of the bacterial community (particle-attached and free-living bacteria) in the water samples: Eub338-II-III for most *Eubacteria* (Amann et al. 1990; Daims et al. 1999), Gam42a for most *Gammaproteobacteria* (Manz et al. 1992), CF319 for many clades belonging to the *Bacteroidetes* group (Manz et al. 1996), Ros537 for the *Roseobacter* clade (Eilers et al. 2001) and SAR11-441R for the SAR11 cluster (Morris et al. 2002). Cells were first permeabilized with lysozyme (37°C, 1h) and achromopeptidase (37°C, 0.5 h) and hybridizations were carried out at 35°C overnight. Counterstaining of CARD-FISH filters was done with 4,6-diamidino-2-phenylindole (DAPI, 1μg ml^{-1}) and a minimum of 10 fields (500-800 DAPI-stained cells) were manually counted in an Olympus BX61 epifluorescence microscope.

For microautoradiography, we essentially followed the protocol described in Alonso and Pernthaler (2005) modified as in Vila-Costa *et al.* (2008). Only 0.22 μm filters were used, which were developed after 6 days of exposure for ^{3}H-leucine and 18 days for ^{35}S-DMSP in Arctic samples, or after 5 days for ^{3}H-leucine and 2 months for ^{35}S-DMSP in Antarctic samples. Filters were then stained with DAPI and between 500 and 700 hybridized cells were counted within a minimum of 10 fields.

RESULTS

Phytoplankton biomass in the Arctic stations was generally dominated by *Phaeocystis pouchetti,* an haptophyte that forms large colonies and produces high concentrations of DMSP. In the Antarctic stations, conversely, the highest abundances corresponded to apparently non-DMSP producing diatoms such as *Thalassiosira* spp. (station AN1) or *Pseudonitzschia* spp. (station AN2). As a consequence, much higher DMSPt to chlorophyll *a* ratios (DMSPt:Chl *a,* an indicator of the occurrence of DMSP-producers within algal assemblages) were found in the Arctic (31-173 nmol μg^{-1}) than in the Antarctic waters (2-15 nmol μg^{-1}; Table 1).

Prokaryotic abundances ranged between 0.2 x 10^6 cells ml^{-1} in Antarctic stations to up to 2 x 10^6 cells ml^{-1} in station AR4 (Table 2). Instead, the highest bacterial leucine incorporation rate was recorded at station AN1 (~180 pmol leucine l^{-1} h^{-1}) with the other stations presenting lower values (~30 to 90 pmol leucine l^{-1} h^{-1}; Table 3).

Exposure to natural sunlight reduced post-exposure (measured in the dark for 2 to 3 hours after light incubation) leucine incorporation (Table 3), but this reduction was only significant (Tukey's test, $p < 0.05$) in comparison to the dark treatment when UVB was included. Only in station AN1 a significant inhibition was also present in samples exposed to PAR+UVA. Similarly, the trace ^{3}H-leucine assimilated during incubations by organisms 0.22- 5 μm (mainly prokaryotes; Fig. 1b,) declined by 18% (station AR4) to 85% (station AN1) upon exposure to full sunlight radiation conditions. No significant differences were found between dark and PAR+UVA treatments except in stations AR7 and AN1, where PAR+UVA accounted for most of the observed decrease. Samples from AR3 and AR5 from the Arctic and AN1 from Antarctica received the highest UVB doses during incubations (7 to 9 kJ m^{-2}, Table 1) and a positive correlation was found between the inhibition of trace ^{3}H-leucine assimilation due to UVB exposure (with respect to PAR+UVA incubation) and the UVB doses measured during each experiment (Spearman's $r = 0.81$, $p < 0.05$, $n = 6$). In contrast, no correlation was apparent between UVB doses and the degree of inhibition of the bacterial leucine incorporation measured after exposure.

A		Fraction (%) of total DAPI counts detected with CARD-FISH probe in 0.22 µm filters				
Station	BA (10^6 ml^{-1})	Eub338-II-III	Gam42a	CF319a	Sar11	Ros537
AR3	0.9	96 ± 5	6 ± 3	7 ± 4	68 ± 3	5 ± 4
AR4	2.0	95 ± 7	3 ± 2	10 ± 4	64 ± 10	5 ± 2
AR5	0.8	94 ± 6	6 ± 4	2 ± 2	61 ± 7	3 ± 2
AR6	1.1	92 ± 4	6 ± 3	5 ± 4	71 ± 6	3 ± 2
AN1	0.2	93 ± 3	27 ± 6	42 ± 7	20 ± 4	2 ± 2
AN2	0.2	95 ± 5	19 ± 6	18 ± 4	48 ± 7	4 ± 3

B		Fraction (%) of total DAPI counts detected with CARD-FISH probe in 5µm filters			
Station	BA (10^6 ml^{-1})	Gam42a	CF319a	Sar11	Ros537
AR3	0.03	-	94 ± 7	-	-
AR4	0.02	-	95 ± 8	-	-
AR5	0.02	-	94 ± 6	-	-
AR6	0.02	-	95 ± 4	-	-
AN1	0.02	35 ± 12	22 ± 12	2 ± 2	2 ± 3
AN2	0.04	11 ± 6	40 ± 14	3 ± 2	2 ± 2

C

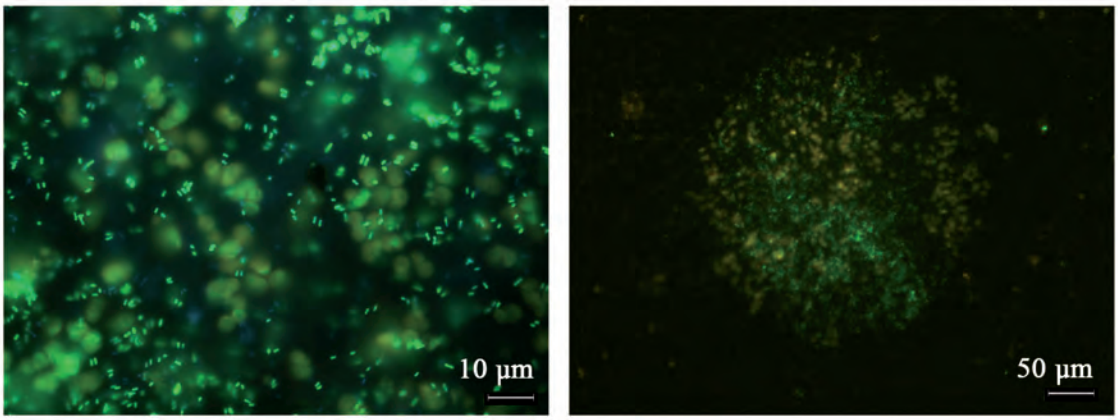

Table 2. (A) Initial abundances of bacteria (BA) and free-living (0.2 - 5 µm) bacterial assemblage structure described as percentage of hybridized cells with specific probes by CARD-FISH at the beginning of each experiment. (B) Particle-attached (> 5 µm) bacterial assemblage described by CARD-FISH at the beginning of the experiments. Eub, Eubacteria (EUB338-II-III); Gam, *Gammaproteobacteria* (Gam42a); Bcdt, *Bacteroidetes* (CF319a); SAR11, SAR11 cluster (SAR11-441R); Ros, *Roseobacter* (Ros537). CARD-FISH values represent means ± standard deviations. (C) Epifluorescence images of *Bacteroidetes* cells (hybridized with the CF319a probe) associated to colonies of *Phaeocystis pouchetii* on 5 µm filters. Left panel: green *Bacteroidetes* and orange *Phaeocystis cells*. Scale bar represents 10µm. Right panel: view of a whole colony of the flagellate where the green fluorescence of hybridized *Bacteroidetes* is visible.

The recorded percentages of assimilation of added ^{35}S-DMSP (0.2-2.3%; Fig. 1a) were lower than those of ^{3}H-leucine, and showed a variable behavior with regard to light, with no significant differences among light treatments in 3 stations (AR4, AR5 and AN2), significant increase by PAR+UVA in station AR7 and significant inhibition by UVB (station AN1) or by both light treatments (station AR7). No significant correlation was found between inhibition and UVB doses.

'Free living' (0.22-5μm) and 'particle-attached' (> 5 μm) bacterial assemblages were characterized by CARD-FISH. Most prokaryotic cells hybridized with the eubacterial probe EUB338-II-III (92-96% of DAPI counts). Hybridization with specific probes showed that the free living Arctic bacterial communities were largely dominated by the SAR11 clade (*Alphaproteobacteria*), which accounted for 61-71% of total DAPI counts (Table 2) whereas the rest of the groups showed much lower percentages. Conversely, Antarctic stations presented higher numbers of *Gammaproteobacteria* and *Bacteroidetes* and less SAR11 cells, and interestingly, *Bacteroidetes* dominated the community at station AN1, associated with the extremely high chlorophyll concentration (~20 μg l^{-1}) of a dense diatom bloom.

	Bacterial heterotrophic activity (pmol ^{3}H-leucine l^{-1} h^{-1})					
	Stn. AR3	Stn. AR4	Stn. AR5	Stn. AR7	Stn. AN1	Stn. AN2
Initial	55 ± 2	77 ± 1	92 ± 2	80 ± 20	182 ± 24	31 ± 1
DARK	93 ± 3^{a}	107 ± 7^{a}	162 ± 16^{a}	160 ± 13^{a}	227 ± 30^{a}	34 ± 7^{a}
PAR+UVA	84 ± 13^{ab}	93 ± 4^{a}	161 ± 21^{a}	152 ± 10^{ab}	137 ± 18^{b}	26 ± 3^{ab}
PAR+UVR	72 ± 4^{b}	71 ± 4^{b}	155 ± 9^{a}	138 ± 9^{b}	119 ± 11^{b}	23 ± 3^{b}

Table 3. Bulk bacterial activity measured as ^{3}H–leucine incorporation rates before and after exposure to the following radiation conditions: PAR+UVA, PAR+UVR and darkness. Values are means ± standard deviations. Letters refer to results with a post hoc Tukey's test ($p < 0.05$). Different letters indicate significant differences among treatments.

As for the bacteria retained in the 5 μm fraction, they were almost entirely comprised by members of the *Bacteroidetes* cluster in the *Phaeocystis*-dominated Arctic stations (Table 2b). Microautoradiographic examination of integral *Phaeocystis* colonies revealed that *Bacteroidetes* accounted for almost all DAPI counts within the colony mucus, and very high numbers were also found associated with other particles. The particle-attached bacteria from the 2 Antarctic stations were less group-specific, but still appeared to be enriched in *Gammaproteobacteria* and *Bacteroidetes* whereas barely any other group was found.

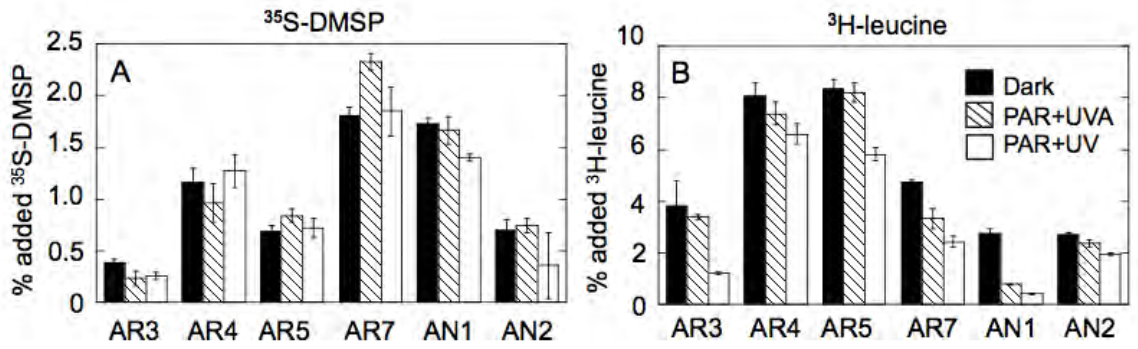

Fig. 1. Percentages of assimilated ^{35}S-DMSP (A) and ^{3}H-leucine (B) by organisms 0.2 - 5 µm during exposure to the following radiation conditions: PAR+UVA (dashed bars), PAR+UV (white bars) and darkness (black bars). Values are averages ± standard errors.

	% of hybridized *Eubacteria* active in ^{35}S-DMSP uptake					
	Stn. AR3	Stn. AR4	Stn. AR5	Stn. AR7	Stn. AN1	Stn. AN2
DARK	37 ± 8^{a}	35 ± 7^{a}	44 ± 9^{a}	45 ± 8^{a}	44 ± 5^{a}	-
PAR+UVA	30 ± 7^{a}	33 ± 5^{a}	45 ± 5^{a}	44 ± 8^{a}	42 ± 5^{a}	-
PAR+UVR	31 ± 3^{a}	30 ± 4^{a}	33 ± 7^{b}	47 ± 7^{a}	32 ± 7^{b}	-
	% of hybridized *Eubacteria* active in ^{3}H-leucine uptake					
DARK	79 ± 5^{a}	77 ± 5^{a}	83 ± 5^{ab}	79 ± 5^{a}	65 ± 7^{a}	65 ± 4^{a}
PAR+UVA	80 ± 3^{a}	71 ± 3^{a}	85 ± 4^{a}	83 ± 4^{a}	59 ± 12^{a}	66 ± 8^{a}
PAR+UVR	82 ± 3^{a}	71 ± 7^{a}	79 ± 4^{b}	79 ± 7^{a}	37 ± 5^{b}	62 ± 6^{a}

Table 4. Percentage of positively hybridized cells with EUB338-II-III probes for *Eubacteria* taking up ^{35}S-DMSP and ^{3}H-leucine (average ± deviation of fields) as measured by MAR-CARD-FISH after exposure to each treatment. Letters refer to results with a post hoc Tukey's test ($p < 0.05$). Different letters indicate significant differences among treatments.

The contribution to both trace ^{35}S-DMSP and ^{3}H-leucine assimilation by the different groups of bacteria was further assessed by applying microautoradiography to the hybridized filters. Samples were incubated with higher concentrations of ^{35}S-DMSP compared to those used for the incorporation measurements, in order to label bacteria sufficiently for autoradiographic detection. Only the free-living bacterial fraction was subjected to analysis; bacteria retained in the 5 µm filters occurred in aggregates and thus silver grains could not be univocally attributed to individual bacteria. High numbers of active cells were found for both substrates, although more bacteria assimilated ^{3}H-leucine (65% to 83% of Eub, dark treatment) than ^{35}S-DMSP (35% to 45% of Eub, dark treatment) at all locations (Table 4). Negligible numbers of cells were labeled with silver grains in the killed controls.

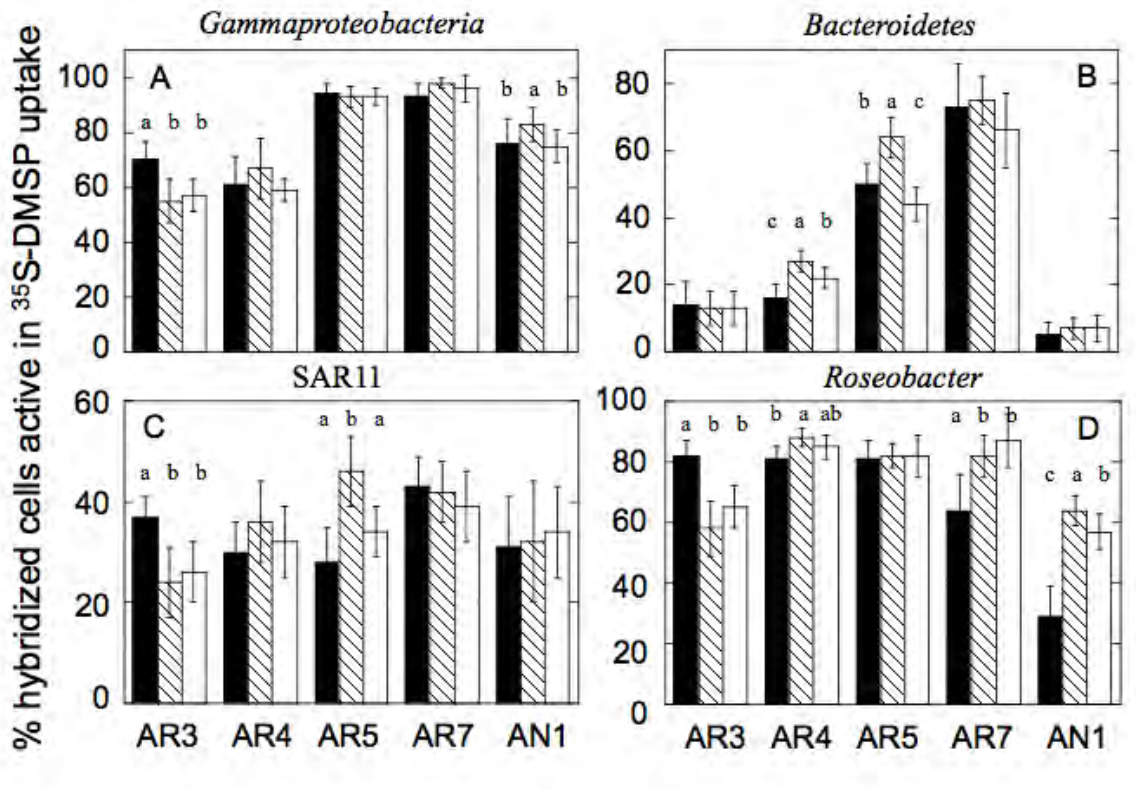

Fig. 2. Percentage of positively hybridized cells with probes for free-living *Gammaproteobacteria* (a), *Bacteroidetes* (b), SAR11 (c), and *Roseobacter* (d) taking up ^{35}S-DMSP (average ± standard deviation of fields) as measured by MAR-CARD-FISH after exposure to the following radiation conditions: PAR+UVA (dashed bars), PAR+UVR (white bars) and darkness (black bars). Note that the Y axes show different scales. Letters refer to results with a post hoc Tukey's test ($p < 0.05$). Different letters indicate significant differences among treatments.

Little differences were found among the total number of active eubacteria exposed to different light conditions. Only bacteria from stations AR5 and AN1 showed significantly smaller percentages of leucine and DMSP-sulfur assimilating cells when exposed to full sunlight radiation compared to PAR+UVA exposure (Tukey's test, $p < 0.05$).

Among the taxonomic groups taking up ^{35}S-DMSP, *Gammaproteobacteria* (Fig. 2a) and *Roseobacter* (Fig. 2d) were the ones showing the highest numbers of active cells (60% - 98% of hybridized cells), followed by the SAR11 clade (Fig. 2c, 24%-46%). *Bacteroidetes* (Fig. 2b) were much more variable among stations, with percentages of active cells in ^{35}S-DMSP uptake ranging from 5% (station AN1) to 75% (station AR7).

With respect to ^{3}H-leucine, both *Gammaproteobacteria* and *Roseobacter* presented numbers of active cells as high as those taking up ^{35}S-DMSP (Fig. 3a and 3d). SAR11 showed

up to 83% of cells taking up ^{3}H-leucine (Fig. 3c), which is twice the values for ^{35}S-DMSP samples and, on the contrary, less *Bacteroidetes* (~10%-50%) were active for ^{3}H-leucine (Fig. 3b).

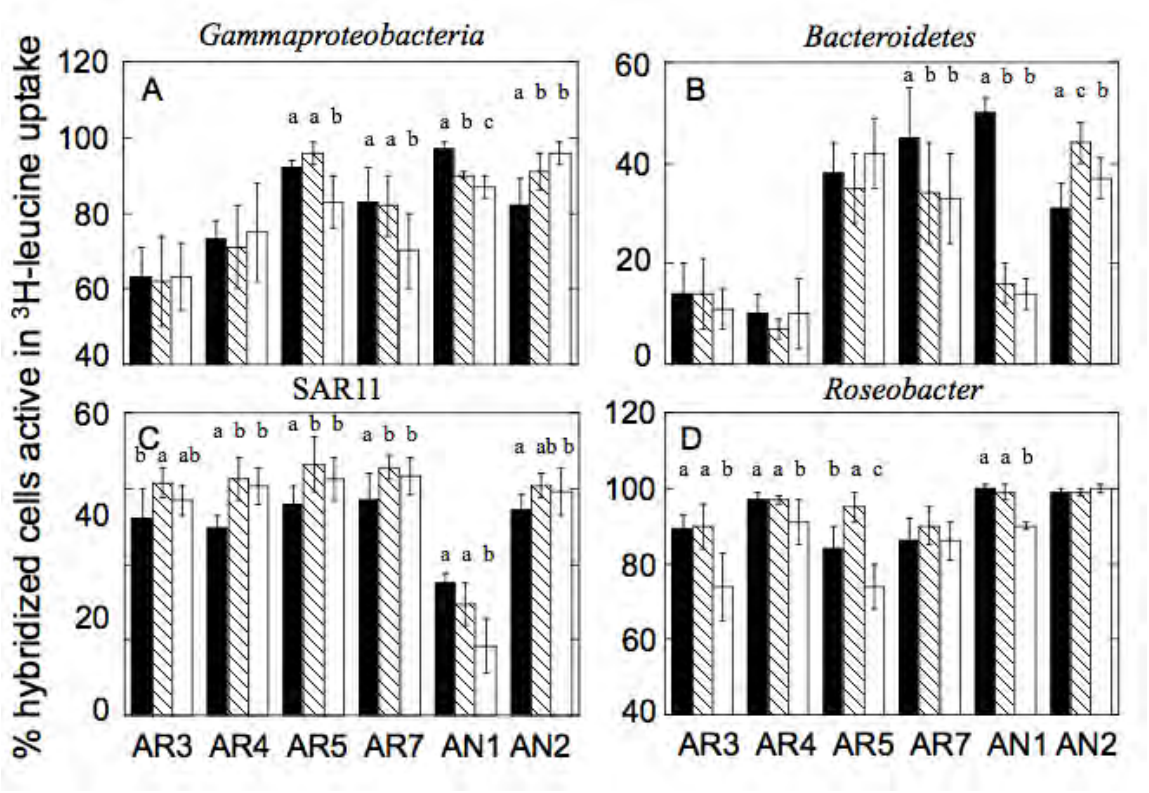

Fig. 3. Percentage of positively hybridized cells with probes for free-living *Gammaproteobacteria* (a), *Bacteroidetes* (b), SAR11 (c), and *Roseobacter* (d) taking up ^{3}H-leucine (average ± standard deviation of fields) as measured by MAR-CARD-FISH after exposure to the following radiation conditions: PAR+UVA (dashed bars), PAR+UVR (white bars) and darkness (black bars). Note that the Y axes show different scales. Letters refer to results with a post hoc Tukey's test ($p < 0.05$). Different letters indicate significant differences among treatments.

When samples were exposed to different sunlight conditions, some differences were detected within bacterial groups, although the general effects of UVR were small and variable among experiments. *Gammaproteobacteria* seemed to be stimulated in their ^{35}S-DMSP uptake due to dark enclosure only in station AR3; no important differences were found in the rest of stations. Instead, inclusion of UVB led to significant lower numbers of *Gammaproteobacteria* labeled for ^{3}H-leucine in stations AR5, AR7 and AN1 (11%, 15%, and 10 % reduction in the percentage of active cells compared to the dark treatment, respectively, Fig. 3a) whereas in station AN2 a significant 17% increase was caused by full sunlight exposure compared to dark treatment.

Bacteroidetes showed no significant differences among treatments in most stations, except in station AR4 and AR5, where their numbers of cells active in ^{35}S-DMSP uptake increased after PAR+UVA exposure compared to the dark treatment and decreased due to UVB (Fig. 2b). ^{3}H-leucine uptake by txhis group was only affected by light in station AR7, showing a 25% decrease in both light incubations compared to the dark treatment (Fig. 3b).

Within *Alphaproteobacteria*, members of the *Roseobacter* cluster were either photoinhibited in their ^{35}S-DMSP uptake (station AR3) or remarkably stimulated by one or both light types in stations AR4, AR7 and AN1 (i.e. 8%, 20% and 55% increase after PAR+UVA exposure, respectively, Fig. 2d). Regarding the uptake of ^{3}H-leucine, significant decreases in active cells caused by UVB were found in stations AR3, AR5 and AN1, the ones with the highest measured doses of UVB (Fig. 3d). Indeed, ^{3}H-leucine assimilating *Roseobacter* was the only group for which a significant correlation could be found between the reduction in the number of active cells after full sunlight exposure and the UVB doses received (Spearman´s $r = 0.81$, $p < 0.01$, and $r = 0.78$, $p < 0.05$ for percentages of the dark and PAR+UVA treatments, respectively, $n = 6$).

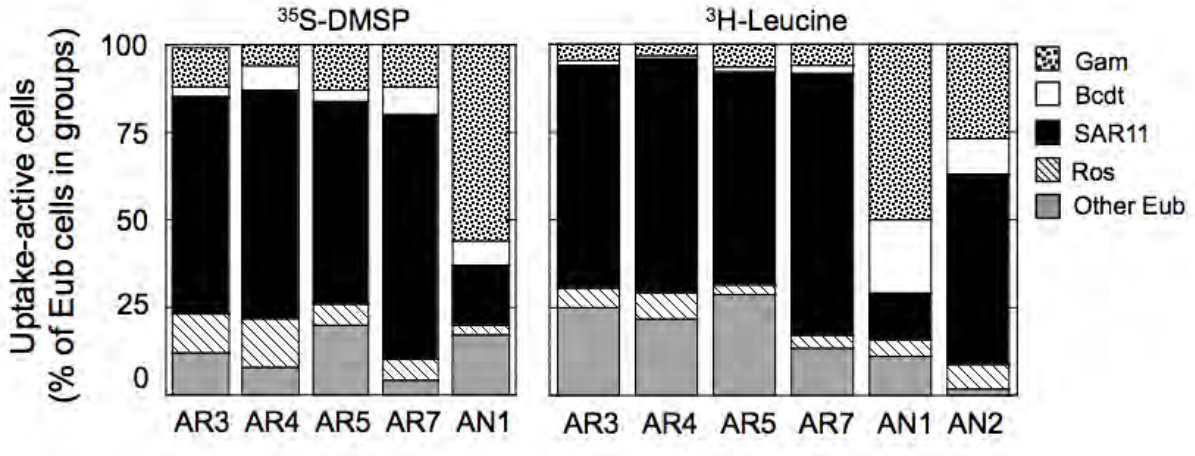

Fig. 4. Relative contribution of each of the analyzed phylogenetic groups (*Gammaproteobacteria* [Gam], *Bacteroidetes* [Bcdt], SAR11, *Roseobacter* [Ros] and other *Eubacteria* [Eub]) to total active bacteria in the uptake of ^{35}S-DMSP and ^{3}H-leucine in the studied stations. Percentages were calculated relative to eubacterial cells (probes EUB338-II and –III) as average of the three treatments.

Assimilation of ^{35}S from DMSP by SAR11 showed no clear trend, presenting dark stimulation in experiment AR3, PAR+UVA-driven stimulation in station AR5 and no effect in the other 3 experiments (Fig. 2c). Instead, the only repetitive pattern was found for this group in the uptake of ^{3}H-leucine (Fig. 3c). In 5 out of 6 experiments, the number of active SAR11

was slight but significantly higher after exposure to both light treatments, showing increases ranging from 12% to 20% after PAR+UVA exposure. Note that the PAR-only treatment was not done in these experiments.

To examine the importance of each bacterial group to substrate uptake, we estimated the contribution of each phylogenetic group to the total number of cells assimilating each substrate. This was calculated from the fraction of active cells within one group and its abundance (with respect to total eubacterial cells) relative to the percentage of total eubacteria active at substrate uptake. This relative contribution (average of the 3 treatments) is shown in Figs. 4a and 4b. The numbers of cells assimilating ^{35}S-DMSP or ^{3}H-leucine were both largely dominated by the SAR11 group in all Arctic stations (on average 69% and 76% of active bacteria, respectively), whereas in station AN1 *Gammaproteobacteria* was the major contributor to active cells, accounting for 56% and 50% of the ^{35}S-DMSP and the ^{3}H-leucine-assimilating bacteria, respectively. Besides the numeric abundance of *Bacteroidetes* in station AN1, their low numbers of active cells led to a small contribution to total active cells.

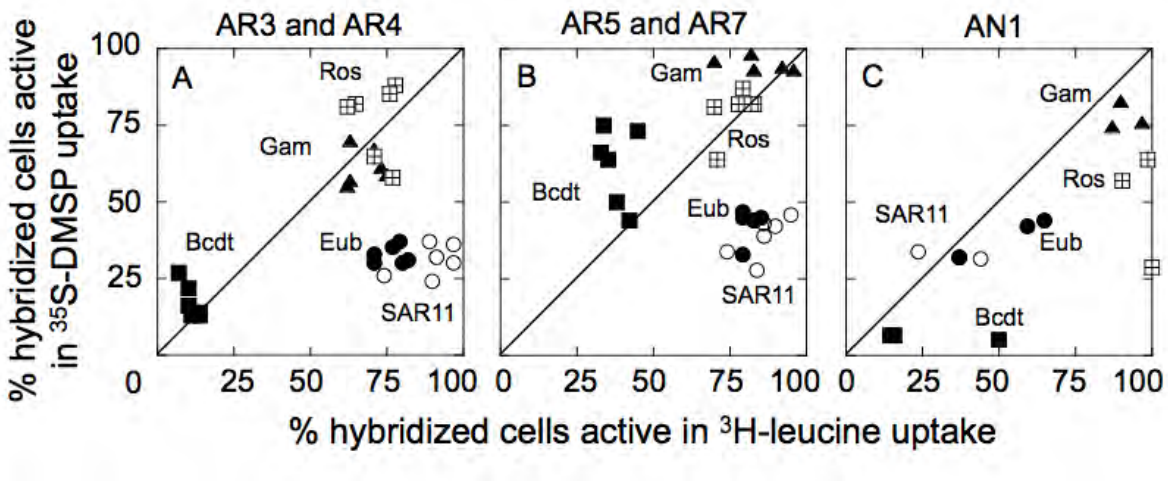

Fig. 5. Comparison of ^{35}S-DMSP versus ^{3}H-leucine labeled cells (as % of total DAPI counts) for each phylogenetic bacterial group from stations AR3 and AR4 (A), AR5 and AR7 (B) and AN1 (C). Each data point represents one single treatment. Line indicates a 1:1 relationship (same number of active cells for both substrates). *Gammaproteobacteria* [Gam], *Bacteroidetes* [Bcdt], SAR11, *Roseobacter* [Ros] and total *Eubacteria* [Eub]).

Fig. 5 shows the percentage of total DAPI-stained prokaryotic cells that hybridized with each probe and incorporated each substrate, pooled from the different treatments and stations. Stations AR3-AR4 and AR5-AR7 were plotted together due to similar behavior of groups (Figs. 5a and 5b, respectively). In the Arctic, *Roseobacter* and *Gammaproteobacteria* showed similar percentages of ^{35}S-DMSP and ^{3}H-leucine labeled cells (i.e. they appear very close to the 1:1 line), and in stations AR5 and AR7 *Gammaproteobacteria* presented more active cells (Fig. 5b). Within the *Bacteroidetes* group, conversely, the percentage of ^{35}S-DMSP labeled cells was often higher than that of ^{3}H-leucine labeled cells, and, like *Gammaproteobacteria,* much higher percentages of active cells were found in stations AR5 and AR7 (Fig. 5b). In the Antarctic station AN1, all these groups were more active in assimilating ^{3}H-leucine than ^{35}S-DMSP (Fig. 5c). SAR11 behaved distinctly: in the Arctic stations they showed on average 50% fewer ^{35}S-DMSP labeled than ^{3}H-leucine labeled cells (Figs. 5a and 5b), but in AN1 they fell close to the 1:1 line (Fig. 5c).

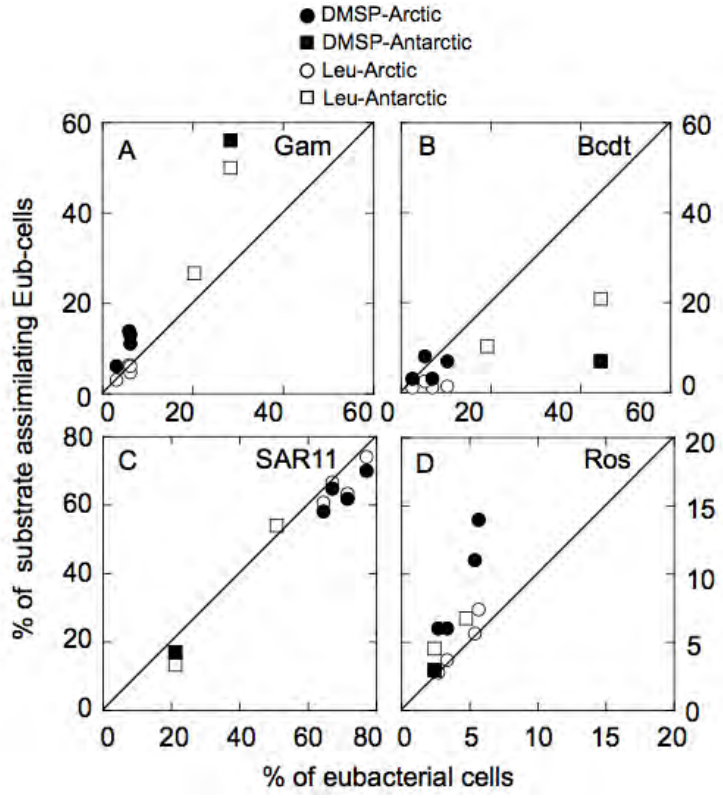

Fig. 6. Contribution of (A) *Gammaproteobacteria* [Gam], (B) *Bacteroidetes* [Bcdt], (C) SAR11 and (D) *Roseobacter* [Ros] from Arctic and Antarctic stations to the number of cells assimilating ^{35}S from DMSP (solid symbols) or ^{3}H-leucine (open symbols) versus the relative abundance of the group (calculated with respect to *Eubacteria* (EUB) cells). Values are expressed as average of the three treatments. Data points on the 1:1 line indicate contribution to cells assimilating substrate corresponding to their contribution to abundance. Note that the X and Y axes show different scales.

The contribution of each taxonomic group to ^{35}S-DMSP and ^{3}H-leucine assimilating eubacteria was compared with their relative abundance in the bacterial community (Fig. 6). In general, both *Gammaproteobacteria* (Fig. 6a) and *Roseobacter* (Fig. 6d) accounted for a higher fraction of ^{3}H-leucine and ^{35}S-DMSP-assimilating cells than that expected from their relative abundance, and SAR11 fell close to the 1:1 line (Fig. 6c), suggesting that they contributed to the number of cells assimilating both substrates accordingly to their relative abundance. Instead, *Bacteroidetes* were underrepresented among DMSP and leucine assimilating bacteria, specially in Antarctic samples (Fig. 6b).

DISCUSSION

Despite the low temperatures and extreme conditions, the Arctic and Antarctica can support high prokaryotic heterotrophic activities due to the spring and summertime development of localized massive phytoplankton blooms when the ice retreats (Fogg, 1977; Harrison and Cota, 1991; Sakshaug, 2004). Our values of heterotrophic bacterial abundance and activity (measured as ^{3}H-leucine incorporation) fell within the ranges previously reported for polar areas (e.g. Rich *et al.*, 1997; Straza *et al.* 2010; Wheeler *et al.*, 1996) and are similar to those measured in other oceanic regions (Alonso and Pernthaler, 2006; Church *et al.*, 2004; Gasol *et al.*, 1998).

High DMSPt:Chl *a* ratios were observed in the Arctic due to the dominance of the colonial haptophyte *Phaeocystis pouchetii* (Lasternas and Agustí, 2010), a well-known DMSP producer (Liss *et al.*, 1994), whereas in Antarctica, where mainly diatoms comprised the phytoplankton assemblage, lower ratios were found. The DMSP:Chl *a* ratio is a good indicator of the relative occurrence of DMSP producers among the phytoplankton assemblages (Kiene *et al.*, 2000; Simó *et al.*, 2002), and the values found in the Antarctic stations are typical of low-DMSP producing phytoplankton (Kiene *et al.*, 2000).

Exposure of samples to natural sunlight caused a significant reduction of both the post-exposure ^{3}H-leucine incorporation rates and the percentage of trace ^{3}H-leucine assimilated during incubations (Table 3; Fig. 1b), suggesting that the UVR levels received were actually affecting bacterial heterotrophic activity, with UVB generally accounting for most inhibition.

This deleterious effect of UVB radiation on leucine uptake by marine bacteria has been already reported for many different non-polar systems (Aas *et al.*, 1996; Alonso-Sáez *et al.*, 2006; Herndl *et al.*, 1993; Pakulski *et al.*, 1998; Sommaruga *et al.*, 1997) yet its effects on the heterotrophic activity of polar free-living bacterial assemblages are still rather understudied (e. g. Pakulski *et al.*, 2008). The large discrepancy between the ^{3}H-leucine incorporation rates and the trace ^{3}H-leucine assimilations measured in station AN1 might be due to the presence of high numbers of large leucine-assimilating diatoms, such as *Thalassiosira* spp, *Eucampia* spp and *Chaetoceros* spp. (**Chapter 4**), which were removed by 5 μm pre-filtration for the trace assimilation measurements but not for the incorporation measurements.

Remarkably, a significant correlation between inhibition and UVB doses was found for the trace ^{3}H-leucine assimilation but not for the incorporation measured after exposure. Reasons for this discrepancy can be found in the fact that post-exposure ^{3}H-leucine incorporation was determined by standard 2-3 h dark incubations during which bacteria might have had time to recover from photodamage (Kaiser and Herndl, 1997). This further suggests that incubation of samples with added substrates under natural light may give more realistic estimates of the in situ incorporation rates.

The low assimilation percentages of trace ^{35}S-DMSP (Fig. 1a) might be due to the dilution of the radiolabeled tracer within the potentially high (and highly variable) concentrations of dissolved DMSP released by *Phaeocystis* cells. For this reason, comparisons should be done among light treatments within stations but not among stations. No correlation was found between the light-driven effects on trace DMSP assimilation and UVR doses. Instead, variable responses to the light spectrum were recorded. These results agree with those of Slezak *et al.* (2001), who observed that the degree of bacterial inhibition due to UVR differed between ^{3}H-leucine incorporation and ^{35}S-DMSP consumption in the Mediterranean Sea. Thus, while bacterial assimilation of leucine in either high or trace concentrations seems to be directly affected by photodamage, trace DMSP-sulfur assimilation seems to follow more complex, yet still unknown, physiological responses to light, or be highly sensitive to light-driven DMSP release by phytoplankton.

The CARD-FISH description of the bacterial communities revealed differences between particle-attached and free-living bacterial assemblages (Table 2), as previously reported by DeLong *et al.* (1993) and Riemann *et al.* (2000). Nearly all DAPI-stained cells hybridized with the EUB338-II-III probe, suggesting that the summer prokaryotic assemblages from

both poles were almost entirely comprised by bacteria, agreeing with the observed low numbers of *Archaea* towards the summer in surface samples from the Arctic (Alonso-Sáez *et al.*, 2008) and Antarctica (Murray *et al.*, 1999). For this reason, only bacteria were analyzed in the present work.

Whereas the free-living Arctic bacteria were numerically dominated by the SAR11 clade, the particle-attached assemblages were almost entirely comprised of cells assigned to the *Bacteroidetes* cluster, in agreement with some studies that have shown *Alphaproteobacteria* and *Bacteroidetes* groups to be associated to *Phaeocystis* blooms (Brussaard *et al.*, 2005; Janse *et al.* 2000; Simon *et al.*, 1999). Similarly, Lamy *et al.* (2009) found a strong positive correlation between *Bacteroidetes* and particulate organic carbon, suggesting that particle-attached *Bacteroidetes* are important during *Phaeocystis* blooms.

In Antarctica, however, members of *Gammaproteobacteria* and *Bacteroidetes* were the dominant in the aggregate fraction, whereas either *Bacteroidetes* (AN1) or SAR11 (AN2) dominated the free-living assemblages. Such high abundances of free-living and attached *Bacteroidetes* had been observed by Fandino *et al.* (2005) and Riemann *et al.* (2000) during a mesocosms bloom of *Thalassiosira* sp., the same diatoms dominating in AN1, suggesting that this bacterial group, besides being predominant on particles, can also be major components of free-living marine bacterial communities under nutrient-rich conditions.

Relatively few studies have examined the abundance of bacterial groups in polar waters. Within Arctic bacterial assemblages, *Alphaproteobacteria* has often been reported as the most abundant group (15% - 42% of cell counts), with SAR11, when probed, accounting for the majority of it, particularly in summer (Alonso-Sáez *et al.*, 2008; Bano and Hollibaugh, 2002; Elifantz *et al.*, 2007; Garneau *et al.*, 2006; Malmstrom *et al.*, 2007; Vila-Costa *et al.*, 2008). However, none of these authors found such high numbers of SAR11-positive cells before. Interestingly, high proportions of *Bacteroidetes* have sometimes been found in Arctic waters too (Elifantz *et al.*, 2007; Wells and Deming, 2003). In agreement with our results, Antarctic and Southern Ocean waters have been found to be dominated either by *Bacteroidetes* or *Gammaproteobacteria* (Gentile *et al.,* 2006; Glöckner *et al.*, 1999; Simon *et al.*, 1999; Straza *et al.,* 2010) and lower numbers or sequences of *Alphaproteobacteria* have been recovered.

MAR-CARD-FISH was further applied to resolve the single-cell, substrate utilization activity of the observed bacterial groups. High percentages of active bacteria were found in all

stations, in agreement with some previous studies in Arctic and Antarctic waters that showed that prokaryotes are an active component of polar microbial communities (Alonso-Sáez *et al.*, 2008; Elifantz *et al.*, 2007; Kirchman *et al.*, 2007; Malmstrom *et al.*, 2007; Straza *et al.* 2010).

Since leucine is a universal substrate for bacteria that is used to estimate bacterial heterotrophic production (Kirchman *et al.*, 1985), we regarded the number of cells assimilating ^{3}H-leucine (60-85%, Table 4) as a measure of the fraction of active bacteria (e.g. del Giorgio and Gasol, 2008). Since approximately half of the active bacteria were taking up sulfur from DMSP, important percentages of ^{35}S-labeled cells were recorded (up to 47%). The only previous study where bacteria assimilating DMSP and leucine were compared in polar waters is that of Vila-Costa *et al.* (2008), conducted in March and May in a coastal region near the Mackenzie river estuary. They found lower percentages of ^{3}H-leucine assimilating cells (47-62%) and much lower proportions of ^{35}S-DMSP labeled cells (4-5%). The authors suggested that these low numbers were due to the low concentrations of labile organic substrates occurring in the ice covered waters prior to the summer phytoplankton bloom which, that year, peaked in July (Alonso-Sáez *et al.*, 2008). This was confirmed for DMSP, which showed very low concentrations throughout the study (1-6 nM in total; Vila-Costa et al. 2008). Consistent with that hypothesis, the high percentages of active cells found in our Arctic stations would be explained by the large concentrations of DMSP and other substrates released by the *Phaeocystis* bloom.

In the Antarctic station AN1, where the dense diatom bloom carried a much lower DMSP:Chl *a* ratio, percentages of ^{35}S-active cells were as high as in the Arctic samples. As a result, no significant correlation was found between DMSP:Chl *a* ratios and the number of ^{35}S-labeled cells across stations. This does not support the hypothesis that the fraction of DMSP-sulfur assimilating bacteria depends on the contribution of DMSP to total reduced sulfur and carbon sources in the ecosystem (Pinhassi *et al.*, 2005; Vila-Costa *et al.*, 2007). However, the fact that just one station was analyzed for single-cell ^{35}S-DMSP assimilation in Antarctica prompts caution in making generalizations; indeed, to our knowledge, this is the first time that cell-specific uptake of DMSP is probed for Antarctic bacterial assemblages. In any case, DMSP seemed to be an important source of reduced sulfur for a wide array of polar marine bacteria, consistent with the suggested role of this compound in a methionine synthesis shortcut that fuels the protein factory (Kiene *et al.*, 1999).

High percentages of SAR11 cells active in the uptake of both substrates were recorded in the Arctic stations (Figs. 2 and 3). This high activity, together with their numeric dominance, led to a major contribution of this group to total numbers of active cells (Fig. 4). This is in contrast to the results of previous studies in Arctic and other coastal waters where the very abundant SAR11 were not the major contributors to total substrate uptake (Alonso-Sáez and Gasol, 2007; Alonso-Sáez *et al.*, 2008; Elifantz *et al.*, 2005). However, this clade has also shown high activities in oceanic Atlantic waters (Malmstrom *et al.*, 2004a) suggesting a substantial variability of their single-cell activity and/or taxonomic composition changes among oceanic regions. These authors also found that the SAR11 clade dominated the utilization of dissolved DMSP and amino acids due to their high abundances and high percentages of active cells (40-60% and 40-85% of DMSP and amino acid uptake, respectively). Supporting these results, Tripp *et al.* (2008) recently showed that SAR11 cells need exogenous sources of reduced sulfur for growth because they lack the genes for sulfate reduction. Therefore, a DMSP-rich environment such as that we encountered during our cruise through *Phaeocystis*-blooming waters might be a suitable environment for these SAR11 to grow actively up to high abundances.

A great increase in the number of active *Bacteroidetes* was observed for both substrates between the Arctic stations AR3 and AR7 (Figs. 2 and 3). Some members of this group are known to be colonizers of aggregates (Simon *et al.*, 2002) and dominate the consumption of large complex carbohydrates (Cottrell and Kirchman, 2000). With the recorded information, we could not resolve whether the differences between the early (AR3 and AR4) and the late stations (AR5 and AR7) were due to differences in the presence of bloom-associated polymers, such as colony mucus, or in taxonomic shifts within the group, or both.

Roseobacter and *Gammaproteobacteria* were also very active in the uptake of both tracers (Figs. 2 and 3) in agreement with previous results from other ecosystems such as the NW Mediterranean Sea (Vila-Costa *et al.*, 2007) or the North Atlantic and the Gulf of Mexico (Malmstrom *et al.*, 2004b). The capacity to degrade DMSP and assimilate its sulfur is known to be common among *Roseobacter* isolates (González *et al.*, 1999) and their abundances have been positively correlated with DMSP concentrations (González *et al.*, 2000) or DMSP consumption (Zubkov *et al.*, 2002) during blooms of DMSP producers.

In the Antarctic station AN2, the SAR11 cells were also the major contributors to both abundance and total ^{3}H-leucine-assimilating cells. The *Gammaproteobacteria*, relatively abundant, contributed more to total ^{3}H-leucine uptake than their Arctic homologs. A different picture was found at station AN1: the lower abundance of SAR11 made them contribute less than 20% to total active cells for both substrates (Fig. 5) even though the numbers of ^{35}S-DMSP-active cells were quite similar to those from the Arctic. Despite their dominant abundance, *Bacteroidetes* accounted for a small proportion of the total active cells. The relative high abundance of *Gammaproteobacteria* and their high percentages of active cells resulted in a major contribution of this group to both ^{3}H-leucine (50%) and ^{35}S-DMSP (56%) uptake in this Antarctic station. Altogether, these results add to previous evidence that DMSP-sulfur assimilation is widespread among the major bacterial taxonomic groups in a variety of marine environments, also in Antarctic waters.

SAR11 was the only group where cells taking up ^{35}S-DMSP were much fewer than those taking up ^{3}H-leucine (Fig. 5), which suggests that not all active SAR11 were using DMSP-sulfur for protein synthesis. *Gammaproteobacteria* and *Roseobacter*, conversely, presented similar proportions of active bacteria for both substrates. *Bacteroidetes* even showed higher affinity for DMSP-sulfur, yet in this case leucine may not be the best indicator of active cells because *Bacteroidetes* have predilection for large complex carbohydrates and usually present low numbers of amino acid assimilating cells (Cottrell and Kirchman, 2000). These results are in accordance to the percentages of active bacteria found for both substrates in coastal temperate waters (Vila *et al.*, 2004; Vila-Costa *et al.*, 2007) but differ considerably from those recorded by Vila-Costa *et al.* (2008) for winter and springtime Arctic bacterial assemblages, where all groups except *Roseobacter* showed higher affinity for ^{3}H-leucine than for ^{35}S-DMSP.

The contributions of each group to substrate-assimilating cells were compared with their relative abundance in the bacterial community. In general, both *Gammaproteobacteria* and *Roseobacter* accounted for higher fractions of the ^{3}H-leucine and ^{35}S-DMSP-assimilating cells than those expected from their relative abundances (Fig. 6), indicating high activities and a significant role of these groups in the processing of DMSP during the polar summer. Instead, SAR11 fell onto the 1:1 line and *Bacteroidetes* were mainly underrepresented among cells assimilating both substrates. This contrasts with the pattern found by Vila-Costa *et al.* (2008) with winter and spring Arctic assemblages, where only spring *Bacteroidetes* were overrepresented among cells assimilating ^{35}S-DMSP and the rest of the groups either contributed equally or less to active cells than expected based on abundance.

In general, the effects of solar radiation on the single-cell activity of these broad taxonomic groups were small and variable, showing no clear trends among experiments. Even though there are a few studies on the effects of UVR on polar bacterial assemblages and isolates (e.g. Davidson and van der Heijden 2000; Hernández *et al.* 2006; Wickham and Carstens, 1998), to our knowledge this is the first time that group-specific sensitivities to UVR are specifically assessed among in situ dominating polar bacterial taxa.

Apparently, light-driven changes in the number of active cells did not reflect the observed variations in either post-exposure incorporation or during-exposure trace assimilation rates (Table 3, Fig. 1), which might be explained by the fact that single-cell activity was assessed in terms of presence/absence (i.e., each cell is identified as labeled or non-labeled) and not as assimilation per cell (as could have been done by measuring the silver grain areas around cells, Sintes and Herndl, 2006). Hence, higher or lower percentages of active cells do not necessarily equal to higher or lower assimilation rates, since a cell surrounded by silver grains will be counted as labeled regardless of the amount of exposed silver grains.

Still some differences were observed, showing either UVB inhibition or light stimulation of uptake (Figs. 2 and 3). In contrast to the results of Alonso-Sáez *et al.* (2006), where SAR11 cells from the Mediterranean were the most sensitive to the detrimental effects of solar radiation, we found a slight but significant increase in the number of ^{3}H-leucine-assimilating SAR11 cells upon light exposure in five out of six stations, yet those experiments in the Mediterranean were performed under the much higher UVR doses typical of spring and summer. The only group showing a significant correlation between the reduction in the number of active cells due to full sunlight and the UVB doses received was *Roseobacter* in trace ^{3}H-leucine assimilation (Spearman´s $r = 0.81$, $p < 0.01$, and $r = 0.78$, $p < 0.05$ for percentages of the dark and PAR+UVA treatments, respectively). This is also opposite to the results of Alonso-Sáez et al. (2006), who found higher resistance to UVB in *Roseobacter* than in SAR11. The level of resolution of these CARD-FISH probes provides no information about taxonomic variations within the groups, although these observations point to distinct phylotypes adapted to different light regimes (continuous light vs. diel cycles) and different trophic conditions (eutrophy vs. oligotrophy).

The largest differences were found in station AN1, where ^{3}H-leucine uptake by all groups appeared to be negatively affected by both UVA+PAR and UVB exposure. Interestingly, the strong photoinhibition of the abundant *Bacteroidetes* in the uptake of ^{3}H-leucine

reflects the observed decrease in both post-exposure ^{3}H-leucine incorporation and during-exposure trace ^{3}H-leucine assimilation. On the other hand, uptake of ^{35}S by *Roseobacter* was significantly greater upon light exposure compared to dark control, while all other groups showed no significant effects among treatments in this Antarctic station. Such a light-driven enhancement of activity has also been reported for Mediterranean *Roseobacter* by Alonso-Sáez *et al.* (2006) in both leucine and ATP uptake, and it could be related to the reported ability of some *Proteobacteria* and *Bacteroidetes* to derive energy from light with the use of proteorhodospsins (Béjà *et al.*, 2000; Gómez-Consarnau *et al.*, 2007; Sabehi *et al.*, 2004) or the presence of bacteriochlorophyll-*a* in some *Alphaproteobacteria* (Béjà *et al.*, 2002; Shiba *et al.*, 1979). It seems that having the ability to derive energy from light would be a useful strategy in these areas with such long light periods, although Cottrell and Kirchman, (2009) did not find any of this photoheterotrophic bacteria to behave as superior competitors during the Arctic summer. The fact that different groups responded distinctly to light depending on the substrate assimilated points, instead, either to differential regulation or damage of the uptake systems (Herndl *et al.*, 1997) or, as previously suggested, to light-driven variations of the available DMSP released by phytoplankton. In any case, considering that the polar bacterial assemblages are continuously exposed to light during the summer months, incubation under realistic solar irradiances is essential for accurate determinations of bacterial heterotrophic activities and production rates. Unreal dark incubation can lead both to overestimates (as is the case for our leucine assimilation rates) or underestimates (as is the case for our DMSP-sulfur assimilation rates) of the measured activities during the long Arctic and Antarctic summers.

Notable amounts of UVB may reach 15 m depth and biological effects may be detected at 20 or 30 m in inshore polar waters (Convey and Fogg 2007). In deeply-mixed waters this may not be so important since individual cells are near the surface for short times only and have time for repair in the shade of deeper waters. In these cases, the effects of UVR measured in experimental tanks under near surface conditions may easily be overestimates. It is possible that such an overexposure of samples was partially responsible for the greatest effects found in station AN1, where the mixed layer was *ca.* 15 m deep and thus our samples were exposed to doses higher than naturally. In contrast, in shallower and strongly stratified waters, such as those of the Arctic marginal ice zone, where actively growing phyto- and bacterioplankton are held in a narrow surface mixing layer (*ca.* 5 m) in the season of maximum UVR levels, these organisms may be continuously exposed to deleterious light with less chances for repair

(Buma *et al.*, 2001b; Convey and Fogg, 2007). In these cases experimental approaches like ours would provide more realistic estimates.

Overall, our results support a high heterotrophic activity during the Arctic and Antarctic summers and widespread DMSP-sulfur and leucine assimilation capabilities among the dominant bacterial groups. Similar percentages of ^{35}S-DMSP assimilating bacteria were found in the Arctic and Antarctic stations despite the variable DMSP concentrations found across stations, generally higher in the *Phaeocystis*-dominated Arctic waters, indicating for the first time a widespread role of DMSP as a reduced sulfur source for marine bacteria in polar ecosystems. The present study also documents substantial impact of UVR on heterotrophic carbon and sulfur fluxes and further suggests that incubations under realistic solar radiation levels and spectrum are necessary in order to obtain meaningful measurements of the contribution of bacteria assemblages and their taxonomic composition to the processing of labile organic compounds.

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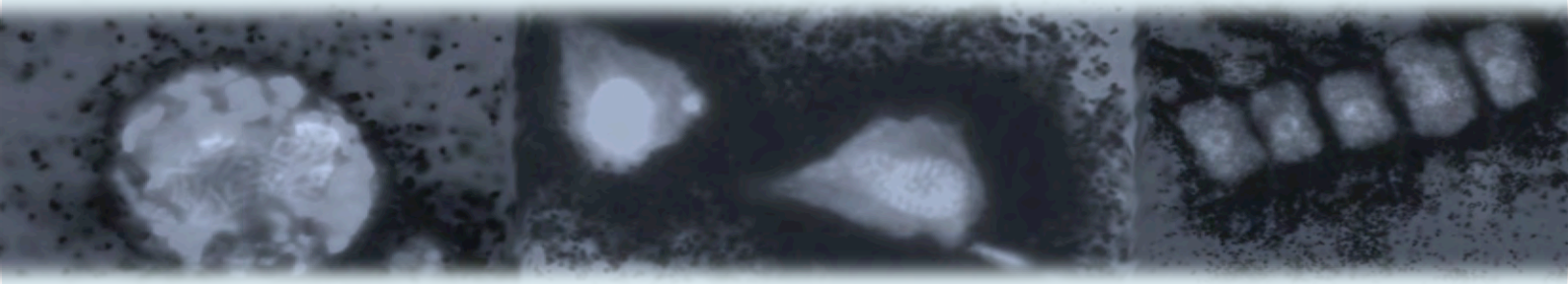

Chapter 4

Sunlight effects on the osmoheterotrophic behaviour of Arctic and Antarctic phytoplankton

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ABSTRACT

Very little is known about the potential ecological role of algal osmoheterotrophy in natural marine communities, however, it is likely to vary depending on the light regimens the algae experience. Measurements of size-fractionated uptake and assimilation of trace additions of ^{3}H-leucine and ^{35}S-dimethylsulfoniopropionate (DMSP) were combined with microautoradiography to assess the extent of heterotrophy in summer phytoplankton assemblages from Arctic and Antarctic waters. The role of solar radiation was investigated by exposing samples to different radiation spectra: the full solar radiation spectrum (280-700 nm), photosynthetically active radiation + ultraviolet radiation A (PAR+UVA, 320-700 nm) and darkness. Assimilation of both substrates occurred in the size fraction containing most phytoplankton (> 5 μm), and light exposure generally increased ^{35}S-DMSP assimilation and decreased ^{3}H-leucine assimilation. Bacteria contributed negligibly to to ^{35}S-DMSP uptake and substantially to ^{3}H-leucine uptake in the >5 μm size fraction. Microautoradiography revealed that the capacity to take up both organic substrates was widespread among the polar algal phyla, particularly in pennate and centric diatoms, autotrophic dinoflagellates and flagellates. Image analysis of the microautoradiograms showed interspecific variability in the ^{35}S-DMSP uptake by phytoplankton depending on the solar spectrum while ^{3}H-leucine uptake was generally lower in light exposure than in the dark. Overall, these results suggest a significant role of polar phytoplankton in the utilization of labile dissolved organic matter and further confirm the central role of solar radiation in regulating heterotrophy in the pelagic ocean.

INTRODUCTION

The uptake and utilization of dissolved organic matter (DOM) as a source of carbon and energy was demonstrated for a wide variety of algal cultures more than three decades ago (see references in Amblard, 1991; Droop, 1974; Neilson and Lewin, 1974). Algal osmoheterotrophy was initially thought to be ecologically irrelevant due to the low substrate concentrations found in natural environments (Wright and Hobbie, 1965; Wright and Hobbie, 1966; Hellebust, 1970) and the inability of the algae to compete with bacteria at these low labile DOM concentrations. Heterotrophic bacteria are regarded as the most efficient consumers of DOM due to their high surface to volume ratio and their efficient uptake systems (Wright and Hobbie, 1966). Consequently, most geochemical models of carbon flow consider bacteria to be the major consumers of DOM (Azam and Cho 1987, Thingstad 2003).

However, some studies have shown that several phytoplankton species are capable of actively taking up substrate from the DOM pool so that they might, in fact, be competitive with bacteria (Allen, 1971; Kamjunke *et al.*, 2008; Kamjunke and Tittel, 2008). Among the organic substrates algae are able to use are pyruvate, acetate, lactate, ethanol, saturated fatty acids, glycolate, glycerol, hexoses and amino acids (e.g. Amblard, 1991; Neilson and Lewin, 1974; Parker *et al.*, 1961). More recently, Vila-Costa *et al.* (2006) discovered that a variety of marine phytoplankton taxa can also take up the ubiquitous algal synthate dimethylsulfoniopropionate (DMSP) and assimilate its sulphur, thus influencing the cycling of organic sulphur in the surface ocean. These evidences, together with the phagotrophy described for many algal groups (Jones, 1994; Raven, 1997), suggest that algae may play a more diverse role in aquatic biogeochemical cycles than just supplying heterotrophs with autotrophically synthesized organic matter.

While most studies have focused on algal cultures (which may not be representative of ecologically relevant organisms) and on freshwater or benthic systems, pelagic marine environments have received less attention and very little is known about the role of algal osmoheterotrophy in natural marine communities.

The uptake and assimilation of organic substrates by algae increase with decreasing light availability (Flynn and Butler, 1986; Hellebust, 1971; Kamjunke *et al.*, 2008; Kamjunke and Tittel, 2008; McKinley, 1977), however, enhanced uptake under light exposure (Bouarab *et al.*, 2004; Kamjunke and Jähnichen, 2000; Paerl, 1991; Paerl *et al.*, 1993; Rivkin and

Putt, 1987; Tuchman *et al.*, 2006) and no effect of irradiance on uptake rates (Nilsson and Sundback, 1996; Znachor and Nedoma, 2010) have also been reported. Studies of algal osmoheterotrophy using cultures have commonly exposed samples to artificial light, and although some have considered in situ light conditions, to our knowledge none has specifically assessed the effect of natural solar ultraviolet radiation (UVR, 280-400 nm).

Research on the effects of UVR (and mainly UVB, 280-320 nm) on aquatic food webs has gained increasing attention in the polar regions, since there is evidence that ozone depletion (Gies *et al.*, 2004; Lebert *et al.*, 2002; Waibel *et al.*, 1999) and the ongoing loss of sea-ice (Johannessen *et al.*, 1999; Rothrock *et al.*, 1999) lead to enhanced underwater levels of UVR. The continuous darkness during the polar winter, the low irradiance under the sea ice layer and the relatively high concentrations of organic nutrients (Hansell *et al.*, 2009) may select for algae with heterotrophic or photoheterotrophic capabilities. Rivkin and Putt (1987) found that Antarctic algae could incorporate amino acids and glucose at ambient concentrations and proposed that this ecological trait might supplement light-limited growth during the polar spring and summer as well as support heterotrophic growth throughout the polar winter.

The first aim of this work was to assess the relevance of DOM utilization in natural marine phytoplankton assemblages during the Arctic and Antarctic summers by tracking the fate of two ubiquitous low-molecular-weight (LMW) dissolved organic compounds: leucine and DMSP. These model substrates were chosen as representatives of the pools of organic nitrogen and sulphur. Our second aim was to address the effect of natural solar radiation on the uptake of these compounds by different algal groups. We combined measurements of size-fractionated radioisotope uptake and assimilation with a microautoradiographic approach to identify the organisms taking up the respective radiolabeled substrate. Image analysis of microautoradiograms allowed us determining group-specific substrate affinities and sensitivities to UVR.

MATERIALS AND METHODS

Study area and sample collection. The study was carried out on board RV Hespérides during the ATOS I and II cruises to the Arctic and Antarctica in July 2007 and February 2009, respectively (Fig. 1). Samples for size-fractionated uptake and assimilation measurements and for microautoradiography were collected at 5 m depth (except for station AN2, sampled at 20 m depth, where high abundances of the diatom *Pseudonitzschia* were found) with a rosette of Niskin bottles mounted on a CTD profiler. Water characteristics of the sampled stations together with the irradiance measurements and time of incubation during experiments are compiled in Table 1.

Experimental design. We performed a number of experiments (seven for size fractionated assimilation and four for microautoradiography, see below) to assess the impact of natural solar radiation on the heterotrophic activity of polar microalgae. Briefly, water samples were incubated in UVR-transparent quartz bottles amended with trace concentrations of ^{35}S-DMSP (donated by R. P. Kiene, University of South Alabama, Dauphin Island Sea Lab, USA) or ^{3}H-leucine (Amersham) under different light conditions. Bottles were either exposed to the full solar radiation spectrum (PAR+UVR), the full spectrum without UVB (i.e., PAR+UVA, covered with Mylar-D foil) or kept in the dark. Samples were incubated inside a black tank with running seawater to maintain *in situ* temperature. To simulate the irradiance level of 5 m depth, samples were placed 5 cm under the surface below an optically neutral mesh that reduced surface irradiances by 40%. Samples from station AN2 (20 m depth) were covered with a double neutral mesh that reduced surface irradiances by 60%.

Radiation measurements. UVR and PAR radiation inside the incubation tank were continuously monitored throughout the incubations with a Biospherical PUV-radiometer 2500 recording irradiances at PAR (400-700 nm) and 6 channels within the UVR range (305, 315, 320, 340, 380, 395 nm). The PAR and UVR doses the individual incubations received are shown in Table 1.

Size fractionated assimilation. Samples of 50 ml were incubated for 7 to 12 h in quartz bottles with added trace concentrations of ^{35}S-DMSP (845 Ci mmol^{-1}, 0.8 pM final conc. for Arctic samples and 120-145 Ci mmol^{-1}, 2.5-3 pM final conc. for Antarctic samples) or ^{3}H-leucine (161 Ci mmol^{-1}, 0.5 nM final conc.). Controls were killed with paraformaldehyde

(PFA, 1% final conc.) before the addition of the radioactive compound. After exposure, the incorporation of substrate was stopped by overnight PFA-fixation (1% final conc.) at 4°C in the dark, and duplicate or triplicate subsamples of 15-25 ml were filtered through 5 μm pore-sized filters (SMWP, Millipore); the filtrate was subsequently filtered through 0.2 μm pore-sized filters (GNWP, Millipore) and rinsed with 0.2 μm filtered seawater. The fraction collected on 0.2 μm filters was mainly comprised by prokaryotic cells, as revealed by microscopy. An intermediate fraction comprising organisms between 0.2 and 1.2 μm was also analyzed in experiments in the Arctic, but since it often yielded very low assimilation percentages and great variability among samples, we did not consider it for the analysis. Macromolecules were precipitated by treating filters with 5 ml of cold 5% TCA for 5 min. The filters were then rinsed with Milli-Q water and their radioactivity determined by placing them into 5 ml of scintillation cocktail (Optimal HiSafe) and counting with a Beckman scintillation counter. The resulting disintegrations per minute (DPM) in the PFA-killed controls of the ^{35}S-DMSP and ^{3}H-leucine amendments were always < 1.5 % of the DPM in the live samples.

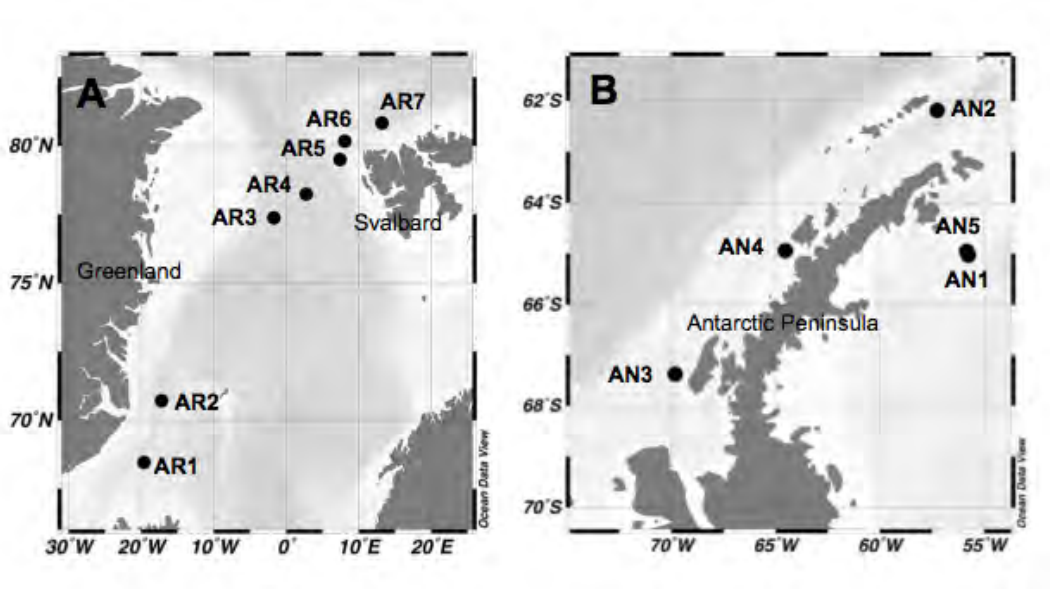

Fig. 1. Map of the stations where different experiments were performed: (A) Arctic stations, July 2007; (B) Antarctic stations, February 2009. Maps were generated with the Ocean Data View software (http://odv.awi.de).

Arctic	Stn	Date (day/mo/yr)	Longitude	Latitude	SW Temp (ºC)	Sampling depth (m)	Incub. time(h)	PAR (E m^{-2})	UVA (kJ m^{-2})	UVB (kJ m^{-2})
	AR1	01/07/07	68º 28.81'W	19º 30.30'N	2.41	5	8.8	-	-	-
	AR2	02/07/07	17º 08.17'W	70º 43.26'N	-0.08	5	10	-	-	-
**	AR3	05/07/07	1º 39.82'W	77º 23.23'N	3.22	5	12	11.1	272.8	9.2
**	AR4	07/07/07	2º51.49'E	78º13.80'N	2.15	5	11.5	5.4	140.8	4.6
*	AR5	12/07/07	7º 29.64'E	79º 30.08'N	0.15	5	11.8	11.4	244.5	7.4
*	AR6	14/07/07	8º 05.16'E	80º 09.93' N	1.36	5	10	10.0	221.1	6.7
*	AR7	19/07/07	13º 14.22' E	80º 49.57'N	0.21	5	9.5	5.3	134.7	4.5
Antarctica										
**	AN1	03/02/09	55º 45.43' W	65º 01.17'S	-0.17	5	7.6	8.4	215.9	9.1
**	AN2	06/02/09	57º 14.42' W	62º 10.63'S	1.67	20	8	1.3	34.8	1.2
	AN3	19/02/09	69º 48.37' W	67º 22.25' S	1.44	5	7.5	-	-	-
	AN4	21/02/09	64º 32.23' W	64º 56.47' S	2.46	5	8.1	-	-	-
	AN5	25/02/09	55º 50.00' W	64º 57.02' S	-0.73	5	7.2	-	-	-

Table 1. Characteristics of the different stations sampled for dark size-fractionated assimilation measurements, time of incubation during experiments and radiation doses received by samples in which light experiments were performed.
(*) Stations where samples for size-fractionated assimilation were also incubated under different light conditions
(**) Stations where, besides size-fractionated assimilation measurements, incubations for microautoradiographic analysis were carried out.

Microautoradiography of algae. Samples of 50 ml were incubated under the different light treatments with added ^{35}S-DMSP (845 Ci $mmol^{-1}$, 0.04 nM final conc. for Arctic samples and 145 Ci $mmol^{-1}$, 0.03 nM final conc. for Antarctic samples) or ^{3}H-leucine (161 Ci $mmol^{-1}$, 0.5 nM final conc.) for 7 to 12 h. Controls killed with PFA were also run simultaneously with all live incubations. After sunlight exposure, live samples were fixed overnight with PFA (1% final conc.) at 4ºC in the dark. Aliquots of 15-20 ml were gently filtered through 5 μm polycarbonate filters (Osmonics, inc.), rinsed with Milli-Q water, air dried and stored at -20ºC until processing. Microautoradiography of ^{35}S-DMSP samples from station AN2 could not be performed due to an insufficient amount of the radiolabeled substrate used.

Microautoradiography was carried out as described by Pedrós-Alió and Newell, 1989 and Vila-Costa *et al.*, 2006. In the dark room, slides were dipped in melted photographic emulsion (NTB-2 KODAK) diluted 2:1 with Milli-Q water. Sections of filters were then placed face-down on the emulsion, and the slides were kept for 10 min on a metal tray in contact with ice for the emulsion to solidify. Finally, they were stored in black boxes at 4ºC for exposure (Arctic samples: for ^{3}H-leucine 6 d, for ^{35}S-DMSP 18 d; Antarctic samples: for ^{3}H-leucine 20 d and for ^{35}S-DMSP 2 mo).

For autoradiographic development, the slides were placed in the developer (KODAK D19) for 3 min, rinsed with Milli-Q water for 30 sec, and then fixed with KODAK Tmax fixer for 3 min followed by 5 min of washing with tap water. Then, the slides were dipped into glycerol (1%) for 2 min and stored inside a dessicator in the dark for 6 h. The filters were gently peeled off so that the cells were transferred to the emulsion and stained with DAPI (1 $\mu g\ ml^{-1}$). Labeled cells were counted under an Olympus BX61 epifluorescence microscope within the major groups showing consistent uptake of any of the substrates. Depending on the abundance of the organisms, between 30 and 700 cells were considered for obtaining the percentages of active cells. Epifluorescence microscopy combined with scanning electronic microscopy was used to identify the eukaryotic microorganisms present in our samples.

Image analysis of the silver grain area surrounding active algal cells. We followed the protocol described by Sintes and Herndl (2006) with modification for algal images. For each sample, three images of the cells were acquired: one of the algae stained with DAPI, one of the fluorescence of chlorophyll (both in epifluorescence mode of a Zeiss Axioplan 2 microscope) and a third image of the silver grains by switching to the transmission mode of the microscope. The images were acquired with a digital camera (AxioCam MRc5) mounted on the microscope. Pictures were taken of 20 to 60 cells per phytoplankton group and treatment. Overlapping signals in the DAPI + chlorophyll images and the transmitted light images (silver grains) indicated cells that had assimilated ^{35}S from DMSP or ^{3}H from leucine. Image analyses were conducted with the KS300 3.0 software (Carl Zeiss), which allowed us to record the area of each cell, as well as the silver grain area around it (see Figs. 4e and 4f). Several, but not all the algal groups were considered for this analysis. In the Arctic experiments, the five groups analyzed were two pennate diatoms (*Pseudonitzschia* spp., *Navicula* spp.), a group of centric diatoms (*Thalassiosira* spp.), autotrophic dinoflagellates (mainly *Prorocentrum* spp., although other species were also included), and the dominant flagellate *Phaeocystis* sp. From Antarctic waters (Station AN1), *Pseudonitzschia* spp., three different species of *Thalassiosira* (spp. A, B and C) with distinct size and chloroplast distribution, and a group of unidentified heterotrophic nanoflagellates were considered. The latter were the only heterotrophic organism analyzed, as they were the ones showing by far the largest ^{35}S-silver grain areas. ^{3}H-leucine samples from station AR3 and AN2 were not analyzed since too few cells were labelled.

Additionally, DAPI-stained bacteria retained onto these filters were counted in order to quantify their contribution to apparent algal substrate uptake. Since many occurred

on aggregates, silver grains could not be attributed to individual bacteria, but an estimated mean silver grain area per bacterial cell was obtained by dividing the silver grain area by the number of bacterial cells in the aggregate.

RESULTS AND DISCUSSION

Effects of sunlight on the bulk assimilation of ^{3}H-leucine and ^{35}S-DMSP by size-fractionated plankton. We observed a widespread capacity for the uptake of leucine and DMSP-sulphur over a wide range of algae. The results of the dark incubations are presented in Fig. 2. Leucine assimilation by organisms > 5 μm was significantly higher than in the size fraction 0.2-5 μm in 8 out of 12 stations (Fig. 2A). Assimilation of ^{35}S-DMSP was always higher in the > 5 μm than in the 0.2-5 μm fraction, mainly comprising heterotrophic bacteria (Fig. 2B). This points to a potentially important role of eukaryotic phytoplankton as low molecular weight (LMW)-DOM consumers and contrasts the common dominant role of bacterioplankton in the uptake of LMW-DOM (Azam and Hodson, 1977; Ellis and Stanford, 1982; Znachor and Nedoma, 2010).

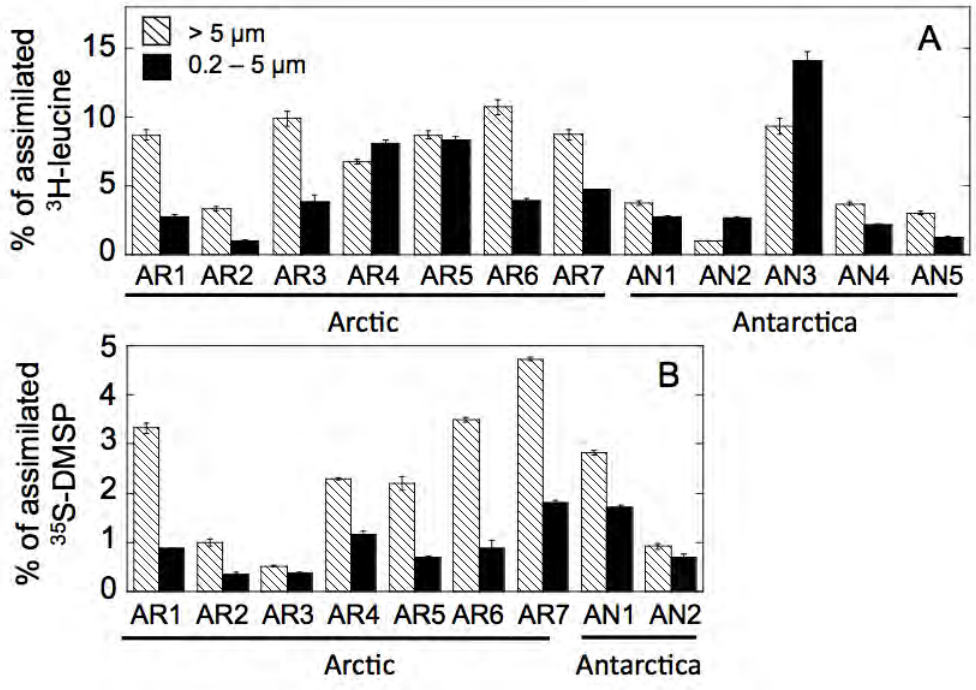

Fig. 2. Comparison between the percentages of assimilated ^{3}H-leucine (A) and ^{35}S-DMSP (B) of total added substrate by organisms > 5 μm (dashed bars) and organisms between 0.2 and 5 μm (black bars) measured at different stations (average ± standard errors). All incubations were performed in the dark

Although the uptake of both leucine and DMSP was initially thought to be specific for heterotrophic bacteria (Kiene *et al.*, 2000; Kirchman *et al.*, 1985; Simó, 2004; Simó *et al.*, 2002; Simon and Azam, 1989; Vila-Costa *et al.*, 2007), several studies have demonstrated that different algal and cyanobacterial species can take up and assimilate either leucine (Hietanen *et al.*, 2002; Kamjunke and Jähnichen, 2000; Kamjunke and Tittel, 2008; Rivkin and Putt, 1987) or reduced sulfur from DMSP (Malmstrom *et al.*, 2005; Vila-Costa *et al.*, 2006). Different amino acids can be used by algae as a carbon source or to meet their cellular nitrogen demands, particularly when inorganic nitrogen is scarce (Bronk *et al.*, 2007; Paerl, 1991; Zotina *et al.*, 2003). However, still very little is known about the magnitude of algal DMSP assimilation in natural communities, its ecophysiological function, and how it influences the cycling of organic sulfur in the surface ocean and whether it can potentially regulate the emission of volatile sulfur to the atmosphere.

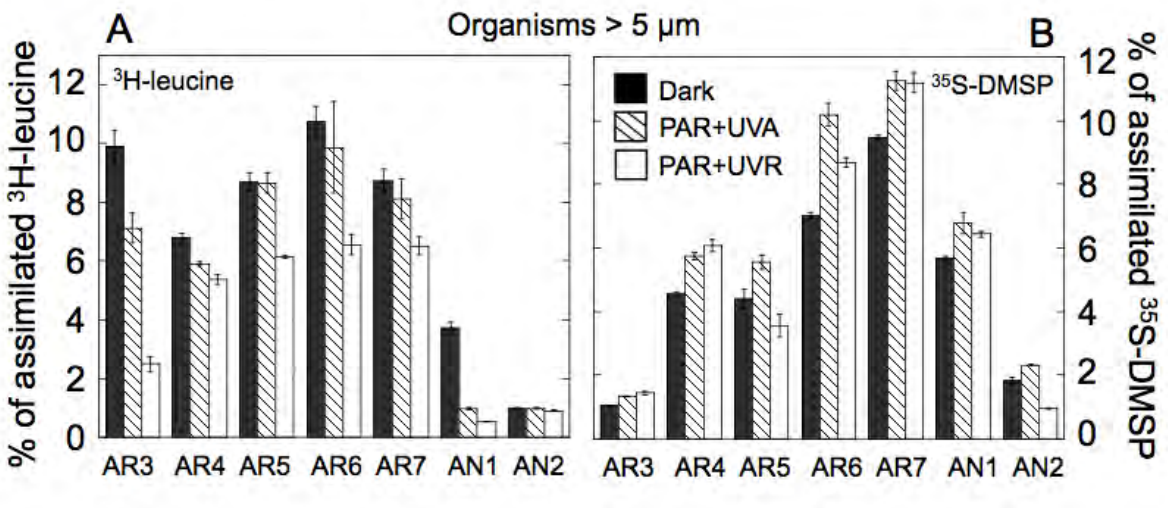

Fig. 3. Percentages of assimilated ^{3}H-leucine (A) and ^{35}S-DMSP (B) of total added substrate by organisms > 5 µm measured after exposure to the following radiation conditions: PAR+UVA (dashed bars), PAR+UVR (white bars) and darkness (black bars). Values are averages ± standard errors.

The results of the subset of incubations performed also under PAR and UVR light are presented in Fig. 3. Exposure to the full spectrum of solar radiation (including UVB) strongly decreased ^{3}H-leucine assimilation by organisms > 5 µm compared to dark treatments in all stations except at AN2 (range 8% to 86% decrease, Fig. 3A). Removal of UVB from the solar spectrum yielded higher assimilation percentages, yet lower than the dark controls at some stations or not significantly different at others. Interestingly, this variability in UVB-induced inhibition of ^{3}H-leucine uptake was significantly correlated with the measured UVB

doses during experiments ($r = 0.88$, $p < 0.01$ compared to both dark and PAR treatments), suggesting that in situ UVR levels affected algae. Several experiments have also shown light-driven effects on the uptake of leucine by phytoplankton cells, although, depending on the species tested, either photostimulation or photoinhibition of uptake or consumption was reported (Church *et al.*, 2006; Kamjunke and Jähnichen, 2000; Kamjunke *et al.*, 2008; Mary *et al.*, 2008; Rivkin and Putt, 1987). Conversely, assimilation of ^{35}S-DMSP by the larger fraction (> 5 μm) seemed to be consistently stimulated by light exposure (Fig. 3B) as also reported elsewhere for diatoms and picophototrophs (Malmstrom *et al.*, 2005; Vila-Costa *et al.*, 2006). At three stations (AR5, AR6 and AN2), the full spectrum of solar radiation inhibited the uptake of DMSP compared to PAR+UVA but none of these responses were directly related to the light doses. Studies on algal osmoheterotrophy have usually exposed samples to artificial light or in situ PAR conditions. Thus, to our knowledge, this is the first report specifically assessing the effects of different doses of solar radiation and the radiation spectrum on phytoplankton heterotrophic activity.

Algal heterotrophic activity by taxonomic groups. Whereas size fractionation may lead to inaccurate estimates of phytoplankton DOM-uptake because it does not completely separate algae from bacterial aggregates, detritus or Protozoa, autoradiographic surveys permit rapid screening of algal populations taking up specific substrates. Microautoradiography was applied to 5 μm filters from four selected stations (AR3, AR4, AN1 and AN2) to determine which phytoplankton phyla were responsible for the measured uptake rates of leucine and DMSP. Most stations revealed that ^{35}S-DMSP and ^{3}H-leucine uptake were widespread among algal groups (Table 2). Percentages of active cells were obtained combining the three light treatments, since generally, it was not the number of active algae, but the size of the silver grain area, which was significantly different among the light treatments. Only some groups displayed changing numbers of active cells among treatments, written in bold in Table 2: *Phaeocystis* spp. (AR3 and AR4) and autotrophic dinoflagellates (AR3) exhibited higher numbers of active cells in ^{35}S-DMSP uptake upon dark incubation compared to the light treatments, whereas *Pseudonitzschia* spp. from stations AR3 and AN1 showed higher uptake after PAR+UVA or both light treatments, respectively. Instead, ^{3}H-leucine uptake by *Pseudonitzschia* spp. from AN1 was inhibited by light incubation. Estimates of abundances and ranges of cell areas within each group are also indicated in Table 2.

Table 2. Most common microorganisms identified in the different stations, cell abundances, microscopic estimates of phytoplankton sizes (expressed as cell area) and percentage of cells showing uptake of organic substrates (average ± standard deviation of the three treatments).

	Percentage of cells active in substrate uptake														
	Arctic								Antarctica						
	Stn. AR3				**Stn. AR4**				**Stn. AN1**				**Stn. AN2**		
	Cells ml^{-1}	Size(μm^2)	DMSP (%)	Leu (%)	Cells ml^{-1}	Size (μm^2)	DMSP (%)	Leu (%)	Cells ml^{-1}	Size (μm^2)	DMSP (%)	Leu (%)	Cells ml^{-1}	Size (μm^2)	Leu (%)
Pseudonitzschia spp.	180	21-69	**87±11**	26±8	1420	28-100	91±5	44±8	270	36-357	**57±47**	**9±8**	2350	113-239	1±1
Navicula spp.	135	102-157	98±3	0	90	83-190	98±2	0	-	-	-	-	-	-	-
Lennoxia spp.	395	110-170	98±3	0	495	179-183	100	0	-	-	-	-	-	-	-
Thalassiosira spp. A	110	57-391	60±8	38±5	190	61-242	94±2	46±4	840	2371-5711	59±5	1±1	120	70-254	32±7
Thalassiosira sp. B	-	-	-	-	-	-	-	-	4360	198-472	95±4	0			
Thalassiosira sp. C	-	-	-	-	-	-	-	-	1470	80-248	70±14	100	-	-	-
Thalassiosira sp. D	-	-	-	-	-	-	-	-	15	876-2473	74±11	100	-	-	-
Chaetoceros spp.	45	118-150	44±3	50±8	55	63-174	17±6	47±7	570	57-943	11±2	43±3	-	-	-
Fragilariopsis spp.	80	5-12	0	0	170	6-13	0	0	530	13-28	0	21±6	-	-	-
Eucampia spp.	25	143-595	100	0	160	277-347	100	0	20	2355-3005	0	100	-	-	-
Lithodesmium spp.	-	-	-	-	-	-	-	-	7	5521-6214	91±9	74±3	-	-	-
Corethron spp.	-	-	-	-	-	-	-	-	3	3200-6300	0	100	< 1	5500-7500	100
Unid. autotrophic dinoflagellates	460	56-300	**47±34**	0	145	61-877	68±21	0	-	-	-	-	-	-	-
Unid. heterotrophic dinoflagellates	180	96-370	98±2	1±1	160	168-440	98±2	0	175	112-314	42±9	0	225	40-148	2±3
Protoperidinium bipes	5	203-380	100	0	20	111-597	100	0	-	-	-	-	-	-	-
Leucocryptos marina	-	-	-	-	90	67-141	100	0	-	-	-	-	-	-	-
Phaeocystis spp.	8320	11-50	**4±7**	0	1940	16-56	**11±15**	0	-	-	-	-	-	-	-
Autotrop. nanoflagellates < 10 µm	2250	5-24	87±10	2±4	550	6-32	95±2	0	270	13-25	12±4	5±4	670	4-13	0
Heterotr. nanoflagellates < 10 µm	-	-	-	-	-	-	-	-	600	13-54	100	98±2	-	-	-

*Data in **bold** indicate significant differences (Tukey's test $p < 0.05$) among light treatments (see Results)

**Thalassiosira* sp. A was so named to differentiate it from species B, C and D from station AN1, but A from different stations was probably not the same species.

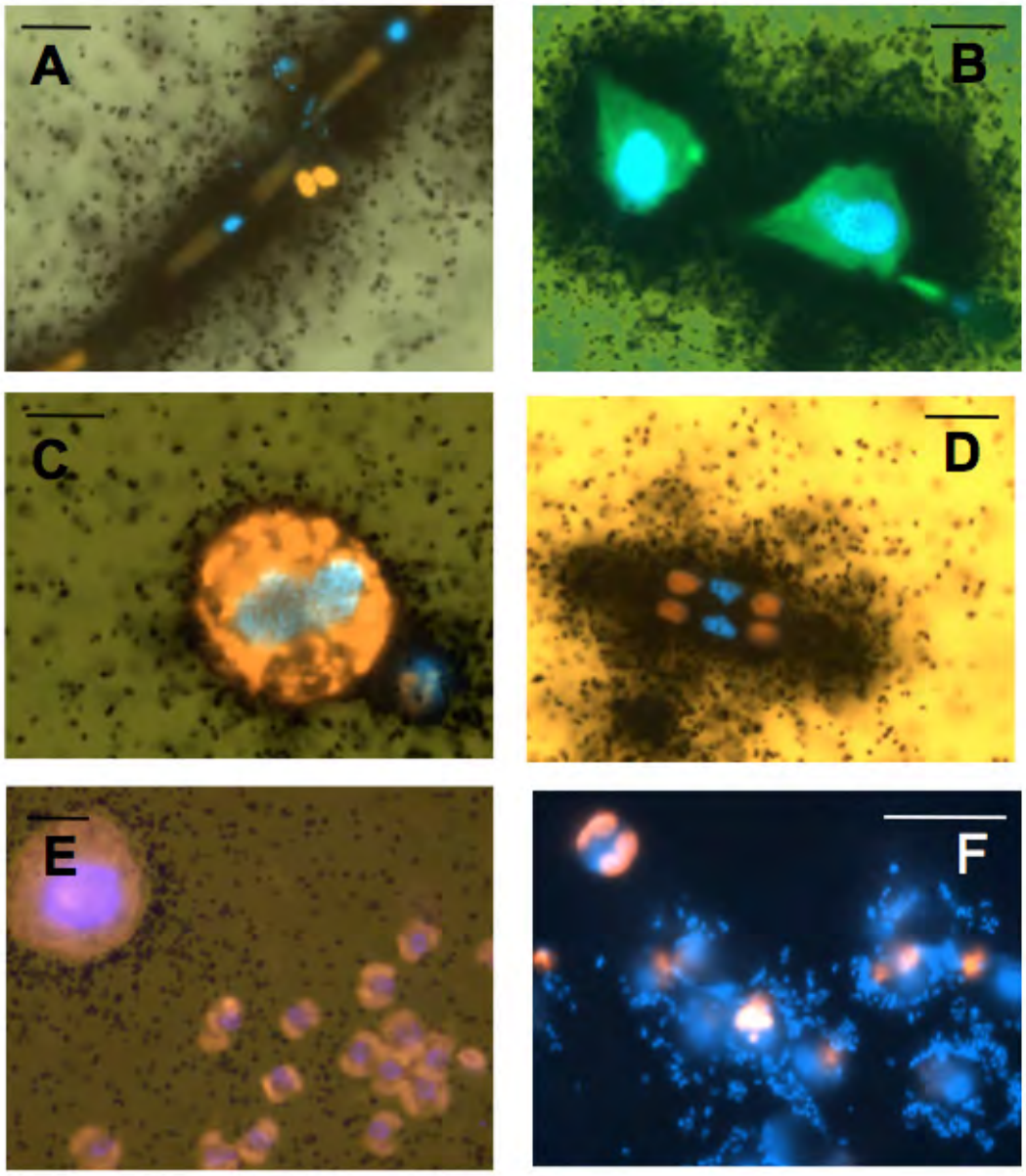

Fig. 4. Microautoradiograms showing uptake of ^{35}S-DMSP by different planktonic organisms in Arctic samples. (A) *Pseudonitzschia* sp.; (B) *Protoperidinium* sp.; (C) unidentified autotrophic dinoflagellate; (D) *Navicula* sp. (E) unlabelled *Phaeocystis* sp. Black dots surrounding cells indicate uptake of the radioactive substrate by algae. (F) Clusters of well-localized silver grains occurred in association with single or clumped bacteria attached to *Phaeocystis* mucus and thus retained onto the 5 μm filters. Scale bar represents 10 μm.

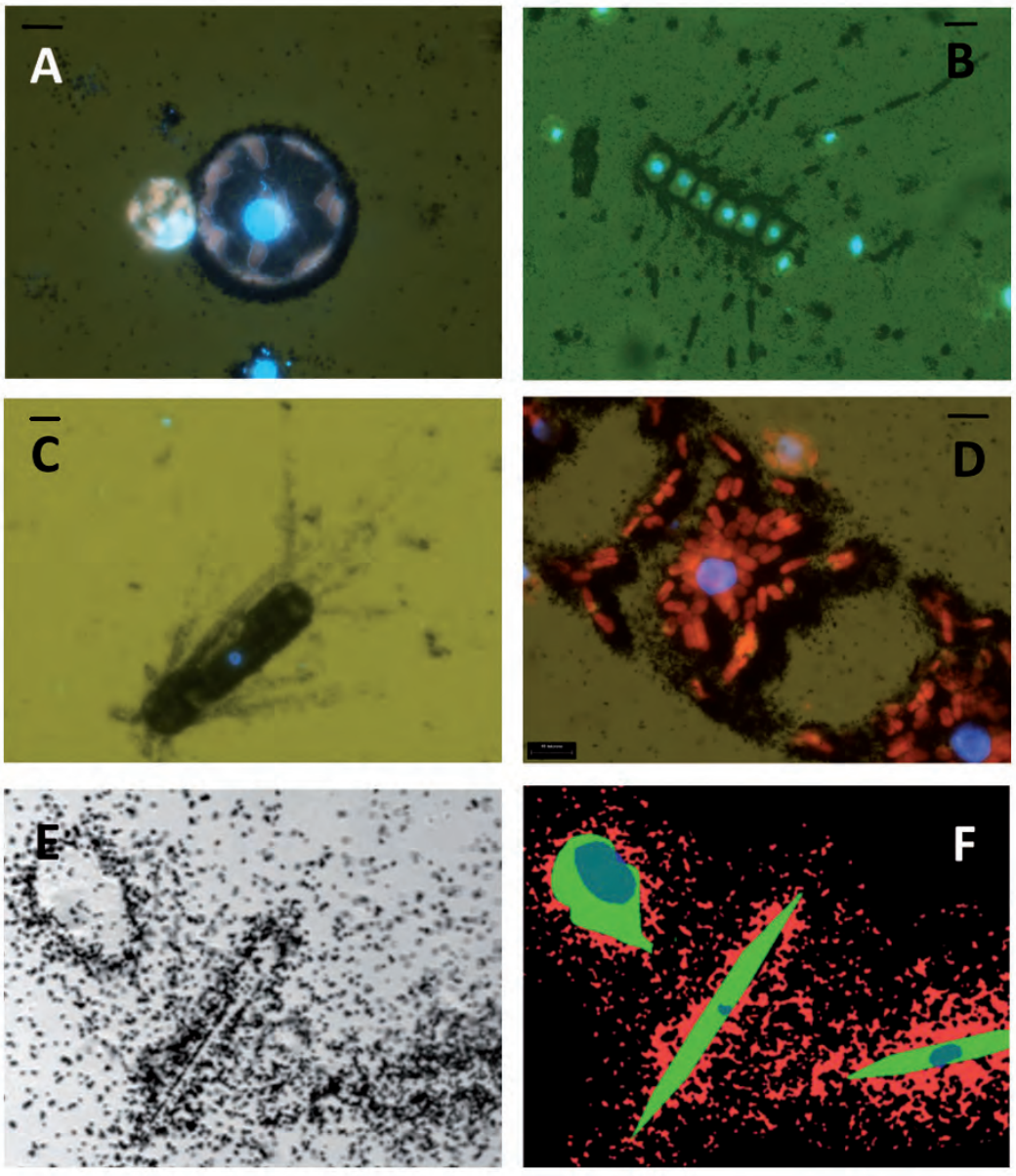

Fig. 5. Microautoradiograms showing uptake of ^{3}H-leucine by different planktonic algae in Antarctic samples. (A) unlabelled *Thalassiosira* sp.B (left) and sp.C (right).; (B) *Chaetoceros* sp.; (C) *Corethron* sp.; (D) *Eucampia* sp. Note that the isotope seems to be specifically incorporated in structures such as chloroplasts (B and D). Scale bar represents 10 μm. (E and F) Examples of image analysis process. Three cells, shown under transmitted light in the left are digitized and nucleus, cell area and silver grain regions are identified, marked with different colors, and then sized. Scale bar represents 10 μm.

In the two Arctic stations (AR3 and AR4) the flagellate *Phaeocystis* sp. dominated the phytoplankton assemblage (Table 2, see also Lasternas and Agustí, 2010), co-occurring with both autotrophic (mainly *Prorocentrum* spp.) and heterotrophic dinoflagellates (AR3), or pennate and centric diatoms (AR4, where *Pseudonitzschia* spp. was nearly as abundant as *Phaeocystis* sp.). Most of these groups showed high uptake of either one or both substrates, although the majority exhibited higher uptake of ^{35}S-DMSP (Table 2). Clearly defined silver grain areas around cells were found upon incubation with ^{35}S-DMSP, whereas the label for ^{3}H-leucine was less intense and generally restricted to fewer groups.

Some typical ^{35}S-DMSP autoradiograms from the Arctic samples are shown in Fig. 4. Diatoms such as *Pseudonitzschia* spp. (Fig. 4A) or *Navicula* (Fig. 4D) appeared heavily labelled. Clusters of well-localized silver grains occurred in association with single or clumped bacteria attached to *Phaeocystis* mucus and thus were retained on the 5 μm filters (Fig. 4F). In all cases, prokaryotic cells were clearly visible due to DAPI staining; bacteria adhering to live microalgae were sometimes present. For the enumeration on DMSP- or leucine-positive algae, only those algae were counted devoid of attached bacteria. Negligible numbers of labelled algal cells in the killed controls were found (< 1 % of cells).

Remarkably, the flagellate *Phaeocystis* sp., the dominant bloom former and DMSP producer in these waters was only weakly labelled with ^{35}S in the dark, the signal was much weaker than that recorded for the other algal groups (Fig. 4F).

The Antarctic stations AN1 and AN2 strongly differed in their phytoplankton composition. While a large number of *Thalassiosira*-like centric diatoms were found in station AN1, station AN2 was almost completely dominated by *Pseudonitzschia* spp. (Table 2). Microautoradiography was performed with both ^{35}S-DMSP and ^{3}H-leucine on samples of station AN1 only, while at station AN2, only ^{3}H-leucine was used. In contrast to what was found in algae from the Arctic, ^{35}S-DMSP uptake was rather low at station AN1 except for the heavily labelled small heterotrophic nanoflagellates. This high uptake of ^{35}S-DMSP (much bigger silver grain areas) in the Arctic might be related to the high DMSP supply rates released by the blooming *Phaeocystis* sp. (Galí and Simó, 2010) compared to the generally lower DMSP concentrations found in Antarctic waters (Galí et al., unpublished data). Supporting this idea, Vila-Costa *et al.* (2006) found higher numbers of ^{35}S-assimilating diatoms in summer, when DMSP contributed a larger share of total sulfur and carbon fluxes. Accordingly, low- or non-DMSP producing diatoms would consume DMSP released by their high producing

phytoplankton partners such as *Phaeocystis* sp., which did not exhibit significant uptake in any of the stations. If this DMSP uptake supplies energy, carbon or sulfur for growth, a DMSP-rich environment like in the Arctic summer might favour algal species capable of utilizing this substrate.

The large diatoms (*Chaetoceros* spp., *Eucampia* spp., *Lithodesmium* spp., *Corethron* spp. and some big *Thalassiosira* (spp. C and D)) showed clear preference for leucine over DMSP as illustrated by the high numbers of active cells (Table 2) and dense silver grain areas around them. Examples of leucine microautoradiograms of Antarctic algae are shown in Fig. 5. At station AN2, however, most of the radiolabel was associated with heterotrophic bacteria. Barely any of the dominant *Pseudonitzschia*, but just a few centric diatoms and heterotrophic dinoflagellates took up leucine.

It is not clear whether microautoradiography reflects actual assimilation (incorporation of the radiolabel into macromolecules) or just uptake. Organic molecules may enter a cell but only support the synthesis of a very limited range of biochemical compounds or may fail to be metabolized at all, in which case they just accumulate in the cytoplasm (Kornberg and Elsden, 1961; Palmer and Togasaki, 1971). Samples were fixed with PFA after exposure to the radioisotopes, a process believed to cause cells to loose cytoplasm (Kiene and Linn, 1999). The fact that the autoradiographic signal remained after fixation points to substrate assimilation. Moreover, the specific labelling patterns observed for some diatoms, which showed silver grains of ^{3}H-leucine specifically associated with structures such as chloroplasts and nucleus, suggests incorporation of the amino acid into cellular macromolecules rather than simple uptake (Figs. 3B and D).

Differential sensitivity to solar radiation among algal groups. In the microautoradiograms of algal samples subjected to image analysis, the differences in the silver grain area around cells reflected the effects of natural sunlight on the assimilation of these two organic compounds. Although not all the groups could be analyzed due to size or abundance limitations, the groups considered comprised ca 70-80 % of the phytoplankton responsible for the uptake of both substrates. We found group-specific responses to either substrate or light (Fig. 6). The pennate diatom *Pseudonitzschia* spp. from the Arctic, the one with the largest silver grain area per cell for ^{35}S-DMSP, showed a significant increase (60% and 72% in Stn. AR3 and AR4, respectively, Tukey's test, $P < 0.05$) in the uptake of DMSP when exposed to PAR+UVA as compared to dark and full solar radiation conditions. Interestingly,

although microautoradiograms from station AN2 are not available, the ^{35}S-DMSP assimilated by the largest fraction, almost entirely comprised by *Pseudonitzschia* spp., also exhibited highest DMSP uptake in the PAR+UVA treatment (Fig. 3B). *Navicula* spp. at station AR4 also showed this pattern. In contrast, both light treatments inhibited ^{35}S-DMSP uptake at station AR3. Generally, dark conditions seemed to stimulate ^{35}S incorporation in the rest of the algal groups from station AR3 and AR4 (Figs. 6A and 6B), while most organisms from AN1 showed a significant photostimulation of ^{35}S-DMSP uptake caused by both light treatments (Fig. 6C). Only heterotrophic nanoflagellates did not show significant differences among different light conditions (Tukey´s test, $P > 0.05$).

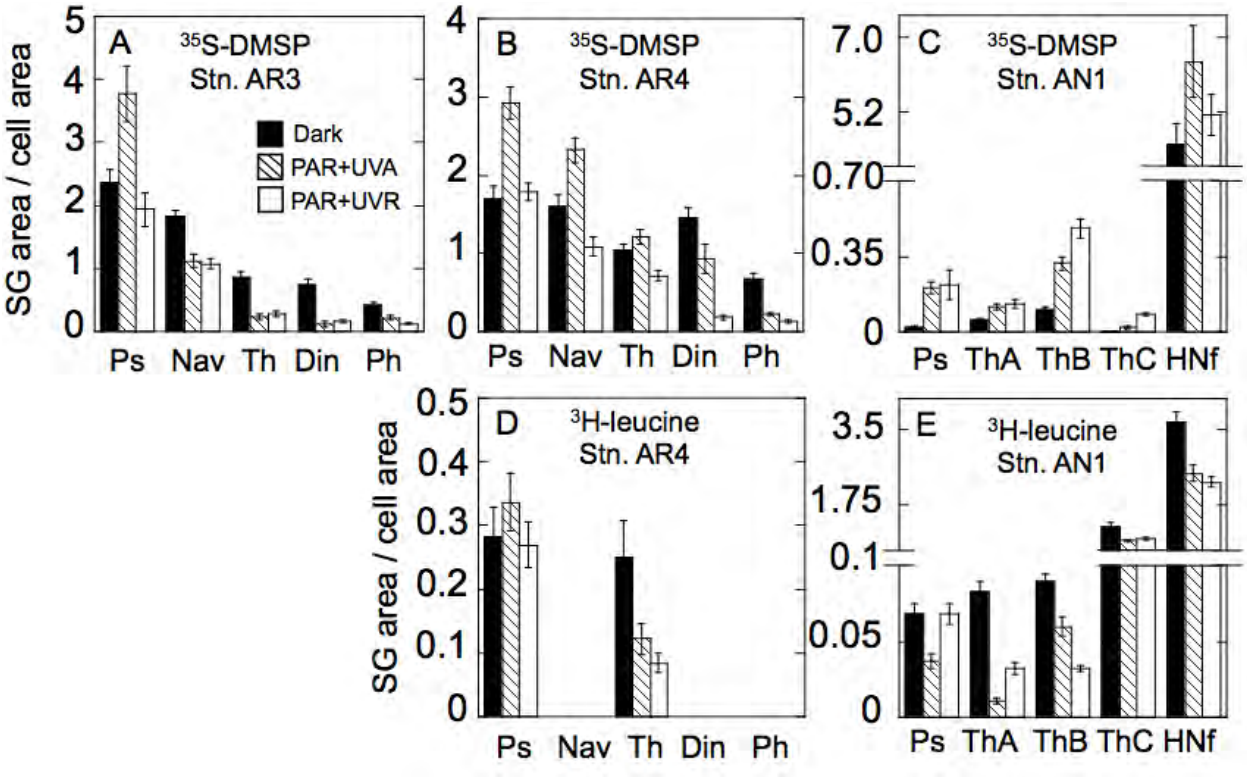

Fig. 6. Average silver grain (SG) area per active algal cell in ^{35}S-DMSP or ^{3}H-leucine uptake as measured by image analysis of microautoradiograms (average ± standard error of 20 to 60 single cells) in 5 μm filters. Stn. AR3, AR4 and AN1, ^{35}S-DMSP samples (A, B and C); Stn. AR4 and AN1, ^{3}H-leucine samples (D and E). Samples were incubated under the following radiation conditions: PAR+UVA (dashed bars), PAR+UV (white bars) and darkness (black bars). Note the break in the Y axis in figures C and D. [Ps] *Pseudonitzschia* spp; [Nav] *Navicula* spp.; [ThA,B,C] *Thalassiosira* spp.A, B, C; [Din] Autotrophic dinoflagellates; [Ph] *Phaeocystis* spp. [HNf] Heterotrophic nanoflagellates.

The uptake of ^{3}H-leucine by *Thalassiosira* spp. at station AR4 was negatively affected by light (Fig. 6D), whereas no significant differences between the light treatments and the dark controls were observed for *Pseudonitzschia* spp. At station AN1, exposure to sunlight either inhibited their activity or did not cause any clear pattern. Only *Thalassiosira* (sp. B) showed a clear negative effect of UVB compared to PAR+UVA exposure (Fig. 6E).

Taken together, it appears that natural UVR levels are influencing the heterotrophic activity of the studied phytoplankton assemblages. However, since a given group can react in different ways depending on the substrate analyzed (i.e., photostimulation for ^{35}S-DMSP and photoinhibition for ^{3}H-leucine) these results cannot be considered as a clear indication of an inhibitory effect of UVR on the activity of the organism. Besides the potentially differential damage of UVR onto different uptake systems (Herndl *et al.*, 1997), several other mechanisms have been proposed. Algal utilization of amino acids has been shown to be most significant in the absence of photosynthesis, e.g., in turbid waters, in dark incubations or at night (Flynn and Butler, 1986; Kamjunke and Tittel, 2008; Schell, 1974; Tuchman *et al.*, 2006). It has been suggested that, during the day, the products of photosynthesis and the uptake of nitrate, ammonium or urea could restrict the uptake of amino acids by increasing the intracellular amino acid pool (Flynn and Butler, 1986). A lower uptake in the light could also be due to dilution of the labelled substrate because of newly photosynthesized substrate, or because of transport systems under repression by photosynthesis catabolites (Hellebust, 1971). Thus, factors other than the light conditions, such as the natural substrate concentration and maybe the past environmental history of the algae affect each species' ability to assimilate organic substrates.

Our observation that ^{35}S-DMSP uptake by various algal groups is enhanced by light could suggest that the photosynthetic apparatus harvests light and transfers this energy into ATP that is used for supplementary powering the active uptake of DMSP. Additionally, increasing DMSP release by UVR-stressed algae (Archer *et al.*, 2010; Sunda *et al.*, 2002) might activate the uptake systems of their low DMSP-producing counterparts. In any case, this light-driven algal DMSP uptake (also observed in the size fractionated assimilation) would lead to higher shares in the hitherto overlooked contribution of phytoplankton as a DMSP sink, particularly in the long daylight of the polar summer.

The group-specific responses shown in Fig. 6 imply different relative contributions to total measured silver grain area (a proxy for total algal uptake) among stations (Fig. 7A, B) and treatments (Table 3). These values were calculated as the mean silver grain area around each group cells multiplied by the abundance of its active cells, and divided by the total sum of silver grain areas of the groups considered for image analysis.

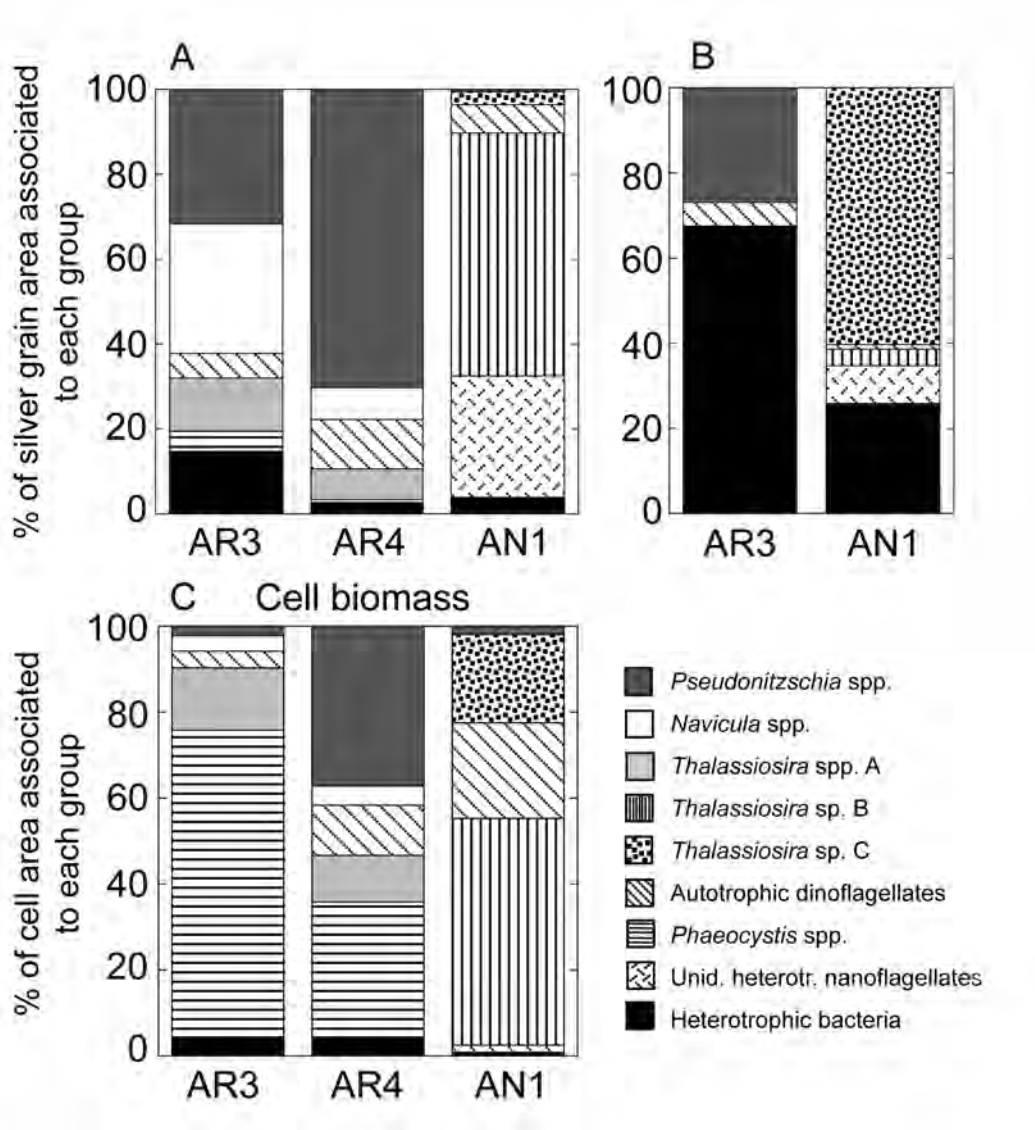

Fig. 7. Average relative contribution of each of the analyzed groups to the total silver grain area (as % of the sum of the silver grain area associated with all the considered groups) for ^{35}S-DMSP (A) or ^{3}H-leucine samples (B). (C) Relative contribution of each of the analyzed groups to total phytoplankton biomass (as % of the sum of cell areas of all the individuals of the considered groups)

Additionally, an estimated contribution of each group to total biomass was calculated by multiplying the average cell area by the abundance of each group divided by the total sum of analyzed cell areas (Fig. 7C). Among these groups, Arctic *Pseudonitzschia* spp. accounted for a significant (stn. AR3) or even dominant (stn. AR4) contribution to total incorporation of ^{35}S-DMSP or ^{3}H-leucine, especially upon light exposure (Figs. 7A, B, Table 3), which was much higher than expected based on their relative abundances (Fig. 7C). Consequently, the contribution of the rest of the groups decreased under PAR+UVA or UVR and PAR +UVA conditions. The less active *Pseudonitzschia* at AN1 contributed only marginally to the uptake of both substrates (Fig. 7A, Table 3). At the Arctic stations, although the specific uptake of ^{35}S-DMSP by *Phaeocystis* sp. was low (or totally absent under light incubations), its high abundance resulted in a substantial contribution (~ 7-12 %) to total uptake in the dark (Figs. 7A, C, Table 3)

Relative contribution of each group to total analyzed silver grain area under different light treatments

		Stn. AR3	Stn. AR4				Stn. AN1	
		^{35}S-DMSP	**^{35}S-DMSP**	**^{3}H-leucine**			**^{35}S-DMSP**	**^{3}H-leucine**
***Pseudonitzschia* spp.**	DARK	16.9	48.4	72.0	***Pseudonitzschia* spp.**	DARK	0.02	0.1
	PAR+UVA	59.0	80.7	89.5		PAR+UVA	1.2	0.0
	PAR+UV	39.5	81.8	93.0		PAR+UV	0.9	0.0
***Navicula* spp.**	DARK	29.3	9.7	-	***Thalassiosira* sp. A**	DARK	8.9	2.1
	PAR+UVA	33.0	6.8	-		PAR+UVA	7.2	0.7
	PAR+UV	43.6	5.9	-		PAR+UV	4.8	1.1
***Thalassiosira* spp.**	DARK	8.9	15.2	28.0	***Thalassiosira* sp. B**	DARK	47.6	8.2
	PAR+UVA	3.5	8.4	10.5		PAR+UVA	62.0	5.0
	PAR+UV	7.9	11.1	7.0		PAR+UV	68.3	3.1
Autotr. dinoflag.	DARK	33.3	19.8	-	***Thalassiosira* sp. C**	DARK	2.8	76.9
	PAR+UVA	4.6	3.9	-		PAR+UVA	3.5	83.0
	PAR+UV	8.9	1.2	-		PAR+UV	2.9	83.8
***Phaeocystis* spp.**	DARK	11.7	7.0	-	**Het. nanoflag.**	DARK	40.6	12.7
	PAR+UVA	0.0	0.2	-		PAR+UVA	26.1	11.3
	PAR+UV	0.0	0.0	-		PAR+UV	23.1	12.0

Table 3. Relative contribution of the analyzed groups to the total silver grain area after exposure to the different light treatments. Percentages were calculated relative to the sum of the silver grain areas associated with all the groups considered for image analysis (excluding bacteria).

At station AN1, the low contribution of heterotrophic nanoflagellates to total cell biomass (3%) contrasts with their high representation among ^{35}S-DMSP- or ^{3}H-leucine-assimilating cells (up to 40% or 10%, respectively, Fig. 7A, B). The highly labelled, large *Thalassiosira* sp. C cells were responsible for most of the ^{3}H-leucine silver grain area around algae (Fig. 7B), even more so under UVR exposure (Table 3). It must be noted, though, that the presence of heavily labelled large diatoms that could not be quantified for uptake, such as *Chaetoceros* spp. or *Eucampia* spp., suggests that the relative contributions of the counted organisms to total ^{3}H-leucine uptake shown in Fig. 7B are most likely overestimates. In any case, our results emphasize the role of light in regulating the amount of organic substrate taken up by specific algal groups.

In an attempt to estimate the contribution of heterotrophic bacteria to the uptake of the >5 μm fraction, we also quantified the silver grain area associated to the filter-retained bacteria. In general, their contribution to ^{35}S-DMSP uptake was small (station AR3) to negligible (AR4 and AN1, Table 4, Fig. 7A) compared to that of the algal groups. Their contribution to ^{3}H-leucine uptake, however, was as high as 70% and 30 % at stations AR4 and AN1, respectively (Table 4). These latter values are overestimates because, as mentioned above, not all eukaryotic organisms were counted for their assimilation. Taken together, our results suggest that phytoplankton were responsible for the vast majority of the ^{35}S-DMSP assimilation in the 5 μm fraction, whereas bacteria retained on these filters seemed to account for a considerable proportion of the assimilated ^{3}H-leucine in all samples but AN1.

	Relative contribution to total silver grain area (% of the sum of silver grain areas associated with all analysed groups)			
	^{35}S-DMSP		**^{3}H-Leucine**	
	Prokaryotic cells	Phytoplankton cells	Prokaryotic cells	Phytoplankton cells
Stn. AR3	14.86	85.14	-	-
Stn. AR4	2.15	97.85	67.64	32.36
Stn. AN1	3.85	96.15	28.49	71.51

Table 4. Comparison between the relative contribution of prokaryotic and phytoplankton cells within 5 μm filters. Percentages were calculated relative to the sum of silver grain areas associated to the analysed algal groups plus heterotrophic bacteria, and values are means of the three treatments.

Part of the radioisotope incorporation by algae could have occurred through bacterivory or phagotrophy. Members of the dinophytes, cryptophytes and haptophytes have been shown to feed on bacteria or other algae (Bird and Kalff, 1989; Jones, 1994; Raven, 1997) whereas diatoms do not. Thus, it might be possible that some of the uptake was not due to uptake of radiolabeled DOM but due to grazing on ^{35}S- or ^{3}H-consuming bacteria or algae; in fact, bacterivory can also be regulated by light (Legrand *et al.*, 1998; Sommaruga *et al.*, 1996). Large hetero- or autotrophic Arctic dinoflagellates such as *Protoperidinium* spp. (Fig. 3b), *Prorocentrum* spp. and the flagellate *Leucocryptos marina* appeared intensely labelled for ^{35}S-DMSP but not for ^{3}H-leucine. This uptake pattern discards bacterivory as a major source of the ^{35}S label and rather suggests osmotrophic uptake (Saló *et al.*, 2009) or grazing on smaller labelled algae. Similarly, small heterototrophic nanoflagellates of the Southern Ocean could have been grazing on bacteria and the observed response to light might actually reflect the effect of UVR on bacterioplankton. Indeed, the responses of these nanoflagellates were similar to those found for some groups of bacteria within the same samples (**Chapter 3**). Remarkably, diatoms showed the greater cell-specific uptake, and they must have directly taken up both substrates from the dissolved pool. Diatoms, particularly polar species that have to survive long winter darkness, are known for their dark survival potentials (Antia and Cheng, 1970; Palmisano and Sullivan, 1982; Smayda and Mitchell-Innes, 1974) with facultative heterotrophy as one of their survival strategies. Therefore, since they were major contributors to ^{35}S-DMSP uptake in most stations (Fig. 7A), an important fraction of the substrate should have been actually taken up by osmoheterotrophy rather than phagotrophy.

UVR-induced shifts in the abundance of algal species have been suggested from experimental results with natural communities (Belzile *et al.*, 2006; Roy *et al.*, 2006). Our incubations were too short to detect changes within planktonic assemblages, but the observed different sensitivities to solar radiation together with the reported interspecific variability in the sensitivity to UVR and recovery from UVR stress among different algae (Davidson *et al.*, 1994; Helbling *et al.*, 1996; Xue *et al.*, 2005) suggest an important role of UVR in determining ecosystem structure through selection of UVR-resistant groups. Indeed, diatoms from polar regions have often been shown to be more resistant to UVR than flagellates and dinoflagellates (Karentz, 1994; Helbling *et al.*, 1994; Davidson and Marchant, 1994). This, together with their osmoheterotrophic potential, might help them to dominate the phytoplankton assemblages in these polar regions.

CONCLUSIONS

Our results support the notion of a major and widespread heterotrophic activity within phytoplankton assemblages during Arctic and Antarctic summers. The use of autoradiography combined with size-fractionated assimilation offers a way to screen phytoplankton populations for heterotrophic potential. This approach has revealed distinct affinities and behavioural trends in polar algae with regard to ^{3}H-leucine and ^{35}S-DMSP uptake and solar radiation. The general trend emerged of a UVR-driven inhibition of ^{3}H-leucine assimilation and photostimulation of ^{35}S-DMSP uptake. The Arctic phytoplankton associated with a dense bloom of colonial *Phaeocystis* sp. were well-adapted to benefit from released DMSP, whereas the lower concentrations of this substrate in Antarctic waters resulted in lower uptake. Both algal assemblages were able of taking up ^{3}H-leucine. While algae (mainly diatoms) accounted for most of the ^{35}S-DMSP assimilation by the > 5 μm fraction, the relative contribution of heterotrophic bacteria to the total assimilated ^{3}H-leucine within this fraction varied depending on the species composition, with lower values when significant uptake by diatoms occurred. These results suggest that light might be strongly affecting the cycling of dissolved labile compounds through different levels of the trophic webs.

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Chapter 5

Solar radiation quality modulates the relative importance of heterotrophic bacteria and picophytoplankton in DMSP-sulphur uptake

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ABSTRACT

There is a large body of evidence supporting a major role of heterotrophic bacteria in dimethylsulphoniopropionate (DMSP) utilisation as a source of reduced sulphur. However, a role for phototrophic microorganisms has only been recently described and little is known about their contribution to DMSP consumption and the potential modulating effects of sunlight. Among the ecological factors that might affect the role of heterotrophic and phototrophic microorganisms, sunlight is particularly relevant. In an attempt to ascertain the relative quantitative roles of heterotrophic bacteria and picophytoplankton in the osmoheterotrophic uptake of DMSP-sulphur upon exposure to natural sunlight, we incubated NW Mediterranean waters under various optical filters and used an array of bulk and single-cell activity methods to trace the fate of added ^{35}S-DMSP. Flow cytometry cell sorting confirmed dark ^{35}S uptake by *Prochlorococcus, Synechococcus,* and heterotrophic bacteria, the latter being the most efficient in terms of uptake on a cell volume basis. Under exposure to full sunlight, however, the relative contribution of *Synechococcus* was significantly enhanced mainly due to the inhibition of heterotrophic bacteria. Microautoradiography showed a strong increase in the proportion of *Synechococcus* cells actively taking up ^{35}S-DMSP, which, after full sunlight exposure, made up to 30% of total active *Eubacteria*. Parallel incubations with ^{3}H-leucine generally showed no clear responses to light. Finally, size-fractionated assimilation experiments showed greater cyanobacterial assimilation during the day than at night. Our results support a major influence of sunlight in regulating the competition among autotrophic and heterotrophic picoplankton for DMSP uptake at both the daily and seasonal time scales.

INTRODUCTION

Dimethylsulfide (DMS) is a biogenic volatile compound that is universally present in seawater (Kettle *et al.*, 1999; Lovelock *et al.*, 1972) and represents the major natural source of sulphur to the global troposphere (Andreae and Crutzen, 1997; Bates *et al.*, 1992). The biogeochemical significance of DMS was first suggested when its emissions were found to be a key step in the global sulphur cycle (Lovelock *et al.*, 1972), and research was further encouraged when marine plankton was proposed to play a significant role in climate regulation through the effects of DMS emissions on cloud formation (Charlson *et al.*, 1987).

The biochemical precursor of DMS is dimethylsulphoniopropionate (DMSP), an osmolyte produced by many phytoplankton taxa that is released into the dissolved organic matter pool through grazing, viral lysis, algal autolysis, or exudation (Simó, 2001; Stefels, 2000), thus becoming available as a significant source of carbon and sulphur for other planktonic organisms. Released dissolved DMSP (DMSPd) also acts as a direct or indirect (by transformation into DMS) chemical signal for plankton microbes (Seymour *et al.*, 2010), marine invertebrates, fish, birds and mammals (Cunningham *et al.*, 2008; DeBose *et al.*, 2008; Nevitt, 2008; Van Alstyne *et al.*, 2001).

Among the marine organisms directly utilizing DMSP, heterotrophic bacteria have been the most extensively studied (Howard *et al.*, 2006; Kiene *et al.*, 1999; Simó *et al.*, 2002; Vila-Costa *et al.*, 2007) and their capacity to assimilate sulphur from DMSP appears to be widespread among different taxonomic groups (González *et al.*, 1999; Malmstrom *et al.*, 2004a; Malmstrom *et al.*, 2004b; Vila *et al.*, 2004; Vila-Costa *et al.*, 2008b; **Chapter 3**). Actually, bacterial uptake of DMSPd and partial assimilation of its sulphur is thought to be the dominant mechanism for DMSP degradation in the pelagic ocean (Kiene et al. 2000; Zubkov et al. 2002). Other transformation processes include cleavage into DMS and carbon products mediated by the DMSP producers themselves and bacteria (Stefels, 2000; Sunda *et al.*, 2002), accumulation or assimilation by zooplankton grazing on phytoplankton (Archer *et al.*, 2003; Dacey and Wakeham, 1986; Saló *et al.*, 2009; Tang and Simó, 2003), and direct uptake and assimilation by eukaryotic phytoplankton (Vila-Costa *et al.*, 2006, **Chapter 4**) and cyanobacteria (Malmstrom *et al.*, 2005; Vila-Costa *et al.*, 2006).

Among the aforementioned transformation processes, little is known about the quantitative role of non-DMSP producing photosynthetic organisms. Due to their autotrophic lifestyle, the contribution of phytoplankton to the turnover of DMSP was expected to be minimal, but *Synechococcus* were reported to be major consumers of DMSP and methanethiol (MeSH) with contributions comparable to other bacterial groups (Malmstrom *et al.*, 2005). Similarly, also *Prochlorococcus*, diatoms, and photosynthetic picoeukaryotes are able to take up and assimilate a remarkable fraction of DMSP (Vila-Costa *et al.*, 2006), suggesting that, in the appropriate circumstances, they could compete with heterotrophic bacteria for this substrate.

An important implication of DMSP use by phytoplankton is the possible effect of light on DMSP consumption processes. Since algae are affected by variations in energy supply due to changes in the quantity and quality of light available, processes such as DMSP-sulphur assimilation may also be coupled to this periodicity. Only in two studies has light been shown to affect the assimilation of DMSP-sulphur by phototrophs, providing variable degrees of light-driven stimulation of the uptake (Malmstrom *et al.*, 2005; Vila-Costa *et al.*, 2006). Therefore, light may be playing an important role in regulating the relative contributions of heterotrophic and phototrophic uptake to total DMSP consumption. So far, however, experiments had been conducted in the absence of ultraviolet radiation (UVR, 280-400 nm) and hence, it is likely that the contribution of phytoplankton relative to that of heterotrophic bacterioplankton (generally less UVR-protected) had been underestimated. UVR has recently been found to be a significant factor modifying the fate of DMSP through either inhibition of microbial consumption (Slezak *et al.*, 2001; Slezak *et al.*, 2007) or stimulation of its production, release (Archer *et al.*, 2010; Slezak and Herndl, 2003; Sunda *et al.*, 2002), or uptake by autotrophs (Malmstrom *et al.*, 2005; Vila-Costa *et al.*, 2006, **Chapter 4**). Incubations under natural sunlight conditions are necessary to properly assess the shares of the different components of the microbial food web in the use of this widespread substrate.

Our aim in this study was to assess the role of sunlight, including UVR, in DMSP uptake by picoplankton through experiments conducted with plankton communities sampled at the Blanes Bay Microbial Observatory and adjacent offshore waters of the northwestern Mediterranean. We used an array of multiple bulk and specific activity methods including

flow cytometry cell sorting, size-fractionated assimilation, and microautoradiography combined with RNA probing after samples were exposed to different light spectrum conditions. Since sunlight has the potential to trigger the autotrophic activity and simultaneously inhibit heterotrophic bacterial activity, our hypothesis was that exposure to enhanced natural solar radiation would favour picophytoplankton in their competition for DMSP uptake against heterotrophic bacteria.

MATERIALS AND METHODS

Study area and sample collection. Water samples were collected either from a shallow (20 m depth) coastal station (the Blanes Bay Microbial Observatory, BBMO) located 800 m offshore, or during a cruise aboard the RV 'García del Cid' between 18 and 26 September 2007 in two stations, one located on the continental shelf in the vicinity of the BBMO (Stn C), and another one offshore over a 2000 m deep water column in the Catalan Sea (NW Mediterranean) between the BBMO and Mallorca (Table 1). Surface samples (0.5 m) from the BBMO were collected with a Niskin Go-flow bottle (5 liters), prefiltered through a 200 μm-mesh-size net, and transported under dim light to the lab for the three experiments carried out on 5 August 2003 (exp. 2), 9 July 2008, and 30 September 2008 (exp. 3 and 4, respectively). Water samples during the cruise (4 or 48 m depth) were taken with a 12 Niskin bottle rosette attached to the CTD (exp. 1, 5 and 6). Incubations were started less than 20 minutes after collection.

Experimental design. Different types of incubations were carried out with added trace ^{35}S-DMSP, kindly donated by R. P. Kiene (University of South Alabama, Dauphin Island Sea Lab, USA). At several occasions, parallel incubations with ^{3}H-leucine (Amersham, 161 Ci mmol^{-1}) were also done for comparative purposes since it is widely used as a measurement of bacterial heterotrophic production (Kirchman *et al.*, 1985). Only in experiments 2, 3 and 4, the PAR and UVR doses were monitored during incubations with a Biospherical PUV-radiometer 2500. UVB doses are shown in Table 1.

Experiment 1: dark incubation for cell sorting. Water was collected around midnight from 48 m depth at the offshore station D. This was the depth where the cell abundance of *Prochlorococcus* (1.8 10^5 cells ml^{-1}, Table 1) was large enough to allow for being sorted. A single 50 ml sample was amended with ^{35}S-DMSP (815 Ci $mmol^{-1}$, 0.03 nM final conc.) and incubated in the dark for 6 h at in situ temperature (ca. 17ºC). After exposure, 5 ml subsamples were fixed with 1% paraformaldehyde + 0.05% glutaraldehyde (final conc.), flash-frozen in liquid nitrogen, and stored at -80ºC. Killed controls were prepared by addition of the fixative 30 min before the addition of the radioisotope, and were simultaneously incubated with the live samples.

Experiments 2, 3 and 4: light manipulation experiments. Spherical quartz glass bottles of 50 or 100 ml (same diameter for a single experiment) were used to incubate surface water samples (0.5 m depth) collected in the Blanes Bay for 4 h under different light conditions. In experiments 3 and 4, samples were amended with trace ^{35}S-DMSP (453 and 23 Ci $mmol^{-1}$, 0.08 and 1 nM final conc., respectively) or ^{3}H-leucine (161 Ci $mmol^{-1}$, 0.5 nM final conc.) immediately before exposure, whereas in experiment 2, they were added after the light incubations. In this experiment (#2), the conditions were: (i) full sunlight spectrum; (ii) full spectrum minus UVB; (iii) full spectrum minus the whole of UVR, i.e., photosynthetic active radiation (PAR) only; and (iv) darkness (wrapped with aluminium foil inside a black plastic bag). In experiments 3 and 4, the conditions were the same except for that treatment (ii) was excluded. For the removal of UVB radiation (i.e., PAR + UVA treatment), one layer of the plastic foil Mylar-D (150 μm thickness, 50% transmission at 325 nm) was used. For PAR-only treatments, bottles from experiment 2 were wrapped with one layer of a vinyl chloride foil (50% transmittance at 405 nm; CI Kasei Co., Tokyo, Japan), and bottles from experiments 3 and 4 were covered with two layers of Ultraphan URUV (0.1 mm thickness, 50% transmittance at 380 nm). All bottles were incubated 4 cm under the water surface inside a black tank with circulating seawater to maintain in situ temperature. After sunlight exposure, 50 - 100 ml subsamples from experiment 2 were incubated for 4 h with trace additions of ^{35}S-DMSP (0.1 nM, specific activity 130-350 Ci $mmol^{-1}$) or ^{3}H-leucine (161 Ci $mmol^{-1}$, 0.5 nM final conc.) in the dark in acid-cleaned glass serum vials. Samples were fixed with 1% paraformaldehyde + 0.05% glutaraldehyde (final conc.), flash-frozen in liquid nitrogen, and stored at -80ºC. Live samples from experiments 3 and 4 were fixed overnight with paraformaldehyde (PFA,

1% final conc.) at 4ºC in the dark. Aliquots of 10-15 ml were filtered through 0.22 μm polycarbonate filters (GTTP, Millipore), rinsed with milli-Q water, air dried and stored at -20ºC until processing.

Experiments 5 and 6: day-night cycles. During the September 2007 cruise, two diel cycle studies of the assimilation of ^{35}S-DMSP by different size fractions were conducted. For that purpose, 50 ml surface water samples (4 m depth) were collected every 4 h during two 24 h periods and trace concentrations of ^{35}S-DMSP (815 Ci mmol^{-1}, 0.8 pM final conc.) were added. Samples were then incubated in 50 ml quartz flasks with the radioisotope for 6 h at in situ light and temperature conditions inside a black tank with circulating seawater. Killed controls were prepared in 30 ml Teflon flasks by adding PFA (1% final conc.) before the addition of the radioisotope. After exposure, the incorporation of substrate was stopped by fixing samples overnight with PFA (1% final conc.) at 4ºC in the dark.

Flow cytometry cell sorting. In experiments 1 and 2, different populations were identified and sorted using a FACSCalibur flow cytometer-cell sorter (Becton Dickinson). Sorted cells were collected onto 0.2 μm nylon filters and assayed by liquid scintillation counting. We used the "single cell sort" mode of the instrument and sorted between 100,000 and 400,000 bacteria, between 30,000 and 130,000 *Synechococcus,* and between 30,000 to 90,000 *Prochlorococcus* (the latter in exp. 1 only). Assimilation of ^{35}S or ^{3}H-leucine in killed samples was 2-3% of the value in live samples.

Microautoradiography combined with catalyzed reporter deposition - fluorescence in situ hybridisation (MAR-CARD-FISH). Filters with retained cells from exps. 3 and 4 were hybridised following the CARD-FISH protocol (Pernthaler *et al.*, 2002). Two horseradish peroxidase (HRP)-probes were used to specifically identify most *Eubacteria* (Eub338-II-III, Amann *et al.*, 1990; Daims *et al.*, 1999) and the cyanobacterial genus *Synechococcus* (Syn405, West *et al.*, 2001). Filters were first permeabilized with lysozyme (37ºC, 1 h) and achromopeptidase (37ºC, 0.5 h) before the hybridisation. Hybridisations were done on sections of the filters at 35ºC overnight, and specific hybridisation conditions were established by addition of formamide to the hybridisation buffers (55% formamide for Eub338-II-III and 60% for Syn405). Smaller pieces from each hybridised section were cut and stained with 4,6-diamidino-2-

phenylindole (DAPI, 1 μg ml^{-1}) to estimate the relative abundance of each group before applying the microautoradiography. Between 500 and 800 DAPI-positive-cells were counted manually in an Olympus BX61 epifluorescence microscope within a minimum of 10 fields. The sections were glued onto slides and embedded in 46°C tempered photographic emulsion (KODAK NTB-2) containing 0.1% agarose in a dark room. The slides were placed face-up on an ice-cold metal bar for about 5 min for the emulsion to solidify, and then stored inside black boxes at 4°C for exposure until development (3 days for ^{3}H-leucine and ^{35}S-DMSP in experiment 3, and 5 days for ^{3}H-leucine and 7 days for ^{35}S-DMSP in experiment 4). Slides were developed by placing them into the developer (KODAK D19) for 3 min followed by fixation with KODAK Tmax fixer for 3 min and 5 min of washing with tap water. Slides were then dried in a dessicator overnight, stained with DAPI (1 μg ml^{-1}) and between 500 and 700 hybridised cells were counted manually within a minimum of 10 fields.

Isotope assimilation during incubations. Triplicate aliquots of samples from exps. 5 and 6 (previously prefiltered through 3 μm pore-sized filters to exclude larger organisms, SSWP, Millipore) were sequentially filtered through 0.65 μm and 0.2 μm pore-sized filters (DAWP and GNWP, respectively, Millipore) and rinsed with 15 ml of 0.2 μm-filtered seawater. Macromolecules were precipitated by treating filters with 5 ml of cold TCA 5% for 5 min. The filters were then rinsed with milli-Q water, placed into 5 ml of scintillation cocktail (Optimal HiSafe), and counted with a Beckman scintillation counter. Incorporation of ^{35}S-DMSP and ^{3}H-leucine in PFA-killed controls was always < 1.5% of that in live samples.

RESULTS

Flow cytometry cell sorting of samples exposed to fractional sunlight (exps. 1 and 2). Flow cytometry cell sorting of samples amended with ^{35}S-DMSP was used to investigate the relative role of heterotrophic bacteria and cyanobacteria in the uptake of DMSP-sulphur. Experiments 1 and 2 were conducted with water from the offshore station D and the coastal BBMO site, respectively. Among cyanobacteria,

Synechococcus occurred in high numbers at both stations, but *Prochlorococcus* only occurred in sufficient numbers at a depth of *ca*. 50 m in station D, experiment 1 (Table 1). Cell sorting in this experiment showed that heterotrophic bacteria, *Prochlorococcus,* and *Synechococcus* had the capability of assimilating ^{35}S from DMSP (Fig. 1a). On a per cell basis, the most important DMSP-sulphur assimilators were *Synechococcus* (Fig. 1b), which showed 8 times more dpm per cell than heterotrophic bacteria. However, when these values were corrected for total cell volume (assuming a volume ratio HB:*Prochl*:*Syn* = 1:5:10), heterotrophic bacteria were the most relevant consumers of ^{35}S-DMSP followed by *Synechococcus* and *Prochlorococcus* (Fig.1c).

Station	Exp.	Techniques	Date (day/mo/yr)	Longitude	Latitude	Depth (m)	HB (10^5 ml^{-1})	Syn (10^4 ml^{-1})	Prochl (10^4 ml^{-1})	UVB (kJ m^{-2})
D	1	Sorting (dark)	24/09/07	2º 51.06'E	40º 39.60'N	48	6.9	2.1	17.8	-
BBMO	2	Sorting (light)	05/08/03	2º 48.03'E	41º 39.90'N	0.5	8.5	7.2	0.2	21.0
BBMO	3	MARCARDFISH	09/07/08	2º 48.03'E	41º 39.90'N	0.5	7.8	3.6	-	22.0
BBMO	4	MARCARDFISH	30/09/08	2º 48.03'E	41º 39.90'N	0.5	7.7	4.8	3.0	11.3
C	5	Diel cycle (Assim)	18-19/09/07	2º 47.58'E	41º 40.08'N	4	4.8	0.5	0.4	-
D	6	Diel cycle (Assim)	23-24/09/07	2º 51.06'E	40º 39.60'N	4	6.0	1.5	1.3	-

Table 1 Sampling locations, methodology,and date of each experiment. The abundances of heterotrophic bacteria (HB), *Synechococcus* (Syn), and *Prochlorococcus* (Prochl) are given for the initial water sample. Assim = Size fractionated ^{35}S-DMSP assimilation. Integrated UVB radiation during experiments was determined with a PUV radiometer (exps. 3 and 4) or estimated from the irradiance values collected at the Malgrat de Mar Metereological station (exp. 1, see Methods).

Incubation of samples from the Blanes Bay under increasing short-wave radiation doses (exp. 2, Fig. 2) resulted in a significant reduction of the contribution of heterotrophic bacteria to total ^{35}S-DMSP uptake (Tukey's test, $p < 0.05$), from 84% in the dark to 17% after full sunlight exposure. Conversely, *Synechococcus* did not seem to be negatively affected by full sunlight exposure, and even a slight photostimulation of their uptake was apparent, yet not significant at $p < 0.05$ (Fig. 2a). As a result, the contribution of *Synechococcus* relative to that of heterotrophic bacteria increased after full sunlight (PAR+UVR) exposure up to 7-fold (Fig. 2c), accounting for 40% of the heterotrophic bacterial ^{35}S-DMSP uptake.

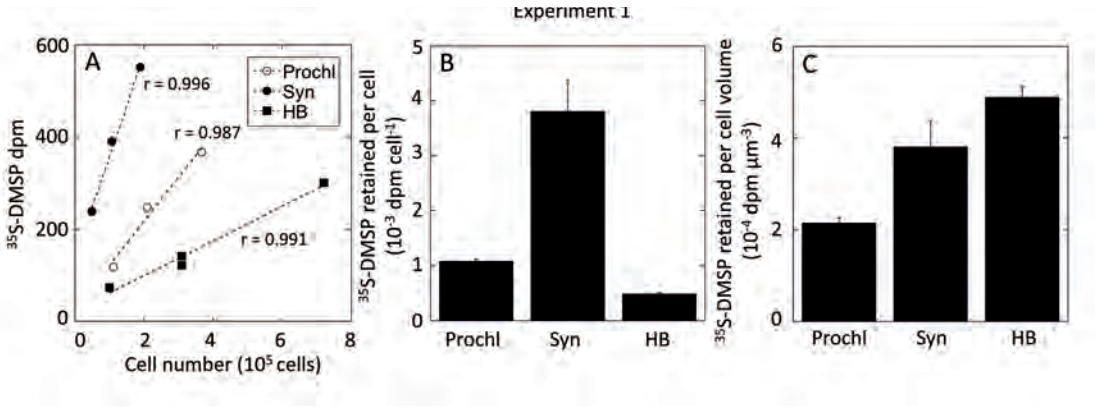

Fig. 1. Experiment 1. (A) ^{35}S-DMSP retained in cells (dpm) as a function of the number of sorted cells: *Prochlorococcus* (Prochl), *Synechococcus* (Syn), and heterotrophic bacteria (HB). (B) Average ^{35}S-DMSP retained per cell (dpm cell^{-1}). (C) Same as B but scaled to cell volume (dpm µm^{-3}), assuming that *Prochlorococcus* and *Synechococcus* are 5 and 10 times larger, respectively, than heterotrophic bacteria. Shown are average ± standard error.

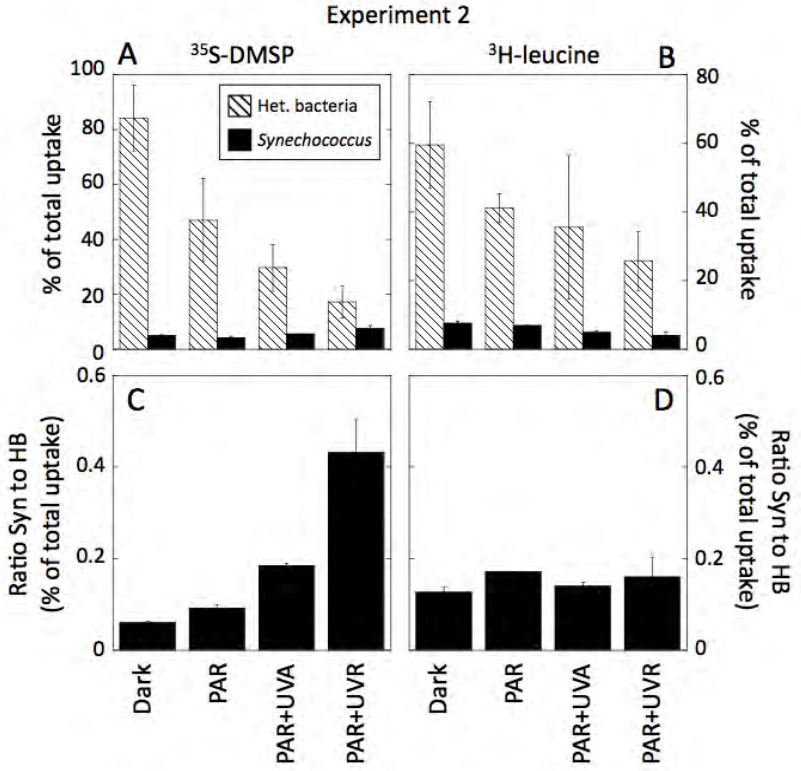

Fig. 2. Experiment 2. Top panels: Contribution of sorted heterotrophic bacteria and *Synechococcus* cells to total uptake of (A) ^{35}S-DMSP or (B) ^{3}H-leucine under different light conditions. Shown are average ± standard error. Bottom panels: ratio between the contribution of *Synechococcus* (Syn) and heterotrophic bacteria (HB) to total uptake of (C) ^{35}S-DMSP or (D) ^{3}H-leucine.

When we further assessed the role of light on the efficiency of ^{35}S-DMSP uptake per cell volume, the differences were even greater (Fig. 3). Exposure to full sunlight caused a drastic decrease in the dpm μm^{-3} in heterotrophic bacteria to the extent that they equalled those of *Synechococcus* (Fig. 3a). Therefore, under full sunlight, *Synechococcus* cells seemed to be, on average, as efficient in taking up ^{35}S-DMSP as the average heterotrophic bacterium, as shown by the high ratio between the uptake per unit of cell volume of *Synechococcus* and heterotrophic bacteria (Fig. 3b).

With ^{3}H-leucine as the added substrate, a decreasing trend in the uptake by both *Synechococcus* and heterotrophic bacteria in all light treatments was observed, though for the latter it was not significant at $p < 0.05$ (Fig. 2b). The resulting pattern of the relative contributions was essentially invariable with the sunlight spectrum (Fig. 2d).

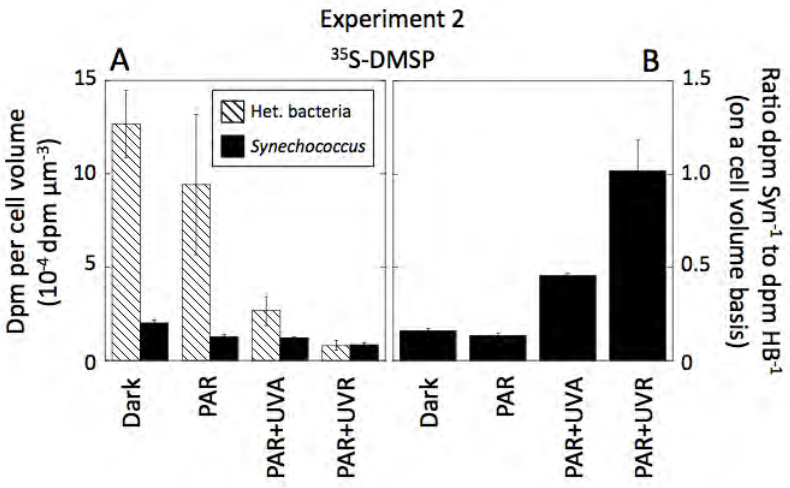

Fig. 3. Experiment 2. (A) Comparison of ^{35}S-DMSP retained per cell volume (dpm μm^{-3}) between heterotrophic bacteria and *Synechococcus* under different light conditions. Values are averages ± standard errors. (B) Ratio between these ^{35}S-DMSP uptakes per cell volume of *Synechococcus* (Syn) and heterotrophic bacteria (HB) under different light conditions.

Single-cell assessment of ^{35}S-DMSP and ^{3}H-leucine uptake by MAR-CARD-FISH. The specific differences in the sensitivity to light of heterotrophic bacteria and *Synechococcus* were further assessed by applying the MAR-CARD-FISH technique to samples from the BBMO (experiments 3 and 4). Hybridisation with specific probes showed that *Eubacteria* accounted for 88% and 67% of total DAPI counts in experiments 3 (summer) and 4 (autumn), respectively, whereas only 1% and 4% were *Synechococcus*

(**Chapter 2.2**). When samples of the experiment 3 amended with ^{35}S-DMSP were exposed to the different light conditions (Fig. 4a), the number of *Eubacteria* active at ^{35}S uptake significantly decreased upon full sunlight exposure compared to both dark and PAR treatments (Tukey's test, $p < 0.05$), whereas the number of active *Synechococcus* strongly increased from less ca. 10% to up to 80%, reaching nearly 30% of total active *Eubacteria* after full sunlight exposure (Fig. 5a) when multiplied by their abundances. In experiment 4, conversely, both *Eubacteria* and *Synechococcus* were stimulated in their ^{35}S uptake upon exposure to light (Fig. 4b); however, the increase of *Synechococcus* (by 8 fold) was much greater than that of bacteria which resulted in a light-driven increased contribution of the former to the total numbers of cells active in ^{35}S uptake (Fig. 5b).

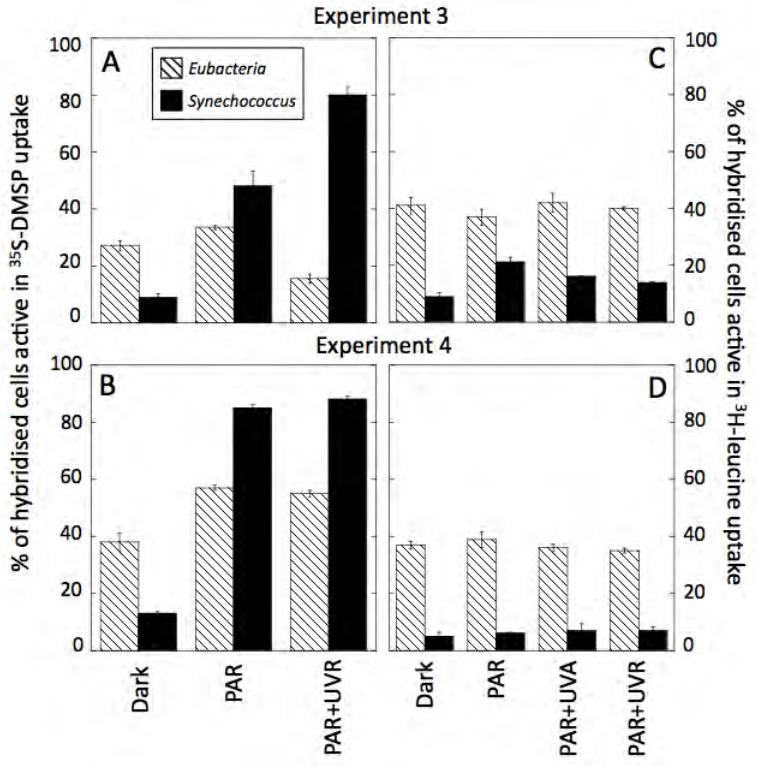

Fig. 4. Experiments 3 (top panels) and 4 (bottom panels). Percentage of *Eubacteria* and *Synechococcus* cells taking up ^{35}S-DMSP (A, B) or ^{3}H-leucine (C, D) as measured by MAR-CARD-FISH after exposure to different light conditions. ^{35}S-DMSP incubations lacked the PAR+UVA treatment.

The number of *Eubacteria* active in the uptake of ^{3}H-leucine in both experiments remained unaffected by light conditions, showing high percentages (70% - 80%) all through incubations (Figs. 4c and 4d). Similarly, *Synechococcus* from the autumn

experiment (exp. 4) were not affected by solar radiation levels (Fig. 4d), whereas in the summer (exp. 3) they were stimulated by PAR exposure compared to darkness. Inclusion of UVR caused a subsequent 30% decrease in the number of active cells (all differences being significant according to the Tukey's test $p < 0.05$, Fig. 4c).

Diel variation in the bulk assimilation of ^{35}S-DMSP by size-fractionated plankton. A further assessment of the relative uptake and assimilation of ^{35}S-DMSP was conducted by size-fractionating samples through 0.65 µm and 0.22 µm filters, both after prefiltration through 3 µm. Results were subtracted to calculate the contribution of organisms sized either 0.65-3 µm (mostly cyanobacteria and picoeukaryotes) or 0.22-0.65 µm (mostly heterotrophic bacteria). Two experiments at sea were carried out during 24 h cycles, one in shelf waters (exp. 5) and the other in open-sea waters (exp. 6). Incubations were done under natural full sunlight conditions. No clear patterns were observed throughout the cycles when plotting single data points measured every 4 h (data not shown); however, when day and night samples were separately averaged for each fraction, we found that, during the day, the larger fraction (containing cyanobacteria) assimilated significantly more substrate than the smaller bacterial fraction (ANOVA, $p < 0.05$), whereas, at night these differences were either lower (cycle 1, Fig. 6a) or null (cycle 2, Fig. 6b).

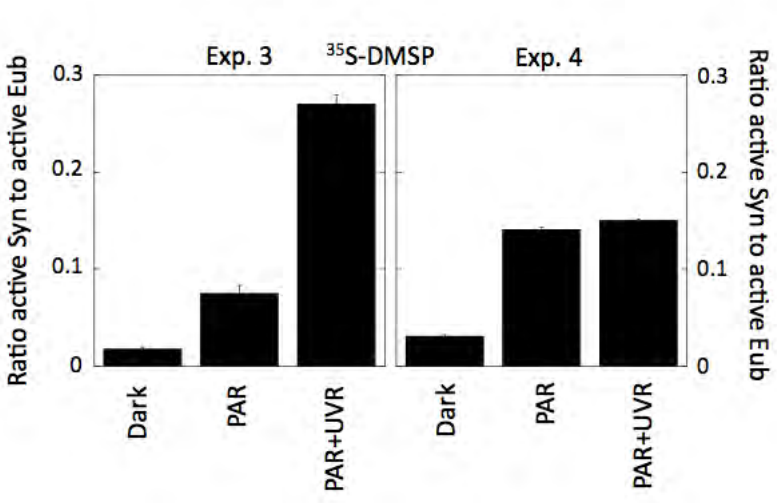

Fig. 5. Experiments 3 (left) and 4 (right). Ratio between the total number of active *Synechococcus* (Syn) and the total number of active *Eubacteria* (Eub) in ^{35}S-DMSP uptake upon different light conditions. Values were calculated by multiplying the percentage of active cells within each group by their total abundances.

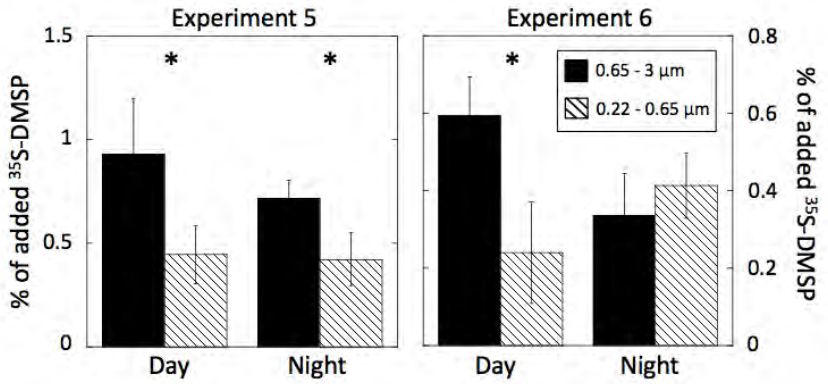

Fig. 6. Experiments 5 (left) and 6 (right). Day- and night-averaged percentages of assimilated ^{35}S-DMSP by organisms sized 0.65-3 µm (black bars) and 0.22-0.65 µm (dashed bars) as measured every 4 h during a 24 h cycle. Values are mean ± standard errors of 3-4 data points. Asterisks (*) indicate significant differences between both size-fractions (ANOVA, $p < 0.05$).

DISCUSSION

It is now recognised that the marine picophytoplankton communities composed of *Synechococcus, Prochlorococcus,* and small eukaryotic phytoplankters dominate the photoautotrophic plankton over vast tracks of the world's oceans. However, whereas their contribution to global primary production is well documented (Partensky *et al.*, 1999; Waterbury et al 1986), their role in the consumption of dissolved organic compounds, although recognized, has been much less intensely studied (e.g. Mary *et al.*, 2008; Michelou *et al.*, 2007; Zubkov and Tarran, 2005).

Results from our dark incubation (experiment 1) further confirmed that, similarly to heterotrophic bacteria, both *Prochlorococcus* and *Synechococcus* may benefit from using a reduced sulphur source such as DMSP, probably because it saves the energy required to reduce the abundant sulphate (Kiene *et al.*, 1999). This is the second study reporting ^{35}S-DMSP uptake by a natural *Prochlorococcus* population, after Vila-Costa *et al.* (2006). Studies with cultured and natural assemblages of heterotrophic bacteria showed that DMSP and glycine betaine share the same membrane transporter (Kempf and Bremer, 1998; Kiene *et al.*, 1998). Putative glycine betaine transporter genes have been found

in the genomes of culture representatives of both *Prochlorococcus* and *Synechococcus* (Palenik *et al.*, 2003; Rocap *et al.*, 2003), thus supporting the observed capacity of these widespread photosynthetic taxa to take up and assimilate DMSP. We found that the amount of radioisotope incorporated per cyanobacterial cell was larger than that per heterotrophic bacterium, consistent with their larger size. On a per biovolume (proxy to biomass) basis, though, and in agreement with the observations of Vila-Costa *et al.* (2006), heterotrophic bacteria were the most efficient at incorporating DMSP, although they were closely followed by *Synechococcus* and further behind by *Prochlorococcus*. Since the sample was collected in the night and it was incubated in the absence of light, it is likely that the observed uptake efficiencies among the studied groups may change in the presence of light because the cyanobacterial heterotrophic uptake of DMSP may be stimulated upon illumination, as suggested by the present and previous works (Malmstrom *et al.*, 2005; Vila-Costa *et al.*, 2006).

Although often considered ecologically unimportant, recent studies indicate that cyanobacterial photoheterotrophy might significantly influence the flux of dissolved organic matter (DOM) in the euphotic zone of marine ecosystems. As an example, Church *et al.* (2004; 2006) attributed their observed light-enhancement of bacterial production to *Prochlorococcus* photoheterotrophy in the North Pacific gyre, and these same organisms were also responsible for about 30% of methionine turnover in the Arabian Sea (Zubkov *et al.*, 2003). Likewise, some *Synechococcus* have been shown to assimilate amino acids (Paerl, 1991; Willey and Waterbury, 1989), yet their contribution to methionine uptake was lower in the Arabian Sea (Zubkov *et al.*, 2003).

By using flow cytometry cell sorting (experiment 2), we found that exposure to diverse sunlight treatments caused differential effects on *Synechococcus* and heterotrophic bacteria. These two groups had previously been studied in Blanes Bay with regard to their UVR sensitivities. Heterotrophic bacterial activity is generally negatively affected by UVR (Herndl *et al.*, 1993; Sommaruga *et al.*, 1997), although this varies among bacterial taxa, whereas *Synechococcus* exhibits high resistance (Alonso-Sáez *et al.*, 2006; Sommaruga *et al.*, 2005). In agreement with the previous observations, we found that upon PAR and particularly full sunlight exposure, heterotrophic bacteria were inhibited in their ^{35}S-DMSP uptake whereas *Synechococcus* were not. Consequently, the relative role of *Synechococcus* as a DMSP sink became more important (Fig. 2). Further, on a per cell

volume basis, *Synechococcus* cells equalled the amount of radioisotope incorporated by heterotrophic bacteria (Fig. 3), suggesting an important role of sunlight in regulating the fate of dissolved DMSP and the physiological use of DMSP-sulphur.

The fraction of assimilated ^{35}S-DMSP that was not retained by *Synechococcus* or by heterotrophic bacteria showed an increased contribution to total uptake towards the full spectrum, comprising up to 75% under PAR+UVR (Fig. 2a). We have no direct hint of which organisms may be responsible for this large proportion of ^{35}S-DMSP assimilation; Vila-Costa et al. (2006) and ourselves (**Chapter 4**) have reported notable uptake activity by large eukaryotic phytoplankton, particularly diatoms. Actually, the amount of ^{35}S-DMSP assimilated by the unknown organisms increased with PAR and PAR+UVA and decreased with respect to those treatments under full sunlight (data not shown), a response similar to that observed in polar diatoms (**Chapter 4**). This suggests an important role of eukaryotic organisms in the DMSP fluxes, which still deserves further research.

Assimilation of ^{3}H-leucine was simultaneously measured for comparative purposes since leucine is considered a universal substrate for heterotrophic bacteria (Kirchman *et al.*, 1985) that can also be incorporated by cyanobacteria (Kamjunke and Jähnichen, 2000; Mary *et al.*, 2008). Unlike with ^{35}S-DMSP-amended samples, solar radiation did not seem to alter the relative contributions of both heterotrophic bacteria and cyanobacteria to ^{3}H-leucine uptake (Fig. 2d). There are studies showing that light can stimulate the uptake of amino acids by phototrophic organisms (Mary *et al.*, 2008; Michelou *et al.*, 2007; Zubkov and Tarran, 2005), but none studied the process in the presence of UVR.

MAR-CARD-FISH allowed for a visual analysis of the single-cell uptake activity of both *Eubacteria* and *Synechococcus* from Blanes Bay in experiments 3 and 4. As a result of light exposure, the relative contribution of *Synechococcus* to the number of ^{35}S-DMSP assimilating cells strongly increased, whereas that of heterotrophic bacteria either decreased upon UVR exposure (exp. 3, early July) or increased, but less than *Synechococcus* (exp. 4, late September). Moreover, active *Synechococcus* accounted for as many as 30% (exp. 3) or 15% (exp. 4) of active *Eubacteria* under full sunlight, a proportion comparable to those observed for other major bacterial groups in diverse ecosystems (Malmstrom *et al.*, 2004b; Vila *et al.*, 2004; Vila-Costa *et al.*, 2007) and for

Synechococcus in the North Atlantic (Malmstrom *et al.*, 2005). Hence, according to this significant assimilation capacity and their widespread distribution (Waterbury *et al.*, 1979), *Synechococcus* are likely to be an important sink for marine DMSP. Interestingly, a higher contribution of *Synechococcus* was observed in experiment 3 than in 4, in accordance with higher UVB doses measured in the former (Table 1). Yet, any hypothesis about *Synechococcus* heterotrophy dependence on irradiance levels remains to be tested. Once again, this pattern was not reflected in ^{3}H-leucine uptake. Only in experiment 3, *Synechococcus* seemed to respond to light changes, showing an increase in the proportion of active cells upon PAR exposure and a further decrease when UVR was included. This light-stimulation of ^{3}H-leucine uptake by *Synechococcus* has been recently observed at the BBMO across different seasons except autumn, and so has the corresponding UVR-induced inhibition, which was stronger in the spring and summer periods coinciding with higher UVR doses (**Chapter 2.2**). Despite the higher percentages of ^{3}H-leucine-assimilating *Synechococcus* cells found in our light incubations, their contribution to total active *Eubacteria* was never higher than 3%, however. Altogether, these results indicate that heterotrophy in *Synechococcus* is strongly dependent on sunlight spectrum and maybe intensity, but also on the type of substrate considered.

Shipboard experiments 5 and 6 served to further explore the potential osmoheterotrophic competition between cyanobacteria and heterotrophic bacteria at the sub-daily scale. Over the two 24 h periods, a trend towards higher ^{35}S-DMSP assimilation by the cyanobacteria-containing, larger picoplankton fraction (i.e., 0.65-3 μm) was found during the light hours, whereas at night, it was more evenly distributed between the two size fractions. Interestingly, in most cases the average ^{35}S-DMSP assimilation in the 0.65-3 μm fraction was significantly higher than that in the 0.22-0.65 μm fraction, pointing to a more important contribution of picophytoplankton (and maybe attached bacteria) than that of free-living heterotrophic bacteria to total DMSP-sulphur assimilation. However, size fractionation is an inaccurate method to assess the distribution of assimilation owing to imperfect size segregation, inclusion of detritus, and overlook of bacterial aggregates. Furthermore, the larger fraction included beside cyanobacteria, picoeukaryotic cells that could have also assimilated ^{35}S-DMSP (Vila-Costa *et al.*, 2006) or could have ingested labelled bacteria. Whether these complementary players also respond positively to light is unknown. In any case, these results support the aforementioned observations

at the single-cell level that light shifts DMSP-sulphur assimilation away from the clear dominance of heterotrophic bacteria usually found in dark incubations.

As a photosynthetic cell, *Synechococcus* is subjected to diel variations in energy supply over the light:dark cycle, and many physiological processes, such as specific enzyme transcription, DNA synthesis, or cell division are coupled to this periodicity (Jacquet *et al.*, 2001; Wyman, 1999). Hence, diel variations in *Synechococcus* and other picophytoplankters' activities are expected to result in shifts in the relative dominance of phototrophs versus heterotrophs in the uptake of DMSP-sulphur throughout the light:dark cycle. Additionally, similar shifts may also occur at the seasonal scale. Considering that in Blanes Bay, both the highest concentrations of dissolved DMSP (Vila-Costa *et al.*, 2008a) and the maximum abundances of *Synechococcus* (Agawin *et al.*, 1998; Mura *et al.*, 1996; Schauer *et al.*, 2003) occur in the highly irradiated waters of late spring and summer, one might expect that competition between cyanobacteria and heterotrophic bacteria (and possibly larger phototrophs, Vila-Costa *et al.*, 2006) for DMSP-sulphur to be maximal in summer and minimal in autumn and winter .

Overall, our results confirm that marine, free-living, unicellular cyanobacteria (i.e., *Prochlorococcus* and *Synechococcus*) from the Mediterranean Sea are able to take up DMSP and assimilate its sulphur, and all our different experimental approaches agreed with an increased contribution of *Synechococcus* and probably some picoeukaryotes to ^{35}S-DMSP uptake relative to heterotrophic bacteria under light exposure compared to dark conditions. All this suggests that the DMSP dynamics in oceanic surface waters are severely influenced by solar UV radiation through differential inhibition or stimulation of the microbial consortia responsible for most of the DMSP consumption. Our findings stress the generally overlooked role of phytoplankton as DMSP consumers under realistic light conditions and the need for further research. Interestingly, the dramatic shifting-role effects of the light-driven activation of *Synechococcus* did not show up in the uptake of ^{3}H-leucine. Determining the reasons for this differential regulation of substrate uptake by light may help better understand and predict the microbial use of labile DOM in the surface ocean.

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SYNTHESIS OF RESULTS AND GENERAL DISCUSSION

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Summary and links between chapters

Framed within the intricate context of sunlight-microbe interactions, this thesis aimed to contribute to the general knowledge of biological and biogeochemical UVR effects by providing data from sunlight impacts on DOM uptake by specific surface ocean microbial groups. Since bacteria are primary consumers of DOM in seawater, they were our primary focus because any direct or indirect effect on them may have implications for carbon and nutrient cycling. However, some effort was also devoted to the impact of sunlight on the osmoheterotrophic use of DOM by phototrophic organisms. For these studies, we carried out several experiments in three contrasting marine systems: the coastal NW Mediterranean throughout the year and polar waters of Arctic and Antarctica during summer.

Within the enormous range of existing marine ecosystems, the comparison among these regions is of particular interest (see Fig. 1). Besides the enormous divergence in temperature and phasing of solar radiation, they also differ substantially in their optical characteristics, trophic status, and even in the stratification processes, which in the Mediterranean are mainly driven by increasing surface water temperatures towards the summer whereas in the polar ice-margin waters, surface stratification is primarily driven by ice-melting. Moreover, the NW Mediterranean is a relatively oligotrophic environment (low nutrient concentrations and low plankton biomass, with production being limited by phosphorous over most of the year, Lucea *et al.*, 2005; Pinhassi *et al.*, 2006), whereas polar nearshore and ice-edge waters often harbor phytoplankton blooms of high biomass and productivity (Fogg, 1977; Harrison and Cota, 1991; Sakshaug, 2004) that may in turn translate into less transparent waters than the Mediterranean (Hargreaves, 2003). However, significant penetration of UVR has also been reported for Antarctic waters, particularly during spring time coinciding with ozone depletion events (Tedetti and Sempéré, 2006). Hence, organisms inhabiting each of the studied regions and periods of the year will have to deal with largely different radiation conditions.

In this scenario, different issues were assessed for each studied ecosystem. In the Mediterranean, microbial responses to sunlight were analysed on both a daily (**Chapter 1**) and a seasonal scale (**Chapters 2.1** and **2.2**), in order to seek for variability in the responses of the different bacterial groups to different scales of sunlight irradiance variation. A broad

knowledge on seasonal patterns and recurrent annual features is available from the Bay of Blanes thanks to the time-series study running with monthly samplings in that site (http://www.icm.csic.es/bio/projects/icmicrobis/bbmo/), thus enabling comparison of our data with a range of physical, biological and biogeochemical variables.

In addition, the wavelength dependence of the responses to sunlight was considered in experiments in both the Mediterranean and the polar regions (**Chapters 2, 3, 4** and **5**). And finally, the effect of sunlight on the use of DMSP as a source of reduced sulfur was assessed in the Arctic and Antarctica (**Chapters 3** and **4**) and in the Mediterranean Sea (**Chapter 6**). We further studied specifically the effect of sunlight on the heterotrophic use of DOM by phytoplankton from the three ecosystems (**Chapters 4** and **5**).

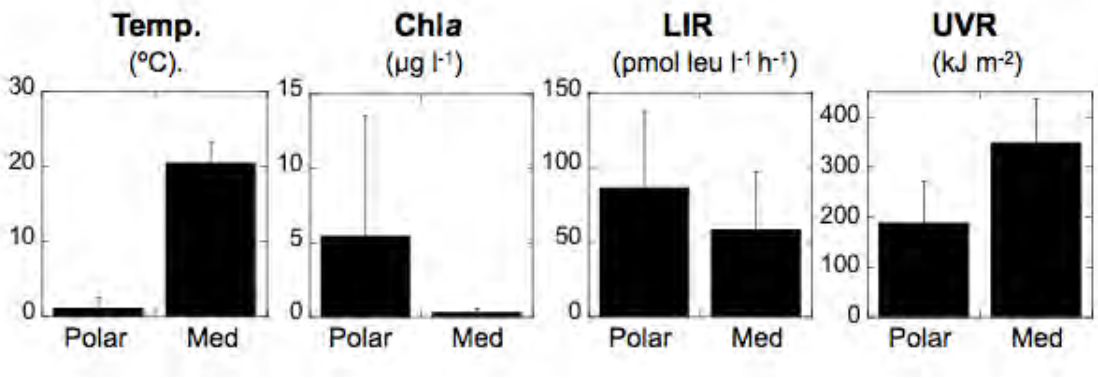

Fig. 1. Compilation of data from all our summertime polar and Mediterranean experiments for comparison between polar and temperate features: temperature (Temp), chlorophyll a concentration (Chla), initial bacterial ^{3}H-leucine incorporation rates measured in the dark (LIR) and UVR doses received by samples during the experiments. The low concentrations of summertime Chla in the Blanes Bay give an idea of its oligotrophic nature in comparison with the highly productive summertime waters of Arctic and Antarctica. Organisms inhabiting the more transparent Mediterranean waters were dealing with higher UVR irradiances. However, since polar organisms are continuously exposed to UVR for months, they might be equally susceptible to sunlight damage.

Taken altogether, our research has been mainly conducted from two different perspectives:

1. From a whole-community point of view, by quantitatively assessing the role of light on bulk bacterial activity, and the assimilation of the studied substrates under the influence of naturally variable (or artificially fixed) light conditions.

2. From a single-cell perspective, by identifying the bacterial or phytoplankton taxa involved in substrate uptake, their specific responses and sensitivities to sunlight, and to which extent these light-driven variations drove the observed bulk results. This question was mainly addressed by using the single-cell technique MAR-CARD-FISH, and only for the last chapter, flow cytometry cell sorting was additionally applied.

Overall, our results evidenced a substantial diversity of microbial responses to light, which seemed to be dependant not just on the irradiance itself, but also on the taxonomic composition of the planktonic communities, the substrate considered, and the accuracy of the light incubation conditions, namely how much did they resemble the natural in situ irradiances. These results can thus be reorganized and re-grouped across chapters to address the fundamental issues discussed in the following sections.

Implications of sunlight variability: scales of variation and light spectral quality

In view of the reported complexity, it is obvious that common trends among experiments will not be easily found. In agreement with the existing literature (see Table 1 from General Introduction) our results identified different degrees of light-driven inhibition, stimulation or no effects due to sunlight exposure. Moreover, when plotting all the results together (merging Mediterranean and polar data), no clear pattern emerged with irradiance as independent variable, indicating that inhibition of bacterial activities is not just a matter of light quantity; in other words, the highest inhibition values were not always related to the strongest doses of UVR. We can conclude, though, that light was actually modulating the flux of DOM through bacteria: by compiling all our results, we found that exposure to full sunlight resulted in an average bacterial activity (leucine incorporation) inhibition of 23% of the dark control, yet with a variability that spanned from 40% stimulation to as much as 60% inhibition (Fig. 2).

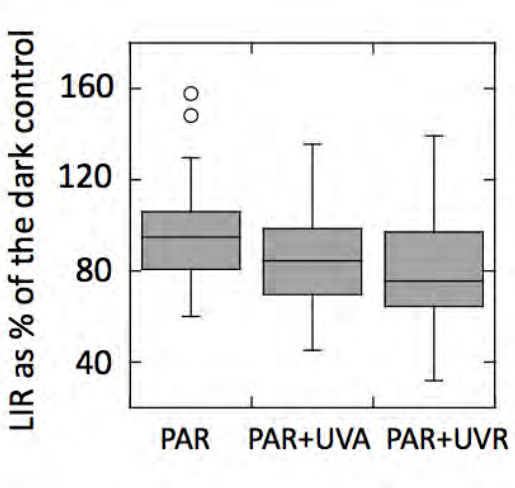

Fig. 2. Leucine incorporation rates (LIR) from all experiments measured under the different light conditions and represented as percentage of the dark control to illustrate the range of variability in the observed responses.

In spite of this wide range, some patterns could still be detected: for instance, the daily variability of bacterial activity reported in **Chapter 1** indicates that light was somehow driving the bacterial behavior in Blanes Bay: not by a direct effect, as reported elsewhere (Jeffrey *et al.*, 1996a), since the irradiances that were registered during the winter experiment were too low to cause such an intense damage, but indirectly, through synchronization of cycles with other microbes (grazers and prey) which would ultimately lead to daily variable supplies of DOM for bacteria. These hourly surveys, purposely intended to catch the often neglected short term variability within days, revealed an hourly variability up to 1.4-fold higher than the annual range of variation. This suggests that the error when extrapolating from a sample obtained at a single time of the day to daily or monthly values may be very significant, calling for careful interpretation of seasonal data.

Moving to a longer scale, in **Chapter 2** we focused on the seasonal variability of LIR responses to sunlight. As a first approach, we measured the year-round bulk bacterial activity both under in situ and controlled (PAR only) light conditions (**Chapter 2.1**). This unveiled an important intra-annual variability that was not always directly related to light intensities, but with some seasonal patterns becoming evident upon averaging (Table 1, **Chapter 2.1**), such as a greater stimulation in spring and inhibition or no effect in summer. Additionally, when we tested the seasonal wavelength dependence of LIR and ectoenzyme activities measured after exposure to different sunlight conditions (**Chapter 2.2**) we observed that, even though the absolute inhibition values were larger in summer, the inhibition per unit of radiation was extremely high in winter. This fact, which initially seemed to point to a more sensitive community in winter, was actually caused by an important overexposure to UVB derived from our experimental setup. Moreover, when comparing the relative contribution to

inhibition of these microbial variables by both UVB and UVA wavelengths, large differences in sensitivity were found among samples from Blanes Bay (Fig. 3). Whereas in some cases UVB was responsible for most of the inhibition compared to the PAR-only treatment, UVA was sometimes found to exert the main effect; indeed, UVA was the major contributor to inhibition in most of the bulk LIR incubations. This higher contribution of UVA than that of UVB has also been seen by other authors (Sommaruga *et al.*, 1997; Tedetti *et al.*, 2009) and might be explained by the fact that even if UVA wavelengths are less energetic that UVB, the amount of UVA energy that reaches the sea surface is much higher than that of the UVB region. Some of these results made sense when the past light history was taken into account and differences could be partially explained by the differences between in situ and experimental light conditions (see below).

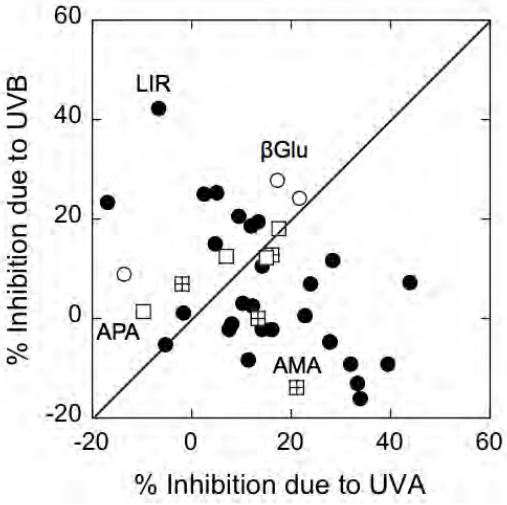

Fig. 3. Scatter plot comparing the relative contribution to inhibition (with respect to PAR-only) of several variables by UVB versus UVA. Only samples from the Mediterranean are considered since UVA effects were not specifically assessed in polar experiments. The variables measured were: leucine incorporation rates (LIR), ß-glucosidase activity (ßGlu), aminopeptidase activity (AMA) and alkaline phosphatase (APA). Values in the 1:1 line indicate samples where both wavelengths contributed similarly to inhibition with respect to the PAR-only values.

A similar experimental design was used in different stations of the Arctic and Antarctica (**Chapter 3**), but focusing mainly on UVB wavelengths, the most damaging region of the spectrum and the only one affected by changes in stratospheric ozone. Here, while no clear correlation was found between the LIR inhibition percentages and the UVB doses, the amount of trace ^{3}H-leucine assimilated by the 'bacterial' fraction was significant and negatively correlated with increasing UVB levels. The explanation for such a difference can be found in the experimental setup: whereas the samples for the size fractionation experiments were exposed to sunlight together with the isotope, LIRs were measured after exposure by 2-3 hours of dark incubation (following the classic protocol), during which bacteria might have recovered partially from photodamage, as previously observed by Kaiser and Herndl (1997).

Besides the temporal and spectrum-based variations, light also fluctuates on a spatial scale not only due to changing solar zenith angle and mixing regimes, but also depending on the optical properties of different regions of the ocean (e.g. Hargreaves 2003). In this regard, the Mediterranean Sea is a highly transparent region where UVR penetrates much deeper than in the productive summertime Arctic and Antarctic waters. Moreover, given that the conditions of temperature and nutrient limitation may also determine the sensitivity and recovery capabilities of cells to UVR (Pausz and Herndl, 2002; Rae and Vincent, 1998; Roos and Vincent, 1998), and since the origin and chemical composition of DOM may influence its photoreactivity and subsequent increased or decreased bioavailability (e.g. Benner and Biddanda, 1998; Herndl *et al.*, 1997; Obernosterer *et al.*, 1999; Tedetti *et al.*, 2009), the responses to UVR of microbes inhabiting these contrasting systems will further vary depending on the particular features of the system.

Unfortunately, bulk activity data did not reveal any clear latitudinal trends among summertime samples from the Mediterranean, the Arctic and Antarctica, which in turn varied significantly even from station to station. Since, in general, literature data allow for minimal comparison of UVR effects from different latitudes due to the fact that different experimental designs or physiological parameters have been considered, little information has been collected to date about differences in the sensitivity of microbes from different ecosystems. Moreover, the use of various types and brands of UVR instruments (spectroradiometers, broad band meters, dosimeters) further complicates comparisons among field studies. In any case, it seems that changing irradiances are differentially affecting bacterial heterotrophic responses on varying scales, and, despite we could not relate that effect to any other parameter such as Chl *a* concentration, primary production, nutrients, temperature or salinity, the presence and activity patterns of specific components of the microbial communities appeared of major importance for understanding the observed light-driven trends (e.g. **Chapters 2.1** and **2.2).**

Notwithstanding, interpretation of this kind of experiments is not straightforward since direct effects on bacterial activity are difficult to discriminate from indirect impacts on DOM availability without specific manipulation of samples, yet such short term incubations are thought to prevent other indirect effects derived from e.g. impacts on viruses or grazers. In any case, we have to bear in mind that the observed bacterial responses are the final balance among all the synergistic and antagonistic effects that are taking place at the same time inside our experimental bottles.

Single cell responses as drivers of bulk patterns

When applying microautoradiography, one must take into account that the results are not directly comparable with bulk quantitative measurements since we enumerate the presence or absence of labeled cells but we don't have information about *how* active each cell is, an issue that could only be roughly addressed through quantification of the silver grain area around cells: the greater the silver grain area around one cell, the higher its uptake (Sintes and Herndl, 2006). It is thus possible that even though we find clear light-driven patterns in bulk measurements, these are not reflected in the numbers of active cells, because one cell surrounded by a silver grain area two fold larger than another will be equally counted as 'active'. However, as shown by some of our results, it is sometimes possible to find some coherence between bulk and single-cell data. As an example, the clear day-night activity patterns found in every group were undoubtedly reflected on bulk bacterial heterotrophic production measurements (**Chapter 1**). Moreover, when these group-specific cycles were suddenly disrupted after the meteorological perturbation episode, the bulk activity also lost its rhythm. Afterwards, *Gammaproteobacteria* alone appeared to drive visible changes in the whole community LIR, pointing to a major contribution of this group to bulk activity despite their low abundances; indeed, their usually bigger silver grain areas compared to those of other groups such as the abundant SAR11 further support this statement (see Fig 4). Given that different bacterial phylotypes are known to display different sensitivities to sunlight (Agogué *et al.*, 2005; Alonso-Sáez *et al.*, 2006; Arrieta *et al.*, 2000; Joux *et al.*, 1999), we had previously hypothesized that some bacterial groups would be less affected than others by temporal variations in the light field. The observed synchronized behaviour of all taxa contradicted this hypothesis. Nonetheless, we do not discard that a differential behaviour of groups may happen in other periods of the year and vary from season to season, as suggested elsewhere (Ghiglione *et al.*, 2007).

On top of the diel variability depicted by hourly samplings, the marine organisms experience seasonally fluctuating light conditions that might affect bacterial groups differentially, as suggested elsewhere (Alonso-Sáez *et al.*, 2006). Indeed, the reported variability in monthly LIR measured in the dark and under constant PAR conditions (**Chapter 2.1**) correlated similarly with changes in the number of active *Gammaproteobacteria* (generally stimulated after light exposure) and with bulk LIR. Neither *Cyanobacteria* nor *Roseobacter,* two groups potentially stimulated by light exposure (Alonso-Sáez *et al.*, 2006;

Mary *et al.*, 2008, **Chapter 2.2**) seemed to contribute to this PAR-driven enhancement of bulk activity. Similarly, the lack of light-driven effects on bulk LIR measured during the spring experiments in **Chapter 2.2**, seemed to be explained by a compensation of the reduction of activity of some groups such as SAR11 by the higher resistance to UVR of other groups, with *Gammaproteobacteria* once again in the forefront. This activity of *Gammaproteobacteria* driving responses visible at the whole community level had also been reported in this sampling site by Alonso-Sáez *et al.* (2008), who found unusually high LIR values concomitant with a drastic shift in bacterial assemblage structure towards dominance of *Alteromonas*-like *Gammaproteobacteria* (Alonso-Sáez *et al.*, 2007). Bacteria belonging to *Gammaproteobacteria* have been shown to react fast to changes in their environment (Eilers *et al.*, 2000; Pernthaler *et al.*, 2001), so it is possible that they were the first responding to the recovery of the diel DOM release cycle (**Chapter 1**), and also the first ones to respond to an sudden increase in light or photosynthate availability (**Chapters 2.1** and **2.2**).

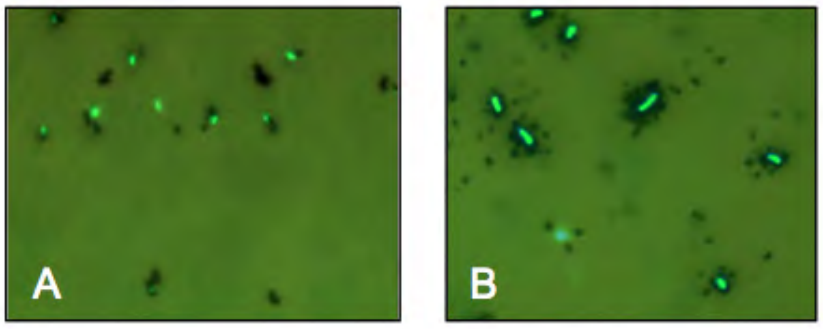

Fig. 4. Microautoradiograms of SAR11 (A) and *Gammaproteobacteria* (B) cells (hybridized with CARD-FISH probes) active for ^{3}H-leucine samples. In this case, silver grain areas around cells are comparable since they belong to the same sample and both the incubation and exposure conditions were the same. Note the much bigger areas around *Gammaproteobacteria* compared to SAR11 cells, which indicates a greater contribution of the former to total ^{3}H-leucine uptake in spite of their lower abundance.

In Arctic samples, conversely, the significant UVR inhibition depicted by bulk measurements was not reflected in group behaviors (**Chapter 3**). This might be due to the aforementioned non-quantitative nature of this technique since the activity was assessed in terms of presence/absence (% of active cells) and not as assimilation per cell (silver grain areas). Only in the Antarctic stations, slightly shorter incubation times seemed to allow detection of light-induced changes in the number of active cells belonging to specific groups.

Remarkably, the bulk responses at station AN1 seemed to be mostly driven by the dominant *Bacteroidetes,* which, similarly to the bulk LIR and trace ^{3}H-leucine assimilation values, were strongly inhibited in their number of cells taking up ^{3}H-leucine by PAR+UVA and UVB treatments.

Seasonal and spatial variability within broad bacterial groups

When applying CARD-FISH for probing broad bacterial groups such as those used in our research, we have to consider that they can include a variety of taxa, which may vary either spatially (e.g. Field *et al.*, 1997) or temporally (Schauer *et al.*, 2003) causing differences in the observed responses and probably masking some effects. As an example, when analyzing the responses of *Alphaproteobacteria* to sunlight, Alonso-Sáez *et al.* (2006) found that they were mainly inhibited by UVA. When going deeper in taxonomic resolution, though, they found contrasting responses between the two alphaproteobacterial groups SAR11 and *Roseobacter* (inhibition vs. stimulation by PAR, respectively, in their uptake of ^{3}H-leucine). Although we might find different responses when we use more narrowly targeted phylogenetic probes, it is also possible to find some common trends within broad phylogenetic groups, at least on a general functional level, such as the common light-driven increases in *Gammaproteobacteria* activity observed in **Chapter 2.1.** Figure 5 shows the magnitude of the changes in the number of active cells of different bacterial types under full sunlight exposure in summertime Mediterranean, Arctic and Antarctic samples. Whereas in general there were no significant differences ($p > 0.05$) among responses of groups from the different ecosystems, some patterns became apparent: for instance, while the SAR11 from the Arctic stations displayed light activation of their ^{3}H-leucine uptake, their Mediterranean counterparts were significantly inhibited under full light exposure (Fig. 5a). Contrastingly, *Gammaproteobacteria*, *Roseobacter* and *Bacteroidetes* displayed a relatively high resistance to UVR, although variable among stations and seasons.

While *Gammaproteobacteria* from Blanes Bay were stimulated in their uptake of ^{35}S-DMSP upon PAR exposure (Fig. 5b), the group was not responding to light in the studied Arctic and Antarctic stations. Similarly, *Roseobacter* from the Antarctic station AN1 were strongly stimulated in their uptake of ^{35}S-DMSP by PAR exposure, a pattern which was not observed in their Arctic and Mediterranean counterparts. All this points to

latitudinal differences in the within-group occurrence of phylotypes or ecotypes adapted to the contrasting light regimes, and prevents a general classification of these broad bacterial groups with regard to their responses to light.

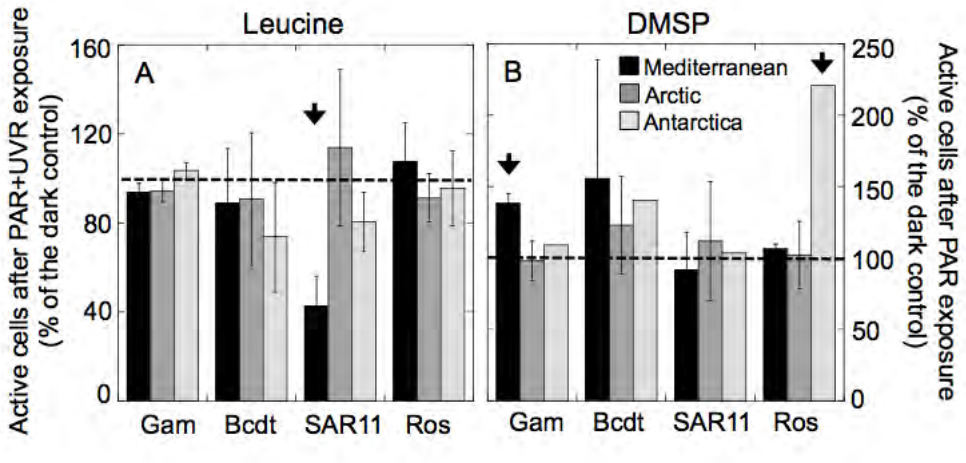

Fig. 5. Comparison of the responses to sunlight of the summertime major bacterial groups from the different ecosystems studied. A) Leucine incorporation. Values above or beyond the 100% line indicate either stimulation or inhibition in the number of labelled cells due to full sunlight exposure compared to the dark treatment. Columns and error bars correspond to averages and standard deviations, respectively, of 2-5 experiments. B) DMSP incorporation (two additional experiments performed in the Mediterranean -not shown in this thesis- are included for comparison with polar samples). Values above or beyond the 100% line indicate either stimulation or inhibition in the number of active cells due to PAR exposure compared to dark treatment. Arrows indicate responses significantly different from their counterparts from other regions ($p < 0.05$).

In most cases, the group specific responses, when occurring, did not seem to be directly dependant on the light levels. We could only find a significant correlation between stimulation/inhibition and irradiance for all SAR11 (Fig. 6a) and for *Roseobacter* from the polar stations (Fig. 6b). Both groups showed lower numbers of cells active in ^{3}H-leucine uptake with increasing UVB doses, yet most other groups responded independently from the doses received. Therefore, even though the use of these broad phylogenetic groups provides an essential first approach to the bacterial communities and their general responses to light, finer levels of resolution will certainly reveal distinct features and may help to understand some of the observed results. For example, a study of the gammaproteobacterial subgroup NOR5 revealed that in spring, when they comprised up to 90% of the *Gammaproteobacteria*, they were responsible for the PAR-driven increase in both bulk and group activities (**Chapter 2.2**).

The aforementioned greater inhibition found under stronger UVR doses (Fig. 6) seems to reject the hypothesis of photoacclimation or selection for photoresistant taxa under higher irradiance conditions, as suggested elsewhere (Alonso-Sáez *et al.*, 2006). Consistently, a greater reduction in activity in winter or autumn with respect to summer was not evident for any bacterial group in Blanes Bay (**Chapter 2.2**). However, when we considered the light-driven responses on a per photon basis, it seemed that most groups displayed greater inhibition per unit of radiation in autumn and spring than in summer (see text in **Chapter 2.2**). Thus, bacterial taxa naturally exposed to lower radiation intensities were relatively more sensitive to experimental light manipulations than the summertime phylotypes naturally acclimated to higher exposures. However, autumn and winter radiation levels seemed to be too low to cause visible effects, pointing to the idea of a light threshold over which physiological adaptation of groups or shifts towards new photoresistant phylotypes may occur.

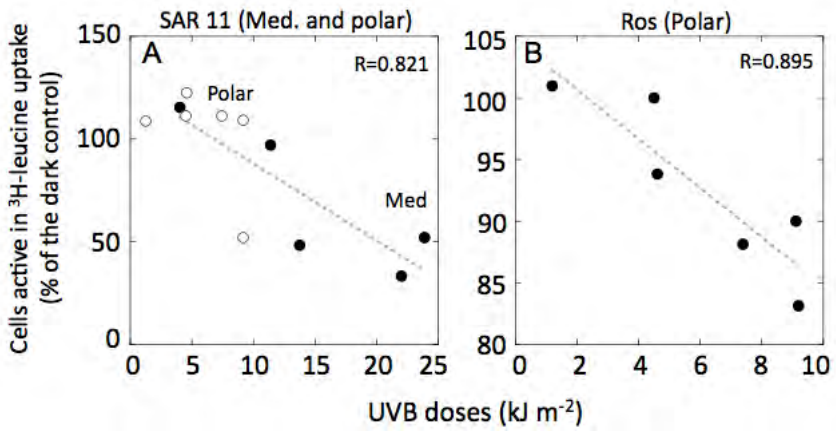

Fig. 6. Relationship between the decreases in the number of cells active in ^{3}H-leucine uptake caused by full sunlight exposure with respect to dark controls (expressed as % of dark controls) and the doses of UVB received by samples. (A) SAR11 from both Mediterranean (solid circles) and polar samples (open circles); (B) *Roseobacter* from Arctic and Antarctic experiments. Only these two groups showed significant ($p < 0.05$) responses with regard to the intensity of light, although for the case of SAR11, the trends were not significant when the ecosystems were plotted separately.

Bacterial photoheterotrophy

Unfortunately, our experiments were not designed to undoubtedly confirm the presence of photoheterotrophic metabolisms such as those of BChl*a*- or proteorhodopsin-containing bacteria (Béjà *et al.*, 2000; Kolber *et al.*, 2000). Nonetheless, in several occasions we detected some light (mainly PAR)-driven enhancements of bacterial activity: *Gammaproteobacteria* in **Chapter 2.1**, *Roseobacter* and NOR5 in **Chapter 2.2** or SAR11 in **Chapter 3**. Among the possibilities for discarding a real photoheterotrophy, indirect stimulation of bacterial activity due to light-enhanced photorelease or availability of DOM through phototransformations need to be considered. However, the fact that *Gammaproteobacteria* abundances were significantly correlated with underwater irradiance conditions but not with any other parameter such as temperature, Chl *a* or primary production data (**Chapter 2.1**) suggests indeed a preference of Blanes Bay *Gammaproteobacteria* for illuminated environments. So far, few studies have addressed the actual advantages that harboring Bchl *a* or PR confers to marine bacteria, and none in natural assemblages. Recent experiments with marine bacterial cultures have begun to reveal some hints on the nature and function of these photosystems (Gómez-Consarnau *et al.*, 2010; Gómez-Consarnau *et al.*, 2007). Anyhow, further research is needed to accurately quantify the ecological relevance of photoheterotrophy in marine ecosystems.

Effects of sunlight on the microbial fate of DMSP

In view of our results, DMSP assimilation seems to be widespread not only among all the dominant bacterial groups from the different ecosystems studied but also among several phytoplankton taxa. Bacteria, in most occasions and as previously reported (Vila-Costa *et al.*, 2007; Vila-Costa *et al.*, 2008b), showed higher affinity for leucine than for DMSP (Fig. 7), suggesting that not all active bacteria were using DMSP as a sulfur source for the synthesis of proteins. Moreover, Arctic and Antarctic bacteria were in general significantly more active in the uptake of both substrates than their Mediterranean counterparts, indicating that despite the low temperatures and extreme conditions, the polar regions can support high heterotrophic activities of prokaryotic assemblages (Cota et al. 1990; Rich et al. 1997; Wheeler et al. 1996).

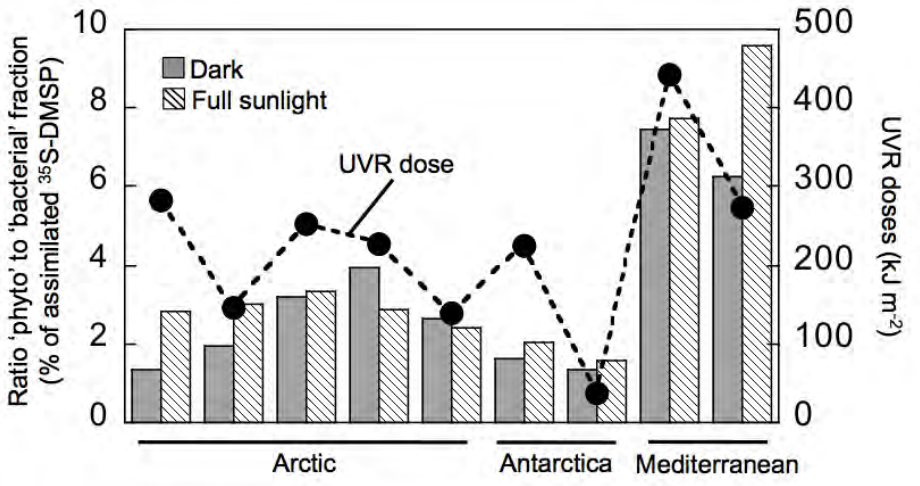

Fig. 8. Relative contribution of the 'phototrophic' size fraction (> 0.65 μm in Mediterranean samples and > 5 μm in polar samples) versus the 'bacterial' fraction (0.22-0.65/5 μm) in the assimilation of ^{35}S-DMSP measured after dark or full sunlight incubations. Bars represent the ratio between both fractions (`phyto' to 'bacteria'), and the dashed line indicates the UVR doses received by the samples during the experiments. Size fractionated assimilation results from two Mediterranean experiments not included in this thesis are also added for comparison. This graph show that an increased contribution of phototrophs to ^{35}S-DMSP uptake is not always the rule, and it does not seem to be directly dependent on the irradiance levels; instead, in view of our single-cell data, the taxonomic composition of the communities seems to play a relevant role.

In contrast to leucine assimilation, which often appeared to be inhibited by full sunlight, bacterial DMSP-sulfur assimilation presented a less clear pattern (**Chapters 3** and **5**). In the different light conditions, bacterial ^{35}S-DMSP assimilation was either stimulated, inhibited, or there was no noticeable effect. This might be partially explained by differential UVR-effects on diverse uptake systems (Herndl *et al.*, 1997), but probably also by the co-occurrence of light-influenced processes (such as DMSP release) that ultimately control the availability of DMSP for microbes (Hefu and Kirst, 1997; Sakka *et al.*, 1997; Slezak *et al.*, 2001; Slezak and Herndl, 2003; Slezak *et al.*, 2007; Sunda *et al.*, 2002).

Contrasting to the DMSP-sulfur assimilation by heterotrophic bacteria, uptake by eukaryotic and prokaryotic phytoplankton showed a common stimulation trend in the light. This was particularly shown by the bulk assimilation data, although it varied when individual organisms were considered (**Chapter 4** and **5**). In this regard, the widespread occurrence of ^{35}S-DMSP uptake by polar (mainly Arctic) diatoms (**Chapter 4**) was an unexpected and interesting discovery which seemed to be explained by the summertime occurrence of high DMSP concentrations in the polar regions, as observed in the Arctic during that year by Galí

and Simó (2010); this could have shaped a favorable scenario for bacterial and phytoplankton groups able to profit from this substrate, thus explaining the great numbers of active cells found in many different phylotypes.

On the other hand, the outcome of different experiments from Blanes Bay waters where picophytoplankton were large DMSP consumers (**Chapter 5**) suggested that light may enhance their competition for DMSP uptake with respect to heterotrophic bacteria. This would be particularly so in highly irradiated summer waters where the highest annual DMSP concentrations are recorded and *Synechococcus* dominate the picophytoplankton assemblage (Mura *et al.*, 1996; Schauer *et al.*, 2003; Vila-Costa *et al.*, 2008a).

However, when plotting all the size-fractionated assimilation results together, we did not find any clear trends, since sunlight exposure did not always lead to an increased contribution of phototrophs versus heterotrophs as compared to dark conditions, neither these effects seemed to be directly dependant on the light levels (Fig. 8). This indicates that the bulk responses greatly depend on the identity of the organisms involved, and probably on other environmental factors. Also, it is to be noted that size fractionation does not completely separate bacteria and larger eukaryotes, and that our 'phototrophic' fractions (>0.65 μm in Mediterranean samples or >5 μm in polar samples) may include heterotrophic eukaryotes also capable of some uptake.

Experimental evidence indicates that both the depth of the mixing layer and the UVR doses work together to produce higher summertime DMSP and DMS production/consumption ratios through favouring the occurrence of high DMSP-producing phytoplankton and inhibiting the utilization of DMSP and DMS by bacteria (Simó and Pedrós-Alió, 1999; Toole and Siegel, 2004). Our findings confirm a major role of solar radiation on DMSP dynamics in the oceanic waters but adding a new pathway through which, depending on the microbial consortia, summertime light levels may favour DMSP-sulfur uptake by a fraction of the phototrophic components, which may ultimately influence the DMS flux to the atmosphere. This suggests that the generally overlooked role of phytoplankton as a DMSP sink under realistic light conditions deserves further exploration.

The relevance of mimicking the natural irradiance conditions

One of the key points of our research was the consideration of the previous light exposure history of the targeted organisms, often neglected in most surveys. In view of our results, it seems that knowledge of the previous in situ conditions is essential to better understand the obtained responses, or even to accurately simulate the light levels naturally received by microbes. We only assessed the light exposure history of the samples in **Chapter 2**, where it appeared to consistently influence the observed responses. First, the relative contribution of UVB or UVA to bulk LIR inhibition (see Fig. 3) was dependent on the UVB/UVA ratio previously experienced by the samples in situ, so that bacteria naturally acclimated to low UVB levels where more inhibited by these wavelengths during our incubations (**Chapter 2.1**). Similarly, year-round LIR measured under controlled and constant PAR-only conditions was significantly stimulated where the difference between the artificial light levels and their in situ PAR conditions was greater, whereas no effect or even inhibition was found when both irradiances were similar. This suggests that bacteria may partially react depending on how much our incubation conditions differed from the natural levels previously experienced. And finally, when assessing the wavelength-dependence of some bacterial processes throughout the seasons (**Chapter 2.2**), the detected greatest wintertime effects seemed to be explained by the high ratio between the UVB dose measured inside the incubation bottles and the UVB doses naturally received by the samples in situ; in other words, our experimental set up led to an overexposure of samples to UVB wavelengths, since cells may have otherwise been subjected to wintertime deep mixing thereby receiving lower damaging doses. In addition, neutral density filters do not accurately mimic the differential spectral attenuation of seawater, enriching the downwelling spectrum in UVB with respect to UVA. It is particularly important to try to realistically mimic the ratios between UVB, UVA and PAR, since the balance between damage and repair depend mainly on these proportions (e.g. Kaiser and Herndl, 1997). Like us, most researchers still expose samples to natural or spectrum-manipulated sunlight ignoring the spectral effects of vertical mixing, which, together with the daily irradiances, determine the doses that organisms are naturally receiving in their environment. Hence, the more similar the incubation conditions to the natural environment, the more realistic the responses obtained will be.

There are a few studies of UVR effects in the context of variable vertical mixing, most of them focusing on phytoplankton productivity (see refs in Neale et al. 2003). So far, the relationship of inhibition of bacterial production to the depth and rate of vertical mixing has

not been studied, although the vertical distribution of damage to bacterial DNA, an indicator of the UVR effect, has been assessed for water columns with different mixing regimes (Boelen *et al.*, 2001; Huot *et al.*, 2000; Jeffrey *et al.*, 1996a), with results suggesting dramatic differences in the vertical profiles of DNA damage depending on whether calm or strongly mixed conditions prevail. To our knowledge, our findings are amongst the first to provide evidence of the role of the past light history on the present behavior of bacterial activity upon light exposure, and call for the need of taking mixing into account when photobiological and photochemical processes are to be measured under realistic conditions. However, in case it is not possible because mimicking mixing conditions implies a certain degree of logistic difficulty, we strongly encourage that at least the analysis of the previous light history is done to avoid misinterpretation of the results.

The limitations of bulk activity measurements.

Certainly, the inherent complexity that characterizes planktonic food webs is a challenge for research on any environmental effect. Due to species-specific responses and interactions among them and other factors, patterns are often highly variable and difficult to predict. In this scenario, knowledge of the identity of the occurring microbes and their specific responses to sunlight is of great importance for a correct understanding of ecosystem dynamics, with most of our results further underlining the importance of the single cell analysis. Size fractionation is a step forward, but still largely incomplete. Both size fractionated assimilation of substrates and bulk bacterial activity measurements may carry some biases: the size fractionation measurements of ^{3}H-leucine, which do not completely separate algae from bacterial aggregates or detritus, were shown to include an important fraction of ^{3}H-leucine assimilating-bacteria in the fraction > 5 μm (**Chapter 4**). Similarly, since dark bacterial leucine incorporation measurements may involve some prokaryotic and eukaryotic phytoplankton taking up ^{3}H-leucine by osmotrophy (**Chapters 2** and **5**), they might lead to overestimations of bacterial secondary production depending on their abundances and their relative contribution to the bulk uptake; indeed, algal uptake of ^{3}H-leucine was often found to be higher in the dark than upon light exposure (**Chapter 4**), so that an increased contribution of these phototrophs to ^{3}H-leucine incorporation might take place in these dark incubations. Our results suggest that bulk measurements, although useful and easy to perform, depend largely on the specific responses of particular components of the ecosystem, and thus, we

should not be able to accurately interpret experimental results without a deep knowledge of the organisms involved.

'Standard' dark measurements: are they the most suitable?

Most bacterial activity measurements are by definition performed in the dark in order to avoid algal stimulation, and to circumvent the aforementioned problems of reproducing ambient light levels (e.g. Kirchman *et al.*, 1985; Smith and Azam, 1992). However, since marine planktonic communities are naturally exposed to varying radiation conditions and obviously light does matter, parallel light and dark incubations seem more accurate to better understand real variations in carbon fluxes (see also Gasol *et al.* 2008). Moreover, although light exposure may sometimes indirectly lead to increased values of bacterial heterotrophic production probably due to an enhancement of bacterial responses to increased photosynthate release or phototrophic metabolisms, dark enclosure may also result in increased uptake values. Morán et al. (2001) proposed that apparent PAR inhibition in samples from the NW Mediterranean was the result of stimulation of bacterial growth in the dark, although the exact mechanisms were not identified. It could also be that some leucine is released as a by-product of photosynthesis (e.g. Braven *et al.*, 1995) therefore diluting the radiolabel and decreasing the detected signal.

On the other hand, we might have misinterpreted some results from dark incubations in **Chapter 1,** since we cannot discard additional UVR negative effects or a less discernible cycle due to photostimulation of LIR during the day. Therefore, incubation of samples for LIR already amended with ^{3}H-leucine in UV-transparent 4 ml-cuvettes such as we did in **Chapter 2.1** seemed an easy method to routinely measure bacterial activity under realistic light conditions. Furthermore, dark incubation may sometimes be totally unreal, such as during the polar summer months, when microbes are continuously exposed to sunlight and dark incubations seemed to lead to overestimates of ^{3}H-leucine incorporation or subestimates of ^{35}S-DMSP uptake (**Chapters 3** and **4**). Our results and those from the existing literature on this topic suggest that measurements under realistic irradiance conditions are necessary for accurate modeling of DOM fluxes throughout microbial food webs; nonetheless, a control measured in the dark seems unavoidable (1) for comparison among different studies, and (2) because, as shown here, inaccurate simulation of light conditions may lead to misleading conclusions.

Consequences of increased UVR and links to global change

In view of the obtained results, it seems that predictability of the ecosystem responses to changes in the light regime will not be obvious. Alterations in UVR intensities derived from natural or anthropogenic causes might modify the rates of metabolism and biogeochemical processes in a cascade of effects that may ultimately affect the functioning of natural ecosystems.

Besides ozone depletion itself, factors such as changes in cloud cover and optical properties, aerosols, air pollution, surface reflection or water attenuation may also affect the amount of UVR penetrating into the water column, and all of these are in turn influenced by global change (Kerr *et al.* 2003; McKenzie *et al.*, 2007). Some experiments in lakes show that the drought derived from warmer temperatures lead to a reduced runoff, DOM load and acidification which resulted in waters of greater transparency to UVR (Schindler *et al.*, 1996a; Schindler *et al.*, 1996b; Yan *et al.*, 1996). Moreover, there is some evidence that the tendency for warmer sea surface temperatures (Hansen *et al.*, 2005; Willis *et al.*, 2004) may influence the timing and strength of stratification (Young and Holt, 2007); should this fact lead to shallower stratification in some areas, marine organisms entrapped there would be exposed to increased radiation levels.

In addition to climate change-derived changes in the oceanic light field, UVR-induced shifts within planktonic assemblages might occur in response to varying conditions, as observed elsewhere (Santos *et al.*, 2010; Belzile *et al.*, 2006; Roy *et al.*, 2006). These variations in species composition would be of importance because they may translate into significant alterations of DOM and nutrient cycling in the marine ecosystem.

Nevertheless, our capacity to predict the dynamics of the community with regard to variations in irradiance levels remains considerably limited. Interpretation from individual experiments are complex and synergies between UVR and other stressors need to be further addressed before we can extrapolate from our small-scale, short-term experiments involving simplified scenarios to real complex systems for which we would like to offer some reliable predictions.

Main conclusions of the thesis

Specific conclusions

The main conclusions that arise from each chapter are:

1. During the winter period of higher primary production in winter in Blanes Bay, all major bacterial groups followed diel cycles of activity showing more active cells at night than during the day, which were reflected on bulk activity measurements such as ^{3}H-leucine and ^{3}H-thymidine uptake.

2. Throughout an annual cycle, bacterial activity in the Blanes Bay was negatively affected by in situ UV irradiances, although the relative contribution of UVB to inhibition seemed to be influenced by the experimental Setting. When measured under constant (and artificial) PAR, the general stimulation in bulk bacterial activity was shown to be mostly caused by light-driven enhancements of *Gammaproteobacteria* activity. This principal role of *Gammaproteobacteria* was probably due to their ability to respond fast to environmental changes or to the potential Photoheterotrophy of some subgroups, such as the NOR5 clade.

3. Major bacterial groups in Blanes Bay showed seasonally different sensitivities to PAR and UVR as assessed at the single-cell level. *Gammaproteobacteria* and *Bacteroidetes* displayed higher resistance than SAR11 cells, which were often inhibited in their activity by PAR and UVR exposure. *Roseobacter, Synechococcus* and the NOR5 clade were often stimulated mainly by PAR. The absolute light-driven changes in the number of active cells were generally greater during the periods of higher radiation intensity (summer), but the inhibition per unit of solar radiation was generally lower during this season, suggesting a certain degree of photoacclimation of bacteria.

4. High percentages of ^{3}H-leucine and ^{35}S-DMSP-assimilating cells were recorded for all the studied bacterial groups from Arctic and Antarctic summer waters. SAR11 dominated both the bacterial abundance and the number of cells assimilating both substrates in the Arctic stations, whereas either *Gammaproteobacteria* or SAR11 were the largest contributors to active cells in Antarctica. Bacterial use of leucine seemed more sensitive to UVR than the use of DMSP-sulfur, yet this sensitivity was more apparent in assimilation rates than in active cell numbers.

5. Microautoradiography revealed a widespread capacity to take up both ^{3}H-leucine and ^{35}S-DMSP among a variety of algal phyla in summer Arctic and Antarctic waters. These included pennate and centric diatoms, autotrophic dinoflagellates and flagellates, and their joint assimilation rates were comparable or even higher than those of bacteria. Different algal taxa displayed different sensitivities to UVR, and, while ^{35}S-DMSP uptake variability did not show any clear trend with the light conditions, ^{3}H-leucine uptake was generally lower in light than in the dark.

6. Flow cytometry cell sorting and microautoradiography analyses of Blanes Bay samples showed that the relative contribution of *Synechococcus* to ^{35}S-DMSP uptake was significantly enhanced under full sunlight exposure relative to that of heterotrophic bacteria, mainly due to inhibition of the latter. Moreover, size-fractionated assimilation experiments showed greater relative assimilation by bacteria during the day than at night. Taken together, these results suggest a major influence of sunlight in regulating the competition among autotrophic and heterotrophic picoplankton for DMSP-sulfur utilization.

General conclusions

From a broader perspective, the results obtained in this thesis allow to further conclude that:

1. Light can modulate the flux of organic substrates through marine food webs at different scales, but the overall effects will vary depending to a great extent on the organisms involved and their specific responses to sunlight. The different affinities found for the studied substrates as well as differential behaviors of groups with regard to light conditions indicate that these interactions are far from simple.

2. The degree of similarity/dissimilarity between the past light exposure history of the organisms and the experimental incubation conditions has a large influence on the effects of manipulated UVR on natural communities. This highlights the limitations of static incubations.

3. High numbers of bacterial cells active in both leucine and DMSP-sulfur uptake were found in summertime polar and Mediterranean waters, and sunlight seemed to play a major role in regulating the total amount of assimilated substrate. Generally, the numbers of ^{3}H-leucine-assimilating *Eubacteria* cells were larger than those of ^{35}S-DMSP-assimilating cells.

4. Phytoplankton osmoheterotrophy may sometimes be of relevance in the fluxes of labile DOM, as shown by incubations of polar and Mediterranean waters with ^{35}S-DMSP. Uptake of DMSP-sulfur by phytoplankton seems to be enhanced when concomitant with naturally-occurring high DMSP concentrations. Sunlight can sometimes allow phototrophs to compete more efficiently for DOM uptake, thus ultimately influencing the cycling of organic sulfur in the surface ocean.

5. Dark incubations are used as the standard method for substrate uptake and assimilation measurements from which to derive bacterial activity and secondary production. These are easier and more practical given the difficulty in accurately mimicking natural light conditions. However, in view of our results, which show that light is an essential modulator of DOM fluxes through bacteria and phytoplankton, we suggest that a key characteristic of the ecosystem functioning and biogeochemical balance, such as bacterial heterotrophic production, should be measured in parallel in the dark and under realistic light conditions. Both a mechanistic understanding and a quantitative assessment of the effects of sunlight on microbial activity are essential to accurately model carbon fluxes through marine microbial food webs.

Future perspectives and open questions

The concern for UVR impacts on marine ecosystems has increased over the past decades in parallel with the development of underwater UV instruments (see refs. in Tedetti and Sempéré, 2006) and growing knowledge on UVR effects on different organisms and ecosystems (Häder *et al.*, 2007). However, there is still a remarkable dearth of data addressing UVR impacts on individual groups in natural communities. Although we have aimed to address some of these effects on the heterotrophic activity of specific surface-ocean microbes, we are aware that this thesis opens many questions that should be addressed in future studies, and we hope that our findings will stimulate further research in this field. Fast evolving techniques such as molecular approaches bring an opportunity for answering more detailed questions with regard to sunlight effects on community structure and on differential UVR-driven expression of functional genes. We believe that trying to answer some of the following remarks would ameliorate our understanding of the role of sunlight on marine ecosystems:

1. Needless to say, a good characterization of the drivers of UV attenuation and their seasonality in one's study area will lead to a better knowledge of the light-related dynamics of planktonic organisms. Such a study is already under way for the Blanes Bay Microbial Observatory sampling site (Galí *et al.*, in prep). This will also increase our capacity to accurately mimic light exposure conditions in order to avoid severe overexposure or underexposure of samples.

2. The development of technological systems for the simulation of vertical mixing in the ocean would allow incubation of samples under realistic sunlight conditions thus avoiding the aforementioned misexposure problems often related to static incubations.

3. New and more specific CARD-FISH probes would allow a deeper insight into particular phylotype responses to UVR without the need for culturing, substantially improving our understanding of the reported intra-group variability at both spatial and temporal scales. High-throughput sequencing of 16SrRNA genes, particularly if not biased by PCR amplification, could also allow more detailed analyses of the responses of marine communities to varying light levels.

4. A careful study of the relative roles of phototrophic organisms in DOM uptake under light exposure seems necessary in order to correct and redraw some of the DOM models were bacteria are considered major players; it might be possible that in some areas the proportion of DOM flowing through chemoheterotrophic bacteria will decrease in the presence of light in comparison to the flux through some photoheterotrophs.

5. Likewise, a deeper dive into the relevance of other phototrophic behaviors in nature is needed in order to unveil to which extent they do confer some advantages to light-harvesting organisms. For example, the combination of single-cell analysis with techniques to identify bacterial photoheterotrophy (i.e. infrared microscopy for bacteriochlorophyll *a* detection or expression of proteorhodopsin genes) might reveal whether light harvesting helps the organisms by enhancing their uptake of DOM, for example, a fact that has not been proven so far.

6. Since different responses are obtained depending on the substrate analyzed, a deeper knowledge of the light-driven regulation of different uptake systems would allow a better understanding of the relevant processes.

7. Finally, a multidisciplinary analysis of the simultaneous effects of light on the different components of the microbial food webs is needed in order to define ecological interactions and antagonistic and synergistic effects more comprehensively.

RESUMEN DE LA TESIS

(*Spanish summary)*

Introducción general

La radiación solar: el motor de la red trófica planctónica

A pesar de que la práctica totalidad del volumen oceánico se halla permanentemente sumida en la oscuridad, los procesos que tienen lugar en la relativamente estrecha capa superficial alcanzada por los rayos solares (zona fótica) son esenciales para el mantenimiento de la biosfera del planeta. La región visible del espectro solar (o radiación fotosintéticamente activa, PAR, 400-700 nm) que penetra en esta capa fótica propulsa cerca de la mitad de la creación fotosintética de materia orgánica global, favoreciendo así la existencia de los demás habitantes de la superficie del océano y de gran parte de los organismos de las zonas más profundas. Como consecuencia, la mayoría de la biomasa oceánica se concentra en este 3% del volumen oceánico total, y los organismos que allí habitan interactúan entre sí creando un complejo entramado de relaciones que conforman la denominada cadena trófica planctónica.

Hasta 1974 se pensaba que estas redes tróficas oceánicas se ceñían al paradigma clásico de tres niveles (productores primarios, secundarios y descomponedores); sin embargo, posteriores descubrimientos comenzaron a insinuar que dicha descripción excluía una parte importante de los flujos reales de materia y energía. En 1974, Lawrence Pomeroy postuló que los pequeñísimos microbios, hasta entonces ignorados, eran en realidad mucho más importantes y diversos de lo que se creía, y propuso la elaboración de un nuevo modelo más completo donde se incluyeran las bacterias como responsables indispensables de la remineralización del gran reservorio de carbono orgánico disuelto (DOC) del océano. Desde aquel momento, subsecuentes hallazgos tales como la regulación de las poblaciones bacterianas por predadores (Borsheim 1984; Fenchel 1982; Pedrós Alió y Brock, 1983) o por infecciones víricas (Bratbak *et al.*, 1992; Bratbak *et al.*, 1994) han ido añadiendo paulatinamente niveles de complejidad a este "bucle microbiano", así denominado desde Azam et al. (1983). En la figura 1 se esquematiza la visión actual de la estructura de las redes tróficas oceánicas.

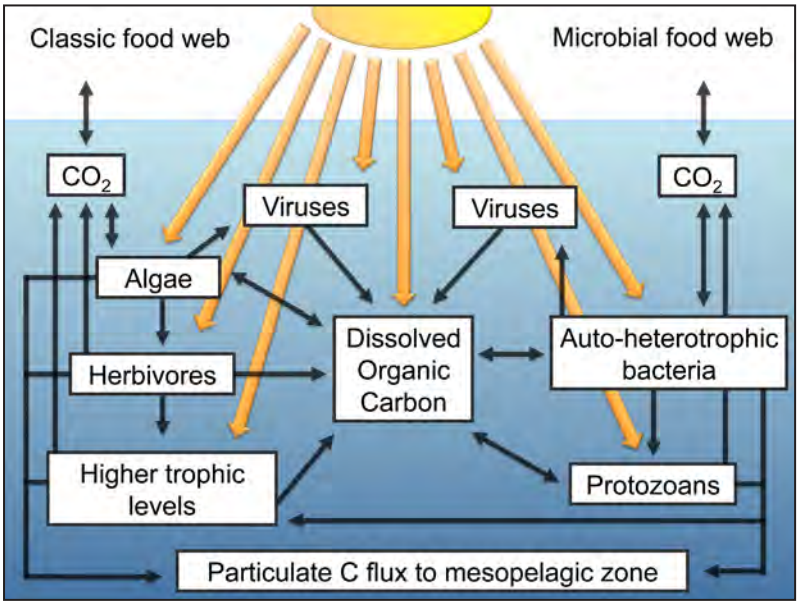

Fig. 1. Diagrama esquemático de las redes tróficas pelágicas ilustrando el flujo de carbono y energía a través de los sistemas. La gran mayoría de estos componentes pueden verse afectados directa o indirectamente por la radiación solar. Adaptado de Häder et al. (2007).

Por tanto, en un principio se asumía que la radiación solar ingresaba en el sistema principalmente a través del fitoplancton, modulando así los flujos de materia orgánica disuelta (DOM) a lo largo de este complejo entramado, ya que cualquier variación en la disponibilidad de la luz alteraría consecuentemente la cantidad de materia orgánica generada por fotosíntesis. Sin embargo, la reciente aparición en escena de la radiación ultravioleta (UVR, 280-400 nm) ha revelado un papel mucho más activo y complejo de la luz en los ecosistemas marinos superficiales.

La radiación UV en los ecosistemas marinos

La inquietud por la disminución de la concentración del ozono estratosférico y los consecuentes aumentos en la radiación UVB (280-320 nm) incidente motivaron, hacia mediados de los 80, múltiples estudios sobre sus efectos en distintos organismos. El descubrimiento posterior de que esta fracción del espectro penetraba en los océanos más profundamente de lo que se había pensado hasta entonces (Karentz y Lutze 1990; Worrest y Häder 1989), comenzó a dar pistas sobre el hecho de que la radiación UV podría estar modulando de manera significativa los ciclos de materia orgánica en el mar a través de

sus efectos no sólo sobre las algas, sino sobre todos los componentes del bucle microbiano (Fig. 1). Sin embargo, pese a los considerable avances desde aquellos hallazgos, aún son muchos los aspectos que desconocemos acerca del papel de la UVR sobre los flujos globales de materia y energía. Además, a pesar de que la reducción en las emisiones de sustancias nocivas para el ozono parece haber promovido parcialmente su recuperación, algunas estimas indican que aún transcurrirán varias décadas hasta que estos niveles puedan restablecerse por completo. En consecuencia, se prevé que las altas dosis de UVR en las áreas afectadas se prolongarán durante ese período (Mckenzie et al. 2007). Por otra parte, debido a que otros muchos factores también pueden influir en la UVR, y a su vez verse afectados por el cambio climático (p. ej. la cobertura de nubes, los aerosoles, la polución del aire), la existencia de interacciones impredecibles y desconocidas entre las dinámicas del ozono y el cambio climático sugieren un futuro todavía bastante incierto (Andrady et al. 2007).

Hoy tenemos la certeza de que la radiación solar puede afecta a diversos organismos y procesos de los sistemas marinos superficiales y que, dependiendo de las regiones o longitudes de onda del espectro solar, sus efectos pueden variar. Aunque la radiación UVB (280-320 nm) es más energética y por tanto más dañina que la UVA (320-400 nm), la mayor proporción de ésta última en el espectro solar hace que pueda considerarse una fuente significativa de daño biológico (Karentz et al. 1994).

En un principio, la mayoría de los estudios sobre el impacto de la UVR se centró en el fitoplancton (ver referencias en Xue et al. 2005), y hoy sabemos que esta radiación afecta no sólo a la fotosíntesis (Smith *et al.* 1992, Neale *et al.* 1994, Cullen & Neale 1997, Villafañe *et al.* 2004, Yuan *et al.* 2007), sino también a la motilidad, al crecimiento y desarrollo de las células, su contenido en pigmentos, la respiración, la toma de nutrientes o el metabolismo de las células fitoplanctónicas (ver referencias en Rai y Mallick 1998; Xue et al. 2005). Por otro lado, cada vez más estudios confirman la evidencia de que la UVR puede afectar a cualquier componente de las redes tróficas marinas, incluyendo tanto a los virus acuáticos (Jacquet y Bratbak 2003; Regan et al. 1992; Suttle y Cheng 1992; Wilhelm et al. 2003) como a flagelados heterotróficos (Ochs 1997; Ochs y Eddy 1998; Sommaruga et al. 1996) o bacterias, entre otros (Gustavson et al. 2000; Herndl et al. 1993; Jeffrey et al. 1996a; Kaiser y Herndl 1997). No obstante, una exposición a la UVR no implica necesariamente que sus efectos sean deletéreos: esta radiación también puede modificar indirectamente los flujos de carbono y nutrientes a través de las redes tróficas estimulando o inhibiendo la biodisponibilidad de DOM para los microorganismos (Benner y Biddanda 1998; Herndl et al. 1997; Morán y Zepp 1997; Obernosterer et

al. 1999; Obernosterer et al. 2001; Tedetti et al. 2009; Tranvik y Kokalj 1998; Wetzel et al. 1995), o regulando algunas interacciones entre virus y hospedadores (Jacquet y Bratbak 2003; Maranger et al. 2002), predadores y presas (Scott et al. 1999; Van Donk y Hessen 1995), simbiontes (Dionisio-Sese et al. 2001), etcétera. Por tanto, dependiendo de la vulnerabilidad de cada compartimento trófico, cualquier efecto negativo de la UVR podría compensarse, o incluso revertirse, debido a algún efecto positivo indirecto (por ejemplo, el daño por UVR sobre las bacterias frente a una estimulación bacteriana debida a transformaciones fotoquímicas de la DOM hacia formas más lábiles). Por último, ya que las regiones UVA y PAR pueden estar implicadas en mecanismos fotoenzimáticos de reparación de DNA (Sancar y Sancar 1988), el balance entre el daño y la reparación variará dependiendo de la exposición relativa al UVB frente a longitudes de onda más largas (Herndl et al. 1997; Kaiser y Herndl 1997), complicando más, si cabe, cualquier tentativa de modelado o predicción.

La UVR como moduladora de los flujos de DOM a través de las redes tróficas marinas

Entre todas las posibles dianas de la UVR que penetra en el océano, las bacterias heterotróficas han despertado una creciente atención. Actores claves en el reciclaje de nutrientes y energía, cualquier impacto sobre ellas podría modificar significativamente los flujos de DOM que son canalizados hacia niveles tróficos superiores. Debido a su falta de pigmentos y a su pequeño volumen celular, las bacterias heterotróficas marinas se encuentran entre los grupos planctónicos más susceptibles al daño por la radiación (García-Pichel 1994; Jeffrey et al. 1996a). Algunos estudios recientes en este campo demuestran que una exposición corta de las bacterias a dosis de UVR naturales o artificiales puede, además de afectar a muchos otros procesos metabólicos, reducir significativamente la incorporación de sustratos marcados radiactivamente como ^{3}H-leucina o ^{3}H-timidina, ambos indicadores de la producción bacteriana (Aas et al. 1996; Alonso-Sáez et al. 2006; Herndl et al. 1993; Kaiser y Herndl 1997; Müller-Niklas et al. 1995; Sommaruga et al. 1997). A pesar de que en general el efecto dañino de la UVR se acentúa hacia longitudes de onda más cortas como el UVB, también se han documentado efectos negativos importantes debidos tanto a PAR como a UVA (Aas et al. 1996; Morán et al. 2001; Pakulski et al. 2007; Sommaruga et al. 1997). Los efectos causados por estas regiones del espectro se atribuyen a procesos fotodinámicos en los que se forman especies reactivas de oxígeno o

radicales libres que a su vez pueden afectar a las células (Harrison 1967), mientras que la radiación UVB puede causar también daño directo sobre las moléculas. Sin embargo, lo cierto es que en la mayoría de los casos se desconoce por qué los efectos dependen más de unas longitudes de onda que de otras, y existen evidencias de respuestas variables según la región, la profundidad, la estación o la hora de muestreo, así como según el sustrato considerado, la intensidad de la radiación y otras variables. En la tabla 1 se resumen los trabajos existentes acerca de los efectos de la radiación solar sobre la actividad heterotrófica de las bacterias marinas (medida generalmente a partir de la incorporación de ^{3}H-leucina o ^{3}H-timidina), ilustrando la remarcable variabilidad de los resultados obtenidos.

Examinando con atención la Tabla 1, podemos apreciar que el efecto de la radiación solar sobre la actividad bacteriana no es siempre negativo. ¿Cómo se explicarían, por ejemplo, la consistente estimulación de la producción bacteriana debida a la PAR observada por Church et al. (2004), o el incremento en la incorporación de ^{3}H-leucina y ^{3}H-timidina que encontraron Pakulski y cols. (2007) al exponer las muestras a las distintas regiones del espectro? Aparte de la posibilidad de que se dé una estimulación indirecta de las bacterias causada por un incremento en la producción de fotosintato o por una mayor biodisponibilidad de la DOM fototransformada, algunos estudios recientes sugieren que la existencia de estrategias metabólicas mixtas conocidas conjuntamente como fotoheterotrofía y aparentemente comunes en los océanos, podrían ser responsables de una fracción importante de la producción bacteriana medida en aguas superficiales (Béjà et al. 2000; Kolber et al. 2001; Kolber et al. 2000; Zubkov et al. 2003).

Table 1. Literatura sobre los efectos de la radiación solar en la actividad heterotrófica de las bacterias marinas. Por simplificar, se incluyen únicamente medidas de incorporación de ^{3}H-leucina y ^{3}H-timidina, los dos trazadores más communes; sin embargo, tanto estos como otros autores también han considerado a veces otros sustratos o incluso actividades ectoenzimáticas.

Authors	Year	Location	Light source	Wavelenghts	Time of exposure	Main reported effects Substrate incorporation	
						Leucine	Thymidine
Herndl et al.	1993	Adriatic Sea	Artificial	UVB	30 min/4h	↓	↓
			Sunlight	PAR+UVR	4h		↓
				PAR+UVA	4h		↓
				Darkness	4h-8h		Recovery
Müller-Niklas et al.	1995	Adriatic Sea	Artificial	UVB	6-12h		
				Darkness			
			Sunlight	PAR+UVR	4h		↓
				Darkness			Recovery
Aas et al.	1996	Gulf of Mexico	Sunlight	PAR	1-11h	↓⇧	↓⇧
				PAR+UVA		↓⇧	↓⇧
				PAR+UVR		↓	↓
		St. Rosa Sound (Florida)		PAR	2-10h	↓⇧	↓
				PAR+UVA		↓	↓
				PAR+UVR		↓	↓
Kaiser & Herndl	1997	N Adriatic Sea	Artificial	UVB	2-4h		↓
				UVA			Recovery
				PAR			Recovery
				Darkness			No recovery
			Sunlight	PAR+UVR	3h	↓	↓
				PAR+UVA		Recovery	Recovery
				Darkness		Recovery	No recovery
Sommaruga et al.	1997	N Adriatic Sea	Sunlight	PAR	3-4h	↓	↓
				PAR+UVA		↓	↓
				PAR+UVB		↓	↓
Pakulski et al.	1998	Pickles Reef (Florida)	Sunlight	PAR+UVR	2 days	↓	↓⇧
Shiah	1999	Kuroshio (Taiwan)	Sunlight	PAR	1day		↓(day)
			Artificial	PAR	4-6h		↓
Visser et al.	1999	Caribbean Sea	Sunlight	PAR	3h	↓	↓
				PAR+UVA		↓	↓
				PAR+UVR		↓	↓
Gustavson et al.	2000	Gullmar Fjord (Sweden)	Sunlight	*respect to PAR+UVA*	1-11 days		
				PAR+UVR			↓⇧
			+artif. UVB	PAR+UVR+UVB			⇧
Ziegler & Benner	2000	Laguna Madre (Texas)	Sunlight	PAR	1h	⇧	
				PAR+UVR		⇧	
Chatila et al.	2001	St. Lawrence estuary (Québec)	Sunlight	PAR	7 days		-
				PAR+UVR			-
			+artif. UVB	PAR+UVR+UVB			↓
Morán et al.	2001	NW Mediterranean	Sunlight	PAR	2h	↓	
			artif.UVB	PAR	2h	↓⇧	
		N Atlantic	Sunlight	PAR	3-6h	↓⇧	
Pausz & Herndl	2002	North Sea	Artificial	PAR+UVR	4h	↓⇧	
				PAR+UVA		Recovery	
				PAR		Recovery	
				Darkness		Recovery	
Visser et al.	2002	Caribbean Sea	Sunlight	PAR+UVR	8h	↓	↓
Church et al.	2004	North Pacific	Artificial	PAR	1-2h	⇧	
Alonso-Sáez et al.	2006	NW Mediterranean	Sunlight	PAR	4h	↓	
				PAR+UVA		↓	
				PAR+UVR		↓	
Hernández et al.	2006	Coliumo Bay (Chile)	Sunlight	PAR	4-11 h	↓	↓
				PAR+UVA		↓	↓
				PAR+UVR		↓	↓
Hernández et al.	2007	Coliumo Bay (Chile)	Sunlight	PAR	5-20h	↓⇧	↓⇧
				PAR+UVA		↓⇧	↓⇧
				PAR+UVR		↓⇧	↓⇧
Michelou et al.	2007	N Atlantic	Sunlight	PAR	1h, 6h	⇧	
Pakulski et al.	2007	E Pacific	Sunlight	PAR	4h	↓	↓⇧
				PAR+UVA		⇧	↓⇧
				PAR+UVA(370nm)		↓⇧	↓⇧
				PAR+UVR		↓	↓⇧
Conan et al.	2008	New Caledonia	Sunlight	PAR	6h	↓	
				PAR+UVA		↓	
				PAR+UVR		↓	
Pakulski et al.	2008	Palmer Station (Antarctica)	Sunlight	PAR	12h	↓	
				PAR+UVR		↓	
Joux et al.	2009	NW Mediterranean	Sunlight	PAR	9-10h	-	-
				PAR+UVR		↓	↓
Santos et al.	2010	Ria de Aveiro (Portugal)	Artificial	UVB	9h	↓	

Fotoheterotrofía en los océanos

A diferencia de nuestra visión terrestre de los ecosistemas según la cual consideramos que las plantas y los animales se comportan ecológicamente como tal, los límites entre distintos microorganismos marinos no están tan claramente definidos. La mayoría de ellos pueden alternar o utilizar simultáneamente distintos tipos de metabolismo de modo que, dependiendo de las condiciones ambientales, son capaces de aprovechar distintas fuentes de energía y de carbono. Así podemos distinguir tres tipos mayoritarios de fotoheterótrofos: bacterias que contienen proteorodopsinas (PR), bacterias con bacterioclorofila *a* (denominadas fotótrofos aeróbicos anoxigénicos, AAnPs) y fitoplancton eucariota o procariota capaz de fagocitar presas o de incorporar osmoheterotróficamente distintos compuestos orgánicos. Además, algunos radiolarios, foraminíferos y ciliados pueden adquirir capacidades fototróficas mediante la endosimbiosis con algas o la retención de plástidos, aunque su relevancia en los ecosistemas marinos es aún desconocida (Porter 1988; Stoecker 1998; Stoecker et al. 2009; Stoecker et al. 1987).

Bacterias que contienen proteorodopsinas

Hasta el año 2000 se consideraba que la mayoría de los metabolismos fototróficos se basaban en moléculas de tipo clorofila, paradigma que se derrumbó con el descubrimiento en aquel año de unas proteínas bacterianas de tipo-rodopsina (proteorodopsinas, PR) aparentemente comunes en el océano. Estos fotosistemas simples, que durante largo tiempo se habían sabido presentes en algunas arqueas halofílicas (Oesterhelt y Stoeckenius 1973), eran estructuralmente similares a las rodopsinas de la retina de eucariotas superiores como los humanos, pero tenían un origen evolutivo distinto. Estas proteorodopsinas son proteínas integrales de membrana que contienen retinal y que funcionan como bombas de protones impulsadas por la luz. Se descubrieron inicialmente en unas *Gammaproteobacteria* marinas muy abundantes, el grupo SAR 86 (Béjà et al. 2000), hecho que motivó el nombre de "proteorodopsinas". Poco tiempo después de este hallazgo, estas PRs comenzaron a identificarse en muchos otros grupos bacterianos distintos y hoy las encontramos en filotipos como las *Alpha- Beta-* y *Gammaproteobacteria, Bacteroidetes, Actinobacteria* y *Planctomycetes,* así como en ciertos grupos de arqueas (Béjà et al. 2000; Giovannoni et al. 2005; Mccarren y Delong 2007; Sharma et al. 2008; Stingl et al. 2007), lo que sugiere un papel importante de estas PR en la ecología de los procariotas que habitan las superficies iluminadas del océano. Sin

embargo, aún hoy se desconoce enormemente la magnitud del papel de estas proteínas en las comunidades naturales. De hecho, hasta el momento sólo dos estudios han ofrecido evidencias directas de que las PR funcionan activamente como pigmentos que utilizan la luz en el agua de mar (Béjà et al. 2001; Giovannoni et al. 2005), y únicamente otros dos trabajos han demostrado, utilizando aislados marinos, algunas ventajas directas de las PR tales como la promoción del crecimiento (Gómez-Consarnau et al. 2007) o de la supervivencia (Gómez-Consarnau et al. 2010), especialmente en condiciones ambientales adversas. Sin embargo, a pesar de esta falta de evidencias, algunos resultados recientes sobre la expresión de genes de PR inducida por la exposición a la luz (Béjà y Suzuki 2008; Gómez-Consarnau et al. 2007; Lami et al. 2009; Poretsky et al. 2009) sugieren un papel activo (aunque de relevancia aún desconocida) de las PR en los ecosistemas naturales.

Bacterias fotosintéticas aeróbicas anoxigénicas (AAnPs)

Antiguamente se consideraba que las bacterias que contenían bacterioclorofila *a* (Bchl *a*), también conocidas como bacterias púrpura, requerían condiciones anóxicas para realizar la fotosíntesis (Pfennig 1967). Esto cambió cuando Shiba et al. (1979) y Shiba y Simidu (1982) demostraron que una bacteria aeróbica obligada (*Erythrobacter longus*) podía producir su aparato fotosintético en presencia de oxígeno y de luz. Algo más tarde, estos autores descubrieron abundancias importantes de estas AAnPs en las costas de Australia (Shiba *et al.* 1991), pero no fue hasta 2000, año en que se descubrió que las bacterias con Bchl *a* abundaban en el océano, cuando se les empezó a reconocer una cierta relevancia a escala global. Desde entonces, diversos trabajos de campo han confirmado una extendida presencia de la Bchl *a* entre diversos miembros de las comunidades bacterianas tales como las *Alpha-*, *Beta-, Gammaproteobacteria* y *Bacteroidetes* (Cottrell et al. 2006; Rusch et al. 2007; Sieracki et al. 2006; Venter et al. 2004; Yutin et al. 2007).

Del mismo modo que con las PR, todavía se dispone de muy poca información acerca de las ventajas biológicas que podría conferir la posesión de Bchl *a*. Sin embargo, aunque nadie ha logrado crecer ninguno de los aislados existentes en condiciones estrictamente autotróficas, algunas especies han demostrado una cierta estimulación en la toma de CO_2 causada por la luz (Kishimoto et al. 1995; Shiba 1984; Shiba y Harashima 1986; Suyama et al. 2002), si bien en tasas demasiado bajas como para sustentar un crecimiento puramente autotrófico. Si bien podría ser un modo de obtener energía extra como suplemento de su metabolismo heterotrófico, quizá incluso regulable bajo ciertas

condiciones adversas como bajas concentraciones de DOC (Suyama et al. 2002), no seremos capaces de entender cualquier contribución a los procesos fototróficos oceánicos sin un estudio más detallado sobre la fisiología y ecología de estos organismos.

Osmoheterotrofía y fagotrofía del fitoplancton

A pesar de que la toma y utilización de DOM por distintos cultivos de algas se demostró hace varias décadas (ver referencias en Amblard 1991; Neilson y Lewin 1974), en un principio se creyó que era un hecho ecológicamente irrelevante debido a las bajas concentraciones de sustratos presentes en ambientes naturales (Wright y Hobbie 1965; Wright y Hobbie 1966). Hoy se sabe que algunas especies de fitoplancton son capaces de tomar una gran variedad de sustratos orgánicos de la fase disuelta de forma que, en determinadas circunstancias, serían potencialmente competitivas frente a las bacterias (Allen 1971; Kamjunke et al. 2008; Kamjunke y Tittel 2008). Todo esto, unido a la fagotrofía descrita en muchos grupos de algas (Jones 1994; Raven 1997), sugiere que el fitoplancton podría tener un papel más diverso en los ciclos biogeoquímicos marinos que el mero hecho de suplir a los heterótrofos con materia orgánica sintetizada autotróficamente. De nuevo, sin embargo, carecemos de la información suficiente para valorar la relevancia del papel de la osmoheterotrofía del fitoplancton en condiciones naturales.

No sólo el fitoplancton eucariota es capaz de tomar sustratos orgánicos del medio: las cianobacterias, productores primarios mayoritarios en muchos ecosistemas, también han demostrado la capacidad de asimilar DOM (p. ej. Chen et al. 1991; Collier et al. 1999; Paerl 1991; Rippka 1972; Zubkov et al. 2003; Zubkov y Tarran 2005). Estudios recientes sobre las cianobacterias *Prochlorococcus* y *Synechococcus,* tan abundantes en muchas regiones del océano, han desvelado un papel fundamental de estos grupos en los flujos de algunos compuestos orgánicos (Malmstrom et al. 2005; Zubkov et al. 2003; Zubkov y Tarran 2005), sugiriendo que este potencial fotoheterotrófico podría ocasionar sobreestimas o subestimas en medidas de producción bacteriana como el método de incorporación de leucina (Kirchman et al. 1985): en el caso de algunos *Prochlorococcus,* por ejemplo, se ha observado que pueden ser responsables de hasta el 30% de las tasas de incorporación de leucina (Michelou *et al.* 2007; Zubkov y Tarran 2005).

En este contexto surgen algunas preguntas inevitables: ¿hasta qué punto influye la luz en todos estos comportamientos fotoheterotróficos? ¿podría esta potencial estimulación de las actividades fotoheterotróficas ensombrecer los efectos negativos de la UVR sobre

la toma de sustratos por distintos microorganismos? ¿Motivaría la exposición a la luz una competición más eficiente en el uso de la DOM por ciertos organismos fototróficos frente a sus homólogos heterótrofos?

Para responder a estas y a otras cuestiones, parece evidente un hecho fundamental: no será posible una comprensión precisa de los procesos que provocan las distintas respuestas a la luz sin un conocimiento en profundidad de la composición de las comunidades microbianas y del papel de los distintos componentes en ellas.

Análisis de identidad: la importancia de las conductas individuales

Mientras que durante los últimos 20 años se han estudiado extensamente los efectos de la radiación solar sobre las comunidades microbianas en su totalidad (Tabla 1), todavía sabemos muy poco sobre cómo se distribuyen estos efectos entre los distintos grupos de microorganismos. Algunos estudios con aislados marinos expuestos a la UVR han evidenciado una importante variabilidad interespecífica no solo en la acumulación de daño en el DNA (Joux et al. 1999), sino también en su viabilidad tras la exposición, sus actividades específicas o su capacidad de reparación del DNA (Agogué et al. 2005; Arrieta et al. 2000; Helbling et al. 1995). Sin embargo, debido a que la mayoría de las bacterias no son fácilmente cultivables, es posible que dichos aislados sean componentes minoritarios de las poblaciones naturales y que estos resultados no sean representativos de los ecosistemas (Amann et al. 1995). Por el contrario, tan solo un número muy pequeño de trabajos han abordado este tema con el estudio de comunidades naturales, desvelando que, cuanto más nos adentramos en el conocimiento de la microbiología marina, mayor parece ser la complejidad que aflora. Por ejemplo, mientras que Winter et al. (2001), mediante análisis con PCR-DGGE, no halló más que unos pocos filotipos sensibles a la UVR en mesocosmos transparentes , Santos et al. (2010) observaron que la exposición de muestras de agua dulce y de estuario causaba una reducción significativa en la diversidad bacteriana, sugiriendo un papel importante de la UVR en la composición de las comunidades. Por otro lado, combinando microautoradiografía (MAR) con técnicas de hibridación fluorescente in situ (MAR-CARD-FISH), Alonso-Sáez et al. (2006) desvelaron distintos efectos de la UVR en la actividad de diversos grupos bacterianos del Mediterráneo, mientras que un análisis citométrico de las mismas muestras mostró que *Synechococcus* parecía significativamente más resistente a la UVR que *Prochlorococcus* (Sommaruga *et al*. 2005). Más recientemente, Kataoka y colaboradores (2009) demostraron por primera

vez la existencia de bacterias resistentes y sensibles a la UVR en un ambiente oceánico mediante el uso de PCR-DGGE combinada con técnicas de inmunocaptura. Entre todos estos métodos, la microautoradiografía puede ser una buena herramienta cuando se trata de analizar el papel de la luz en la toma de compuestos marcados radiactivamente por organismos específicos de las comunidades naturales (Meyer-Reil, 1978, Fig. 2). Además, si se aplica en combinación con algunos técnicas de identificación como el CARD-FISH, obtendremos información acerca de la identidad y la actividad específica de los grupos de interés.

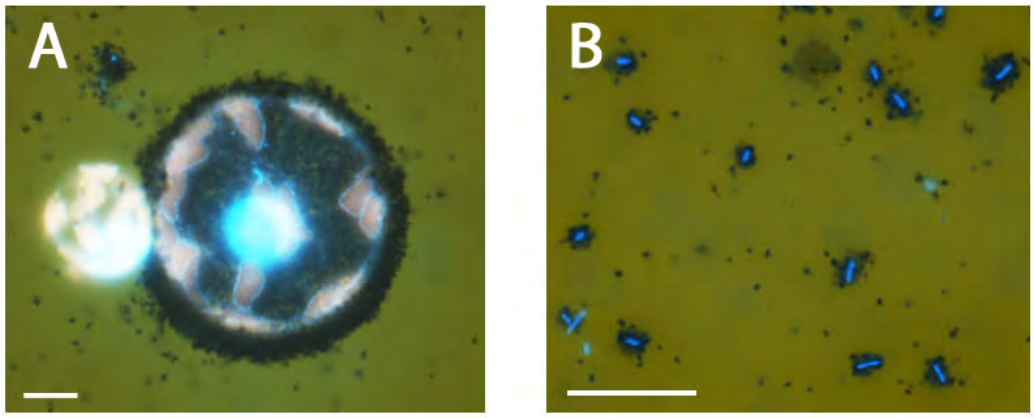

Fig. 2. Ejemplos de microautoradiogramas de muestras marinas de fitoplancton (A) o de bacterias (B). Las regiones negras que rodean a las células reflejan la asimilación de algún sustrato radiactivo, y se forman cuando la radioactividad retenida dentro de las células "activas" impacta sobre una emulsión fotográfica haciendo que precipiten gránulos de plata visibles al microscopio tras el revelado de las muestras. (A) Dos diatomeas céntricas de tipo *Thalassiosira*, una inactiva en la toma de ^{3}H-leucina (izquierda) y otra muy activa (derecha); (B) varias bacterias marcadas para la toma de ^{3}H-leucina mostrando ácidos nucleicos teñidos con 4',6'- diamidino-2-fenilindol (DAPI).

Obviamente, esta variabilidad interespecífica en las actividades metabólicas y sus respuestas a la luz no se restringe a las bacterias, sino que también se han detectado distintas sensibilidades a la UVR y capacidades de reparación variables entre grupos de fitoplancton diferentes, protistas heterotróficos e incluso metazoos pelágicos (Hessen, 2003; Karentz et al. 1991; Llabrés y Agustí 2006; Llabrés et al. 2010; Sommaruga y Buma 2000; Sommaruga et al. 2005). Por tanto es evidente que un buen conocimiento de la

composición de las comunidades microbianas combinado con el análisis de los efectos de la luz sobre especies o grupos particulares mejoraría en gran medida nuestra comprensión del impacto de la UVR sobre los ecosistemas marinos planctónicos.

Escalas de variación en la exposición de los microorganismos marinos a la radiación solar

Sumándose a tan amplio rango de posibilidades, la variabilidad inherente a las condiciones de luz añade algunas láminas más de complejidad al problema. La calidad y la intensidad de la radiación recibidas por cualquier célula fluctuarán continuamente dependiendo mayoritariamente de los cambios en el ángulo solar cenital, pero también de la profundidad y la intensidad de los procesos de mezcla, la atenuación en la columna de agua, la cobertura de nubes e incluso de las superficies de hielo o nieve si se trata de regiones polares. Todo esto se traduce en condiciones lumínicas que varían según escalas tanto temporales (estaciones, ciclos diarios) como espaciales (latitud, profundidad, adhesión a partículas). Obviamente, esta gran variabilidad complica cualquier diseño experimental y dificulta la interpretación de resultados, que a su vez dependerán en gran medida de cuánto difieran las condiciones experimentales de aquéllas que recibían las células en su ambiente natural.

Variabilidad espacial

Variaciones latitudinales: de los trópicos a los polos

En nuestro planeta, el régimen lumínico varía en el espacio como consecuencia directa de la elevación solar, con la consiguiente reducción de la UVR a medida que nos desplazamos desde el ecuador a las regiones polares. Sin embargo, aunque las irradiancias de la UVR son menores en las regiones polares que en latitudes más bajas, se ha dedicado un gran esfuerzo al estudio de los impactos de la UVR en el Ártico y en la Antártida, ya que se consideran las regiones más afectadas por la reducción del ozono y los efectos derivados del calentamiento global (p. ej. pérdida de la cobertura de hielo y una estratificación más acusada de la columna de agua debido a temperaturas más cálidas, Johannessen et al. 1999; Jones y Shanklin 1995; Müller et al. 1997; Rothrock et al. 1999). Asimismo, los organismos que habitan en estas frías aguas polares están permanentemente expuestos

a la luz durante los meses de verano, y además se ha sugerido que pueden ser más sensibles al daño por la radiación debido a un peor funcionamiento de los mecanismos de reparación a muy bajas temperaturas (Vincent et al 2006). En cambio, las aguas tropicales y subtropicales experimentan fuertes calentamientos de la superficie, lo que lleva a la producción de capas superficiales cálidas y saladas que persisten por largos periodos y aíslan las aguas de las superficie exponiendo a los organismos a dosis elevadas de UVR. Y por último y en una situación intermedia, encontramos las áreas templadas donde las estaciones transcurren gradualmente partiendo de una situación de aguas estratificadas cálidas en verano hacia una columna de agua más mezclada en invierno. Por tanto los organismos marinos que habitan distintas latitudes estarán expuestos a muy diferentes regimenes lumínicos, aunque la falta de estudios comparables entre latitudes distintas dificulta cualquier predicción de patrones latitudinales en las respuestas fisiológicas a la radiación.

Variaciones verticales: desplazamientos en la columna de agua

Los procesos de mezcla vertical son otro factor determinante de la exposición de la UVR recibida por los organismos planctónicos. Debido a que las longitudes de onda más cortas (p. ej. UVB) se absorben más rápido que las más largas (UVA o PAR) en la columna de agua (Hargreaves et al 2003), la DOM y los microorganismos están continuamente expuestos a condiciones de luz cambiantes a medida que se desplazan hacia arriba y hacia abajo dentro de la capa de mezcla superficial. Por consiguiente, dependiendo tanto de la atenuación de la luz como de las condiciones de mezcla, la exposición será más o menos larga, y como la proporción relativa de la radiación UVB frente a la UVA o a la PAR disminuye a medida que aumenta la profundidad, cuanto más profundamente se desplacen los organismos, mayor serán sus oportunidades de reparación. A pesar de las dificultades de simular las condiciones de mezcla in situ, algunos estudios han concluido que una mezcla profunda protege a las células de una exposición mantenida en el tiempo mientras se incrementa la reparación (Herndl et al. 1997; Huot et al. 2000; Jeffrey et al. 1996b; Kaiser y Herndl 1997; Neale et al. 2003). En consecuencia, cualquier medición realista de las actividades biológicas y biogeoquímicas debería tener en cuenta esta exposición variable a la luz.

Variabilidad temporal

Variaciones estacionales vs. variaciones diarias

La variabilidad en el campo lumínico no está restringida a la escala espacial, sino que también incluye la dimensión temporal. A lo largo del día, los microorganismos de áreas tropicales y templadas están expuestos a condiciones cambiantes que van desde irradiancias elevadas hasta la más completa oscuridad, excepto aquellos que habitan en latitudes altas durante los solsticios de verano. Muchos parámetros biológicos varían según ciclos diarios en el océano como consecuencia directa de la relación entre la luz solar y la biota marina, y muchos organismos exhiben ciclos sincronizados con los cambios diarios en la disponibilidad de la luz. Como el ejemplo más obvio, los picos diarios en la producción primaria generalmente ocurren alrededor del mediodía, y este abastecimiento diario a su vez puede dirigir ciclos en predadores o consumidores de la DOM (Atkinson et al. 1992a; Atkinson et al. 1992b; Gasol et al. 1998; Hernández-León et al. 2001; Shiah 1999). Del mismo modo, la UVR puede afectar directamente a los organismos a lo largo de los ciclos día-noche: por ejemplo, Jeffrey et al. (1996b) observaron una inhibición en la incorporación de ^{3}H-timidina y un aumento del daño en el DNA en muestras diurnas del Golfo de México, mientras que después del atardecer el daño se eliminaba rápidamente y la producción bacteriana volvía a recuperarse. En concordancia con estos resultados, Both y colaboradores (2001) mostraron que la expresión del gen de reparación del DNA recA también exhibía su máximo al final del día.

Superpuestos a esta escala de variabilidad, los niveles de radiación que alcanzan la superficie del océano fluctúan a lo largo de las estaciones: aumentan desde el invierno al verano, con los consiguientes cambios en la dinámica de la estratificación de la columna de agua. Sin embargo, muy pocos estudios han considerado esta variabilidad estacional en las respuestas a la luz de comunidades cambiantes a lo largo del año. Algunos trabajos sobre comunidades fitoplanctónicas han revelado cambios estacionales en la sensibilidad al UVR (p. ej. menor inhibición por UVR en verano, Gala y Giesy 1991; Hobson y Hartley 1983) mientras que otros autores no observaron distintas sensibilidades en muestras de otoño, primavera o verano (Furgal y Smith 1997). Otros estudios que también indican sensibilidades cambiantes del fitoplancton a lo largo del año han sugerido que factores tales como el tamaño celular, la composición taxonómica, la temperatura, la luz o la disponibilidad de nutrientes podrían estar a su vez influyendo las respuestas observadas (Banaszak and Neale 2001; Villafañe et al. 2004).

Por el contrario, hasta el momento ningún estudio ha analizado la estacionalidad de las respuestas bacterianas a la radiación. Únicamente Alonso-Sáez et al. (2006), que realizaron incubaciones a la luz tanto en primavera como en verano, sugirieron que parecía darse algún tipo de selección de grupos fotoresistentes en los periodos de irradiancias más intensas en la Bahía de Blanes (Mediterráneo noroccidental).

¿Se pueden adaptar las bacterias a condiciones cambiantes de luz?

No existe un consenso claro sobre si las bacterias se pueden adaptar a la UVR; a pesar de la gran variabilidad interespecífica de la sensibilidad a la UVR y de las capacidades de reparación entre distintos grupos de bacterias (Agogué et al. 2005; Alonso-Sáez et al. 2006; Arrieta et al. 2000; Joux et al. 1999; Kataoka et al. 2009), algunos resultados controvertidos ensombrecen la respuesta: mientras que ciertos estudios indican que no hay diferencias entre la sensibilidad de bacterias provenientes de ambientes con distintos regimenes lumínicos, descartando por tanto cualquier estrategia adaptativa provocada por cambios en las condiciones de luz (Agogué et al. 2005; Bailey et al. 1983; Herndl et al. 1993; Hernández et al. 2007, Xenopoulos and Schindler, 2003), otros han hallado evidencias que sugieren que sí que podría darse algún tipo de fotoadaptación. Así, observaciones tales como incrementos en el número de bacterias tras la exposición a la UVR (Thomson et al. 1980), una mayor recuperación de las actividades bacterianas durante el segundo día de exposición respecto al primero (Pakulski et al. 1998), grupos de bacterias más sensibles a la UVR en primavera que en verano (Alonso-Sáez et al. 2006), respuestas de distintos aislados bacterianos a la exposición a la UVB en concordancia con los niveles de radiación de sus ambientes de origen (Fernández-Zenoff et al. 2006), o una menor reducción de la diversidad bacteriana y mayor recuperación en el bacterioneuston que en el bacterioplancton (Santos et al. 2010), apuntan hacia algún tipo de capacidad de ajuste bacteriano ante niveles variables de UVR. Sin embargo, son necesarios aún estudios comparativos de la vulnerabilidad a la UVR a largas escalas temporales o espaciales para poder aceptar con solidez un potencial bacteriano para la fotoadaptación.

Cerrando el ciclo

A pesar de todos los efectos enumerados previamente de la luz sobre los organismos, las interacciones entre estos dos componentes no son siempre unidireccionales, como se ilustraba en la figura 1. El propio plancton puede a su vez modular hasta cierto punto los niveles de radiación que alcanzan la superficie del océano. Entre otras posibilidades, se ha sugerido que lo consigue mediante la producción del compuesto biogénico dimetilsulfoniopropionato (DMSP) y su compuesto de degradación volátil, el dimetilsulfuro (DMS). El hecho que motivó inicialmente el estudio del DMS fue la hipótesis de James Lovelock en 1972 de que las emisiones de este gas desde la superficie del mar hacia la atmósfera podrían cerrar el ciclo global del azufre, línea que ganó interés cuando Charlson y colaboradores postularon en 1987 que este compuesto podría estar implicado en la regulación del clima. Estos autores sostenían que los productos de oxidación del DMS en la atmósfera actuaban como núcleos de condensación de nubes y que por tanto podrían favorecer su formación, reduciendo así el balance radiativo sobre los océanos.

Los procesos que dirigen la síntesis, los flujos y las transformaciones del DMSP y el DMS aún no se comprenden del todo (Simó 2001; Stefels et al. 2007). El ciclo del DMSP/DMS, esquematizado en la figura 3, podría resumirse como sigue: el DMSP es producido por distintos grupos del fitoplancton fundamentalmente como osmoregulador intracelular (Dickson y Kirst 1987) aunque también se han descrito otras funciones como crioprotector o antioxidante (Malin y Kirst 1997; Welsh 2000). El DMSP puede bien degradarse a DMS dentro de las células fitoplanctónicas, o bien liberarse a la fase disuelta a través de procesos de autolisis, ataque vírico, predación o exudación por las algas (Hill et al. 1998; Laroche et al. 1999; Wolfe et al. 1994), donde pasa a ser degradado por las comunidades microbianas. Sin embargo, esta producción de DMS (a través de la denominada vía de fragmentación o *cleavage pathway,* ,Ansede et al. 2001) no es el destino primario del DMSP disuelto, sino que la mayoría de este DMSP disuelto es transformado por las bacterias a través de vías de desmetilación en compuestos no volátiles (Visscher et al. 1992), o bien se incorpora en la biomasa bacteriana (Kiene et al. 1999). Algunos estudios han estimado que este último proceso puede satisfacer entre 1-15% de la demanda total de carbono de las bacterias y prácticamente toda su demanda de azufre (Kiene y Linn 2000; Simó et al. 2002; Zubkov et al. 2001). Por tanto, las bacterias se consideran los consumidores principales de esta fuente de azufre reducido que, aunque menos abundante, resulta energéticamente más económico que el ubicuo sulfato (Kiene et al. 1999). Sin embargo, recientemente se ha descrito incorporación de azufre del DMSP en protozoos herbívoros (Burkill et al. 2002; Saló et al. 2009; Simó

2004; Simó et al. 2002; Tang y Simó 2003; Wolfe et al. 1994) e incluso en fitoplancton no productor (o poco productor) de DMSP (Malmstrom et al. 2005; Vila-Costa et al. 2006b). La predación también puede estimular la conversión de DMSP a DMS si el precursor y los enzimas responsables de la reacción se mezclan físicamente en el medio (Kim et al. 2010; Stefels et al. 2007; Wolfe y Steinke 1996). El DMS puede a su vez ser consumido por algunas bacterias (González et al. 1999; Kiene y Bates 1990; Vila-Costa et al. 2006a), aunque también otros procesos fotolíticos o de ventilación pueden ser causas importantes de pérdida de DMS (p. ej. Toole et al. 2003; Zemmelink et al. 2004). Por tanto, dependiendo de que vía prevalezca y de qué organismos estén implicados, el flujo de DMS a la atmósfera será más o menos importante.

Una vez más, la luz del sol parece ser un modulador clave de este ciclo DMSP/DMS, ya que la mayoría de los procesos de liberación, producción, consumo y fotolisis dependen directa o indirectamente de los niveles de radiación (Kiene et al. 2000; Stefels 2000; Sunda et al. 2002; Vila-Costa et al. 2006b). En este contexto, si este complejo balance de efectos sinérgicos y antagónicos causados por la luz se traducen finalmente en un flujo aumentado de DMS a la atmósfera (Vallina y Simó, 2007), el ciclo se podría cerrar a través de esta retroalimentación negativa entre el plancton y la radiación. Algunas estimas recientes de la magnitud de esta retroalimentación sugieren que no es suficiente para contrarrestar significativamente los incrementos en la temperatura derivados del cambio global (p. ej. Vallina *et al.* 2007b), aunque sí que podría atenuar significativamente la radiación a escalas de tiempo más cortas en áreas del océano alejadas de las influencias continentales (Meskhidze y Nenes, 2006; Vallina et al. 2006, 2007a).

Por tanto, para poder comprender y predecir estas conexiones entre el plancton, el DMS, las nubes y la radiación solar, que en su conjunto constituyen un curioso ejemplo de organismos vivos impactando en la física de nuestro planeta, será necesario avanzar en el estudio de los protagonistas claves de este ciclo del DMS, sus funciones e interacciones en las redes tróficas y sus respuestas ante presiones ambientales tales como la radiación solar.

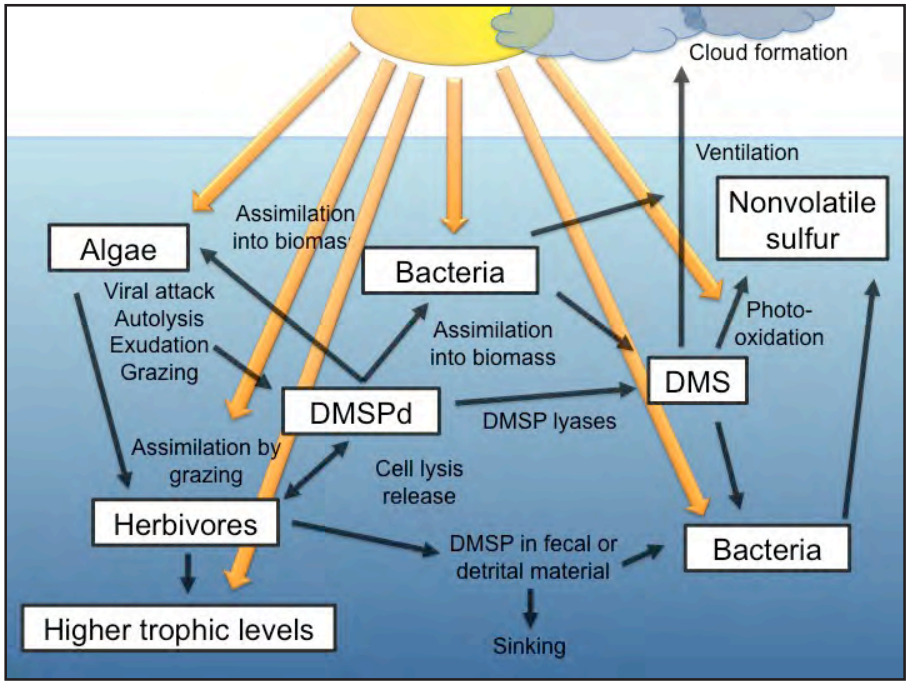

Fig.3. Esquema del ciclo DMSP/DMS en los océanos. Sólo una pequeña fracción del DMS escapa de este complicado ciclo y se ventila a la atmosfera, donde potencialmente podría favorecer la formación de nubes y por tanto una cierta atenuación de la radiación (adaptado de Simó 2001).

OBJETIVOS Y ESQUEMA DE LA TESIS

El objetivo principal de esta tesis es el de avanzar en el conocimiento del papel de la radiación solar natural sobre la actividad heterotrófica de las comunidades microbianas de aguas marinas superficiales, abordado tanto desde un punto de vista global como desde una perspectiva individual. Pretendemos responder a la pregunta de cómo influye la luz del sol en la toma y asimilación de distintos compuestos orgánicos disueltos por grupos específicos de bacterias y algas de diferentes ecosistemas (Mediterráneo costero y aguas del Ártico y de la Antártida), con especial énfasis en comprobar hasta qué punto se explican las respuestas observadas por la intensidad como por la calidad de la luz presente in situ. Con el fin de responder a ésta y a otras preguntas más específicas planteadas a continuación, llevamos a cabo una serie de experimentos de manipulación de la luz en los cuales cuantificamos y analizamos la toma de compuestos orgánicos marcados radiactivamente. Para ese propósito combinamos medidas de asimilación total con un enfoque microautoradiográfico, y además contrastamos las respuestas observadas con los niveles de radiación recibidos por las muestras durante los experimentos. Esta tesis se compone de seis trabajos distintos organizados en cinco capítulos principales que tratan de abordar los objetivos más específicos enumerados a continuación:

Capítulo 1. Cambios diarios en la actividad heterotrófica total y específica de bacterias de aguas superficiales de invierno en el Mar Mediterráneo noroccidental

Las variaciones diarias en los niveles de radiación producen cambios directos en muchos procesos biológicos tales como la fotosíntesis; las bacterias marinas también presentan en ocasiones ciclos diarios en su actividad o abundancia tanto a causa de daño directo por la UVR sobre ellas o como respuesta a cambios en la disponibilidad de DOM (p. ej. fotosintato), predación, etc. Sin embargo, no está claro si los distintos grupos de bacterias se comportan igual a lo largo de los ciclos diarios. En este capítulo quisimos (1) seguir la actividad diaria de los grupos mayoritarios de la Bahía de Blanes y (2) analizar si la radiación solar era responsable directa de alguno de los patrones observados en la incorporación de leucina y timidina.

Capítulo 2. Variabilidad estacional en las respuestas microbianas a las condiciones de radiación solar:

2.1 Variabilidad anual en la modulación por la radiación de la actividad heterotrófica de las bacterias en aguas superficiales del Mediterráneo noroccidental

A pesar de que hay muchos estudios sobre los efectos de la UVR sobre la producción bacteriana total, muy pocos trabajos han analizado este aspecto a lo largo un ciclo estacional completo durante el cual se espera que las respuestas bacterianas fluctúen según la variación estacional en los niveles de luz. En este capítulo pretendimos (1) examinar la existencia de patrones estacionales en las respuestas de las comunidades bacterianas de la Bahía de Blanes a los niveles in situ de PAR y UVR, y (2) comparar esos patrones con las respuestas de estas comunidades medidas bajo condiciones de luz invariables (PAR artificial). Además (3), exploramos si estos patrones se podían explicar por la estacionalidad de otros parámetros físicos o biológicos para discernir lo factores fundamentales que afectaban estas respuestas en una escala anual.

2.2. Patrones estacionales en la sensibilidad a la luz solar del bacterioplancton de aguas costeras superficiales del Mediterráneo

Aunque se sabe que distintos grupos bacterianos de este área han mostrado distintas sensibilidades a la radiación solar, se dispone de muy poca información acerca de cómo reaccionan estos grupos a los niveles de radiación típicos de cada estación del año; sin embargo, se ha sugerido que podría existir una cierta fotoadaptación o una selección de especies fotoresistentes hacia los periodos de más alta radiación. El objetivo de este subcapítulo fue evaluar la variabilidad estacional en la sensibilidad de los grupos de bacterias dominantes en aguas del Mediterráneo noroccidental.

Capítulo 3. Efectos de la radiación solar en la asimilación de DMSP y leucina de bacterioplancton heterotrófico polar

Los organismos marinos que habitan las aguas polares están continuamente sometidos a condiciones de radiación extremadamente variables que van desde la oscuridad más absoluta durante los meses de invierno hasta exposición continuada a la luz durante el verano. Quisimos evaluar el efecto de la radiación solar (y específicamente de la UVB) sobre las comunidades bacterianas del aguas árticas y antárticas en términos de asimilación de DMSP. Debido a que las mayores concentraciones de DMSP a escala

global se dan durante los meses de verano en ambos polos, nuestra hipótesis fue que este hecho podría aumentar la presencia de grupos bacterianos asimiladores de DMSP y que una exposición continua a la luz solar podría afectar sus capacidades y tasas de incorporación de este azufre. Por tanto, el objetivo de este capítulo fue el de comprobar las sensibilidades específicas de cada grupo entre las bacterias que asimilaban tanto DMSP como leucina.

Capítulo 4. Efectos de la radiación en el comportamiento osmoheterotrófico del fitoplancton ártico y antártico

A pesar de reconocerse desde hace años, se sabe muy poco acerca del potencial papel ecológico de la osmoheterotrofía de algas entre las comunidades naturales. Se ha sugerido que las especies polares de fitoplancton podrían cambiar a un crecimiento heterotrófico como una manera para sobrevivir durante el largo verano oscuro. Sin embargo y hasta el momento, ningún estudio ha analizado específicamente los efectos de la UVR sobre este comportamiento de las algas. En este capítulo, nuestros objetivos fueron (1) establecer la frecuencia y la relevancia del uso de la leucina y el DMSP entre comunidades de fitoplancton polares y (2) analizar el efecto de la radiación solar en la toma específica de estos compuestos por distintos grupos fitoplanctónicos.

Capítulo 5. La calidad de la radiación solar modula la importancia relativa de las bacterias heterotróficas y el picofitoplancton en la toma de DMSP

A pesar de que hay muchas evidencias sobre el papel mayoritario de las bacterias heterotróficas en la biogeoquímica del DMSP, éste se ha descrito muy recientemente para microorganismos fototróficos y se sabe muy poco acerca de su potencial contribución a los ciclos de DMSP y cómo la radiación puede influir en ellos. Debido a que la incorporación de DMSP por el picofitoplancton a menudo parece estimularse por la luz, quisimos comprobar si la exposición a la radiación solar provocaba un aumento en la competición por el uso de DMSP del picofitoplancton frente a las bacterias heterotróficas.

Los seis estudios que se presentan en esta tesis tienen un aspecto en común: se centran en el papel de la radiación como modulador de las actividades heterotróficas de las bacterias marinas o el fitoplancton, y fundamentalmente desde una perspectiva individual; sin embargo, también difieren en algunos de los aspectos más específicos que se evalúan en ellos. La tabla 2 resume y esquematiza cuáles de estos temas se abordan en cada uno los capítulos.

		Paper 1	**Paper 2**	**Paper 3**	**Paper 4**	**Paper 5**	**Paper 6**
Temporal varibility	Diel cycles	■					
	Seasonal		■	■			
Geographic variability	Mediterranean	■	■	■			■
	Arctic				■	■	
	Antarctica						
Light quality	Artificial (PAR)	■	■				
	Sunlight PAR or UVR			■	■	■	■
Substrate considered	Leucine	■	■	■	■	■	■
	Thymidine						
	DMSP				■	■	■
Organisms considered	Het. bacteria	■	■	■	■	■	■
	Prok. phytoplankton						
	Euk. phytoplankton					■	

Metodología empleada

Para la elaboración de esta tesis se han empleado por una parte técnicas de medida de procesamiento de carbono a nivel de comunidad, como producción bacteriana y asimilaciones fraccionadas de diversos sustratos radioactivos, y por otra, algunas técnicas de resolución individual (fundamentalmente microautoradiografía) para determinar las respuestas específicas de distintos grupos bacterianos o fitoplanctónicos a la radiación solar. A continuación se describen brevemente y de manera general tanto el diseño experimental de la mayoría de los experimentos como los protocolos de las técnicas más utilizadas.

Incubación de muestras a distintas condiciones de luz. Prácticamente todos los experimentos consistieron en incubaciones de muestras superficiales tanto del Mediterráneo como del Ártico o de la Antártida bajo distintas condiciones de luz y añadiendo siempre un control en oscuridad. Para las incubaciones se utilizaron matraces de cuarzo (por ser transparentes a la UVR) de 50 ó 100 mL que se disponían en tanques exteriores con agua corriente para mantener la temperatura in situ. Las muestras de agua se incubaban sin sustratos añadidos cuando las medidas se realizaban a posteriori, como en el caso del análisis de abundancias de bacterias o fitoplancton y producción bacteriana, o previa inoculación con el sustrato de interés si lo que se pretendía era medir incorporación durante la exposición (asimilación fraccionada, MAR-CARD-FISH). En general los tratamientos que se emplearon fueron: oscuridad (matraces cubiertos con papel de aluminio y con bolsas de basura para evitar la reflexión), espectro total (matraces expuestos directamente al sol), PAR + UVA (matraces cubiertos con una capa del filtro Mylar-D, que elimina la radiación UVB) o PAR (matraces cubiertos con dos capas del filtro Ultraphan URUV farblos para la exclusión de toda la UVR). En algunas ocasiones las muestras se cubrían con una malla neutra para atenuar la radiación y simular en la medida de lo posible las dosis ambientales recibidas por los organismos in situ.

Además, durante todos estos experimentos se monitorizaron las dosis de PAR y de UVR recibidas por las muestras mediante un radiómetro instalado en el centro de los tanques de incubación a la misma distancia de la superficie que los matraces. Una vez finalizadas las incubaciones, las muestras se fijaban si era necesario o se tomaban alícuotas para medir otros parámetros.

Abundancia de distintos grupos del picoplancton. Las abundancias de bacterias, cianobacterias o picoeucariotas fotosintéticos en las muestras se analizaron por citometría de flujo (Gasol y Del Giorgio 2000). Para la cuantificación de las bacterias, se fijaron

alícuotas de 1.2 mL con 1% de paraformaldehído + 0.5% de glutaraldehído (conc. final) y se almacenaron congeladas a -80ºC hasta su posterior análisis mediante tinción con SybrGreen I (1:10,000) en un citómetro de flujo FACSCalibur (Becton-Dickinson). Las bacterias se detectaron y se cuantificaron de acuerdo al tamaño de la célula (*side scatter,* SSC) y a la fluorescencia del SyberGreen I (FL1, fluorescencia verde). Por otra parte, las abundancias de *Synechococcus, Prochlorococcus* y picoeucariotas fotosintéticos se estimaron inmediatamente tras el muestreo a partir de las muestras vivas, analizando alícuotas sin teñir por citometría de flujo. Todos los grupos se detectaron y contaron de acuerdo a su tamaño y a la fluorescencia naranja (FL2) o roja (FL3).

Actividad heterotrófica bacteriana. La actividad heterotrófica bacteriana se estimó generalmente a partir de la incorporación de ^{3}H-leucina pero en una ocasión (**Capítulo 1**) también se utilizó ^{3}H-timidina. Para la ^{3}H-leucina empleamos el método descrito por (Kirchman et al. 1985) con las modificaciones de (Smith y Azam 1992). Brevemente, 4 alícuotas de 1.2 mL y 2 controles fijados con ácido tricloroacético (TCA) al 50 % se incubaron con leucina radioactiva (40 nmol l^{-1} conc. final, 160 Ci $mmol^{-1}$) durante unas dos horas en oscuridad y a temperatura in situ. La incorporación se detenía añadiendo 120 μl de TCA frío al 50% a las muestras vivas, que se almacenaban a -20ºC hasta su procesado por el método de centrifugación descrito por Smith y Azam (1992). Por otra parte, para el método de la timidina se siguió el protocolo descrito por (Fuhrman y Azam 1980) con las modificaciones de (Smith y Azam 1992). Las muestras se incubaron con 10 nmol L^{-1} de ^{3}H-timidina (conc. final) y fueron procesadas igual que las muestras de ^{3}H-leucina.

Asimilación fraccionada de sustratos. Esta técnica se empleó como aproximación de la cantidad de sustrato que se asimilaba por distintas fracciones del plancton. Tras la exposición anteriormente descrita de las muestras a las cuales se había añadido algún isótopo, la incorporación se detenía mediante fijación de las muestras con paraformaldehído (PFA, 1% final conc.) a 4ºC en oscuridad. 2 ó 3 submuestras de entre 15 y 30 mL se filtraron secuencialmente a través de filtros de distinto tamaño de poro (p. ej. 5 μm SMWP y 0.2 μm, GNWP, Millipore), y se aclararon con agua de mar filtrada. Seguidamente las macromoléculas se precipitaron cubriendo los filtros con 5 mL de TCA frío al 5% durante 5 minutos. Los filtros se aclararon después con milli Q y se determinó su radioactividad sumergiéndolos en 5 mL de coctel de centelleo (Optimal HiSafe) y procesándolos con un contador de centelleo Beckman.

CARD-FISH (*Catalyzed Reporter Deposition-Fluorescence in situ Hibridization)*. Para la determinación de la abundancia in situ de los distintos grupos bacterianos se utilizó el protocolo de CARD-FISH (Pernthaler et al. 2002). Las muestras se fijaron con paraformaldehído (PFA) durante la noche a 4ºC (1% conc. final). Distintas alícuotas de entre 10 y 20 mL se filtraron por filtros de policarbonato de 0.2 μm (GTTP, Millipore), se lavaron con milli Q, se secaron al aire y se almacenaron a -20ºC hasta su procesado en el laboratorio. Para las hibridaciones, se emplearon distintas sondas-(HRP, *horse-radish peroxidase*) para caracterizar la composición de la comunidad bacteriana en las muestras de agua: Eub338-II-III para la mayoría de las *Eubacteria* (Amann et al. 1990; Daims et al. 1999), Gam42a para la mayoría de *Gammaproteobacteria* (Manz et al. 1992), CF319 para distintos clados pertenecientes *Bacteroidetes* (Manz et al. 1996), Ros537 para el clado *Roseobacter* (Eilers et al. 2001), SAR11-441R para el grupo SAR11 (Morris et al. 2002), Syn405 para el género cianobacteriano *Synechococcus* (West et al. 2001), NOR5-730 para el grupo NOR5 (Eilers et al. 2000) y CYA339 para *Cyanobacteria* (Nübel et al. 1997). Todas las sondas fueron adquiridas de biomers.net (Ulm, Alemania).

Los filtros se permeabilizaron primero con lisozima (10 mg mL^{-1}, 37ºC, 1h) y después con acromopeptidasa (60 U mL^{-1}, 37ºC, 0.5 h) antes de la hibridación. Las hibridaciones se llevaron a cabo sobre secciones de los filtros at 35ºC durante la noche, y las condiciones específicas de hibridación se establecieron mediante la adición de formamida a los medios de hibridación (45% para la sonda SAR11, 50% para la sonda NOR5, 60% para la sonda Syn405 y 55% para el resto de sondas). Tras la hibridación, se llevó a cabo una reacción de amplificación catalizada por el enzima HRP durante 15 – 20 minutos donde se añadían H_2O_2 y tiramida marcada con un fluorocromo. Tras la amplificación, se disponían los filtros sobre un porta y se teñían con DAPI (1μg mL^{-1}). Alrededor de 500 y 800 células teñidas con DAPI se contaron manualmente en un mínimo de 10 campos por microscopía de epifluorescencia.

MAR-CARD-FISH: Microautoradiografía combinada CARD-FISH. Entre 25-70 mL de agua se incubaron con sustratos radioactivos en concentraciones traza (0.5 nmol L^{-1} para la ^{3}H-leucina y entre 0.8 pmol L^{-1} y 1 nmol L^{-1} para el ^{35}S-DMSP) en los mismos matraces de cuarzo si las incubaciones se hacían a la luz o en tubos Falcon si se llevaban a cabo en oscuridad, como es el caso del **Capítulo 1**, a temperatura in situ. Junto con las incubaciones vivas se exponían controles fijados con PFA (1% conc. final). Transcurridas entre 3 y 12 horas de incubación, las muestras vivas se fijaron durante unas 12 horas con PFA (1% conc. final) a 4ºC en oscuridad. A continuación se procedió según el protocolo de CARD-FISH descrito anteriormente, pero una vez finalizado el proceso,

en vez de montar los filtros sobre los portas, se cortó una pequeña sección de cada filtro y se tiñó con DAPI (1μg mL^{-1}) para estimar la abundancia relativa de cada grupo antes de aplicar la microautoradiografía, ya que es más difícil que penetre el DAPI una vez que los filtros están cubiertos con la emulsión fotográfica. Alrededor de 500 y 800 células teñidas con DAPI se contaron manualmente en un mínimo de 10 campos por microscopía de epifluorescencia.

Para el análisis de la actividad bacteriana individual, seguimos el protocolo descrito por Alonso y Pernthaler (2005) con las modificaciones de Vila-Costa et al. (2007) y de Alonso-Sáez y Gasol (2007). Las secciones de los filtros se pegaron sobre portas y se sumergieron en 10 mL de emulsión fotográfica (KODAK NTB-2) a 46ºC que había sido previamente mezclada con 10 mL de agarosa al 0.1% en una habitación oscura. Los portas cubiertos por la emulsión se dispusieron boca arriba sobre una barra de metal fría durante unos 5 minutos para que solidificara la emulsión, y después se almacenaron en cajas negras a 4ºC hasta su revelado. El tiempo óptimo de exposición se determinó para cada experimento mediante revelados periódicos. Una vez transcurrido este tiempo, los portas se revelaron mediante inmersiones secuenciales en el revelador (KODAK D19) durante 3 minutos, en agua milli Q durante 30 segundos, en fijador (KODAK Tmax) durante 3 minutos y finalmente 5-10 minutos lavándose bajo el agua del grifo. Los portas se secaron en un desecador a oscuras durante unas 12 horas, se tiñeron los filtros con DAPI (1 □g mL^{-1}) y se contaron manualmente alrededor de 500-800 células hibridadas mediante microscopía de fluorescencia. La luz transmitida se usaba para la detección de células activas, es decir, las células que aparecían rodeadas por coronas de gránulos de plata.

Resultados, síntesis y discusión general

Resumen y nexos entre capítulos

Esta tesis pretende contribuir al conocimiento general de las interacciones entre el sol y los microorganismos marinos, proporcionando datos acerca del impacto de la radiación solar sobre la incorporación de algunos compuestos de la DOM por grupos microbianos particulares.

Nuestro estudio se centró principalmente en las bacterias ya que, por ser consumidoras primarias de la DOM en el océano, cualquier efecto de la luz sobre su actividad podría tener implicaciones en los flujos de carbono y nutrientes. Sin embargo, también dedicamos una sección de esta tesis al papel de la radiación sobre el uso de la DOM por los organismos fototróficos, tanto eucariotas como procariotas. Para ello, realizamos distintos experimentos en tres ecosistemas muy dispares: el Mediterráneo NO y las aguas del Ártico y la Antártida. El estudio de estos ecosistemas tan contrastados es de interés no sólo por las tremendas diferencias tanto en temperatura como en sus regimenes de radiación (ver Fig. 1), sino porque estos lugares también difieren sustancialmente en sus características ópticas, estado trófico, e incluso en los procesos de estratificación; éstos, que en el Mediterráneo se deben fundamentalmente al incremento en la temperatura de las aguas superficiales, en zonas cercanas a los márgenes del hielo se originan principalmente por la fusión del hielo al final del invierno. Además, mientras que el Mediterráneo es un sistema relativamente oligotrófico de producción limitada por fósforo a lo largo de casi todo el año (Lucea *et al.*, 2005; Pinhassi *et al.*, 2006) y por lo tanto relativamente transparente, en las aguas polares costeras o próximas al margen del hielo a menudo se desarrollan *blooms* primaverales de fitoplancton de biomasa y productividad elevadas (Fogg, 1977; Harrison y Cota, 1991; Sakshaug, 2004) que podrían atenuar significativamente la radiación. Aún así, hay evidencias de una penetración importante de la UVR en la Antártida, particularmente durante la primavera y coincidiendo con situaciones de baja concentración de ozono (Tedetti y Sempéré, 2006). Por todo ello, los organismos que habiten estas regiones tan diferentes tendrán que lidiar con regimenes de radiación extremadamente distintos.

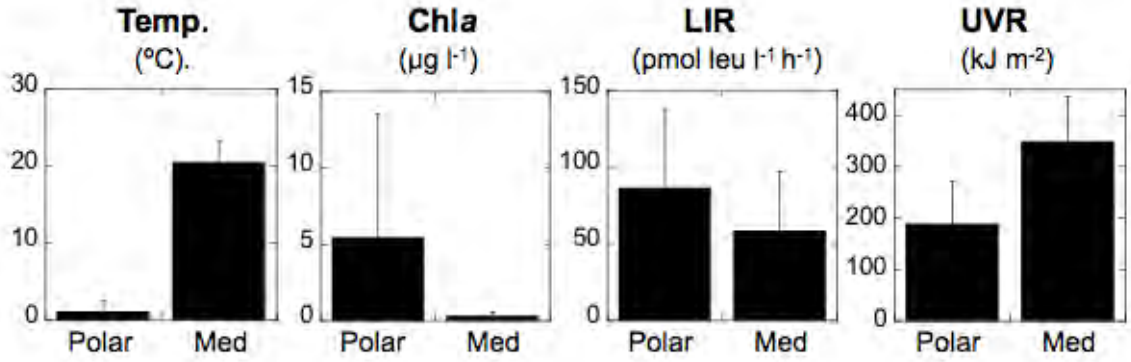

Fig. 1. Compilación de datos de todos nuestros experimentos polares y Mediterráneos (muestras de verano) para la comparación entre distintas características de regiones templadas y polares: temperatura (Temp), clorofila *a* (Chl *a*), tasas iniciales de incorporación de ^{3}H-leucina medidas en oscuridad (LIR) y dosis de radiación UVR recibida por las muestras durante los experimentos.

En cada uno de estos ecosistemas hemos abordado distintos aspectos desde dos enfoques diferentes:

1. Desde un punto de vista comunitario, analizando cuantitativamente el papel de la luz tanto en la producción bacteriana global como en la asimilación de distintos sustratos por toda la comunidad bajo la influencia de condiciones de radiación naturales o fijas (artificiales).

2. Desde una perspectiva individual, identificando los grupos de bacterias o de fitoplancton implicados en la incorporación de los sustratos, sus respuestas específicas y sus distintas sensibilidades a la luz, así como hasta qué punto estas variaciones en los grupos específicos se reflejan en las respuestas globales observadas. Esta cuestión se abordó principalmente mediante el uso de la técnica de resolución individual MAR-CARD-FISH, y solamente para el último capítulo se empleó además la técnica del "cell sorting" por citometría de flujo.

En conjunto, nuestros resultados evidencian una sustancial variabilidad en las respuestas microbianas a la luz, que parecen depender no sólo de los propios niveles de radiación, sino también de la composición de las comunidades planctónicas, el sustrato considerado y la precisión de las condiciones de luz de las incubaciones, es decir, cuán fielmente simulaban éstas las irradiancias presentes *in situ*. Estos resultados se pueden agrupar y reorganizar bajo el siguiente esquema:

Implicaciones de la variabilidad de la luz solar: escalas de variación y calidad espectral

En vista de la complejidad observada, parece obvio que no será sencillo hallar patrones comunes entre los distintos experimentos. De hecho, si representamos todos los resultados juntos (uniendo datos mediterráneos y polares), no emerge ninguna tendencia clara en relación a la intensidad de la radiación, sugiriendo que la inhibición de las bacterias no es simplemente una cuestión de cantidad de luz; es decir, no siempre una dosis más elevada de UVR causará una mayor inhibición. Aún así podemos afirmar que la luz tenía un efecto sobre los flujos de DOM a través de las bacterias: compilando todos nuestros resultados, observamos que la exposición a la radiación total resultó en una inhibición promedio de las tasas de incorporación de leucina (LIR) del 23% (con respecto al control oscuro), aunque se detectó una gran variabilidad en las respuestas que mostraron desde un 40% de estimulación hasta un 60% de inhibición de las tasas medidas (Fig. 2).

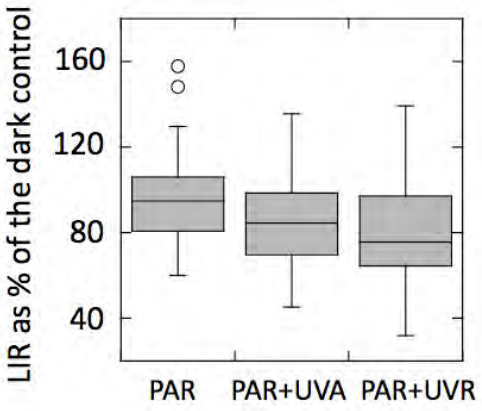

Fig. 2. Tasas de incorporación de leucina (LIR) de todos los experimentos medidas bajo distintas condiciones de luz y expresadas como porcentajes del control oscuro para ilustrar el amplio rango de variabilidad en las respuestas observadas.

A pesar de esta gran variabilidad, se identificaron algunos patrones: por ejemplo, los patrones diarios en la LIR observados en el **Capítulo 1** indican que la luz estaba dirigiendo de alguna forma el comportamiento de las bacterias de la Bahía de Blanes: quizá no directamente, a causa de los bajos niveles de radiación en invierno en este área,

sino indirectamente, a través de la sincronización de los ciclos de otros microorganismos (predadores y presas) que podrían estar causando cambios diarios en el suministro y la disponibilidad de DOM para las bacterias. Estos muestreos cada cuatro horas, diseñados con la intención de detectar cambios (de otra forma inadvertidos) a corto plazo, revelaron una variabilidad diaria hasta 1.4 veces mayor que el rango anual de variación registrado durante ese año. Por tanto, parece necesaria una cierta precaución a la hora de interpretar datos estacionales, ya que el error al extrapolar podría ser sustancial si se consideran medidas puntuales.

Por otra parte, en el **Capítulo 2** nos concentramos en la variabilidad estacional de las respuestas de la LIR a la radiación. Como primera aproximación, estimamos la actividad bacteriana global anual bajo condiciones de luz tanto naturales (y por tanto variables) como constantes (PAR artificial, **Capítulo 2.1**). Así observamos una importante variabilidad intra-anual que, aunque no siempre estaba directamente relacionada con la intensidad, parecía esconder algunos patrones que resultaban visibles al promediar estacionalmente, como una mayor estimulación en primavera o una cierta inhibición en verano (Tabla 1, **Capítulo 2.1**). Además, cuando examinamos la estacionalidad en las respuestas de la LIR y de algunas actividades enzimáticas causadas por las distintas regiones del espectro (**Capítulo 2.2**), observamos que, aunque la inhibición absoluta era mayor en verano, la inhibición por unidad de radiación resultaba más pronunciada en invierno. Este hecho, que inicialmente se achacó a la presencia de una comunidad invernal relativamente más sensible a la UVR, resultó deberse a una importante sobreexposición a la UVB causada por nuestro diseño experimental. Por otra parte, la comparación de la contribución a la inhibición de estos parámetros microbianos tanto por UVA como por UVB reveló una amplia variabilidad en las distintas respuestas de las muestras de Blanes (Fig. 3). Mientras que en algunos casos la UVB era responsable de la mayoría de la inhibición (medida con respecto al valor expuesto al PAR), también la UVA ejercía el mayor efecto en algunas ocasiones; de hecho, ésta era responsable de la mayor parte de la inhibición en la mayoría de las muestras de LIR (Fig. 3).

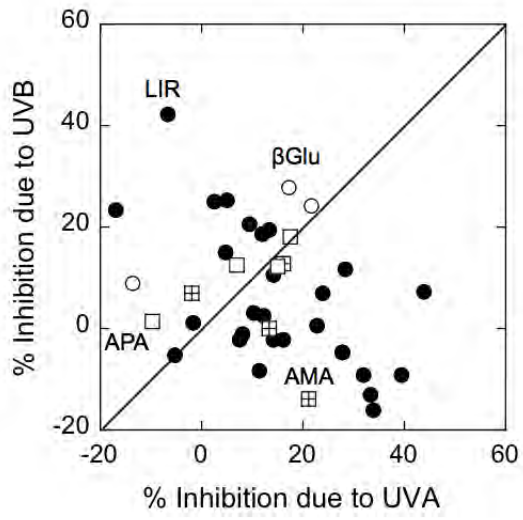

Fig. 3. Comparación de la contribución relativa a la inhibición de algunos parámetros por UVB versus UVA (calculada a partir de los valores expuestos a PAR). Únicamente se incluyen muestras del Mediterráneo, ya que los efectos del UVA no se examinaron específicamente en los experimentos polares. Los parámetros incluidos son: tasas de incorporación de ^{3}H-leucina (LIR), actividad β-glucosidasa (βGlu), aminopeptidasa (AMA) y alcalinofosfatasa (APA). Los valores en la línea 1:1 indican que ambas longitudes de onda contribuyeron por igual a la inhibición calculada respecto a los valores medidos bajo PAR.

Otros autores también han descrito una mayor contribución a la inhibición de la radiación UVA respecto a la UVB (Sommaruga et al. 1997; Tedetti et al. 2009), hecho que podría explicarse porque, a pesar de que las longitudes de onda UVA son menos energéticas que la UVB, la cantidad de energía de UVA que llega a la superficie del océano es proporcionalmente mucho mayor que la de la región UVB. Sin embargo, si teníamos en cuenta la historia previa de exposición de las muestras (es decir, las irradiancias que éstas experimentaban in situ durante los días previos al muestreo), parecía que algunas de las respuestas observadas podían explicarse por las diferencias existentes entre las condiciones *in situ* y las experimentales (ver más adelante).

En las distintas estaciones del Ártico y la Antártida se utilizó un diseño experimental similar (**Capítulo 3**) pero centrándolo en el estudio de los efectos de la radiación UVB, la región más dañina del espectro y la única potencialmente afectada por los cambios en la concentración de ozono. En estos experimentos, mientras que por un lado no pudimos encontrar correlación alguna entre la inhibición de la LIR y las dosis de UVR, por otra parte la cantidad de leucina asimilada por la "fracción bacteriana" sí se correlacionó significativa y negativamente con dosis crecientes de UVB. Esto podría deberse al

hecho de que, mientras que las muestras para los experimentos de fraccionamiento por tamaños se exponían junto con el isótopo, la LIR se midió tras la exposición a la luz en una incubación de unas 2 ó 3 horas en la oscuridad, según el protocolo estándar (Smith y Azam 1992), durante la cual las bacterias podrían haber tenido el tiempo suficiente para recuperarse del daño (Kaiser y Herndl 1997).

Además de las variaciones temporales y espectrales, la luz también fluctúa en una escala espacial, no solo debido a cambios en el ángulo solar cenital y en los regimenes de mezcla, sino también dependiendo de las propiedades ópticas de las distintas regiones del océano (p. ej. Hargreaves 2003). En este sentido, y como se ha comentado anteriormente, el mar Mediterráneo se encuentra entre algunas de las regiones más transparentes donde la UVR penetra más profundamente que, por ejemplo, en las productivas aguas del verano Ártico o Antártico. Además, dado que las condiciones de temperatura y de limitación de nutrientes también pueden determinar la sensibilidad y las capacidades de recuperación de las células frente a la UVR (Pausz y Herndl 2002; Rae y Vincent 1998; Roos y Vincent 1998), y debido a que el origen y la composición química de la DOM pueden influir en su fotoreactividad y subsecuente mayor o menor biodisponibilidad (p. ej. Benner y Biddanda 1998; Herndl et al. 1997; Obernosterer et al. 1999; Tedetti et al. 2009), las respuestas a la radiación de los microorganismos que habitan estos contrastados sistemas variarán en gran medida dependiendo de las condiciones particulares de su ambiente en un determinado momento.

Lamentablemente, los datos de actividad global no revelaron tendencias latitudinales claras entre las muestras del Mediterráneo, el Ártico y la Antártida, que a su vez variaban significativamente incluso entre estaciones. La literatura disponible permite comparaciones muy limitadas entre los efectos de la UVR en distintas latitudes debido al uso de diseños experimentales diversos o parámetros fisiológicos diferentes; es por eso que se sabe muy poco acerca de las diferencias en la sensibilidad relativa de microorganismos pertenecientes a distintos ecosistemas. Además, el uso de varios tipos y franjas de instrumentos de medida de la UVR (espectroradiómetros, radiómetros de banda ancha, dosímetros) complica más aún las comparaciones entre distintos estudios de campo. En cualquier caso, podemos concluir que los cambios en la radiación afectaban las respuestas bacterianas heterotróficas a diferentes escalas y que, a pesar de que no se encontró relación aparente entre estas respuestas y parámetros como la concentración de clorofila, producción primaria, nutrientes, temperatura o salinidad, la presencia y los patrones de actividad de componentes específicos de las comunidades planctónicas resultaron primordiales a la hora de entender las tendencias observadas causadas por la luz.

No obstante, la interpretación de esta clase de experimentos no es sencilla ya que no permite discriminar los efectos directos de la luz sobre las bacterias de otros impactos sobre la DOM, por ejemplo, sin una manipulación específica de las muestras; aún así, se cree que este tipo de incubaciones a tan corto plazo evita otro tipo de efectos indirectos como son los impactos sobre los virus o los predadores. En cualquier caso, no hay que olvidar que las respuestas bacterianas observadas serán el resultado final de todos los efectos sinérgicos y antagónicos que tienen lugar al mismo tiempo en el interior de nuestras botellas experimentales.

Respuestas individuales como conductoras de los patrones a nivel de comunidad

Cuando aplicamos la microautoradiografía debemos tener en cuenta que los datos no son directamente comparables con las medidas cuantitativas totales: al enumerar la presencia o ausencia de células marcadas (ver Fig. 4), no tenemos información sobre cuán activas son esas células, aspecto que únicamente podría calcularse cuantificando las áreas de gránulos de plata alrededor de las células: es decir, cuanto mayor sea el área alrededor de una célula, mayor habrá sido la incorporación del sustrato por ella, siempre que se mantengan las mismas condiciones experimentales (Sintes y Herndl 2006). Por tanto es posible que aunque detectemos claros patrones debidos a la luz en las medidas totales, éstos no se reflejen en el número de células marcadas ya que, por ejemplo, una célula con un área de gránulos de plata dos veces menor que otra se considerará igualmente "activa" por la técnica. Sin embargo, según algunos de nuestros resultados, en ciertas ocasiones sí que es posible encontrar una coherencia entre las medidas a nivel de comunidad (como la LIR) y los recuentos de células activas. Por ejemplo, los ciclos día-noche observados en todos los grupos bacterianos de Blanes (**Capítulo 1**) se reflejaron indudablemente en la LIR total. Incluso cuando estos ciclos específicos se vieron interrumpidos durante el fin de semana entre muestreos, también la LIR perdió su ritmo, mientras que poco después las *Gammaproteobacteria* parecieron dirigir por sí solas los cambios visibles en el ciclo de LIR. Esto apunta hacia una contribución mayoritaria de este grupo a la actividad total a pesar de sus bajas abundancias, aspecto que se refleja también en que sus áreas de gránulos de plata son generalmente mayores que las de otros grupos más abundantes como las SAR11 (ver Fig. 4). Dado que distintos filotipos bacterianos muestran distintas sensibilidades a la radiación solar (Agogué et al. 2005; Alonso-Sáez et al. 2006; Arrieta et al. 2000; Joux et al. 1999), habíamos hipotetizado previamente que algunos grupos se verían menos afectados que otros por estas variaciones temporales en el régimen de

luz, si bien la sincronización de todos los grupos descartó esta sospecha. Sin embargo, no podemos descartar la posibilidad de que en otras épocas del año existan comportamientos diarios diferentes entre grupos, como se ha sugerido para los patrones de actividad en un área no muy alejada de la Bahía de Blanes (Ghiglione et al. 2007).

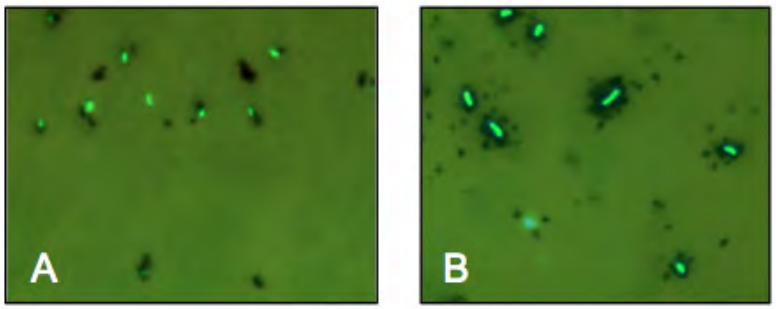

Fig. 4. Microautoradiogramas de células SAR11 (A) y *Gammaproteobacteria* (B) hibridadas con sondas CARD-FISH activas en la toma de ^{3}H-leucina. Las áreas de gránulos de plata alrededor de las células son comparables porque pertenecen a la misma muestra y tanto la incubación y las condiciones de exposición fueron las mismas. Apréciense las mayores áreas alrededor de las *Gammaproteobacteria* en comparación con las de SAR11, lo que podría explicar su importante contribución a la incorporación total de ^{3}H-leucina a pesar de sus frecuentemente bajas abundancias.

Por encima de esta variabilidad a corto plazo descrita por los muestreos cada 4 horas, los organismos marinos experimentan condiciones de luz variables estacionalmente que podrían afectar diferencialmente a distintos filotipos bacterianos (Alonso-Sáez et al. 2006). De hecho, la variabilidad observada en la LIR mensual medida en oscuridad y bajo condiciones de luz (sólo PAR) constantes (**Capítulo 2.1**) se correlacionaba bien con los cambios en el número de *Gammaproteobacteria* activas, que generalmente se estimulaban por la luz en la misma medida que la LIR total. Ni *Cyanobacteria* ni *Roseobacter,* dos grupos potencialmente estimulados por la radiación (Alonso-Sáez et al. 2006; Mary et al. 2008, **Capítulo 2.2**) parecían contribuir a este aumento de la actividad motivado por la exposición a la PAR. Del mismo modo, la ausencia de efectos causados por la luz sobre la LIR total medida durante los experimentos de primavera del **Capítulo 2.2** parecía deberse a una compensación del efecto negativo de la UVR sobre algunos grupos como SAR11 por la elevada resistencia de otros, como *Roseobacter* y, una vez más, *Gammaproteobacteria*. Esta actividad de las *Gammaproteobacteria* causando respuestas visibles a nivel de toda la comunidad ya se había observado previamente en Blanes por Alonso-Sáez et al. (2008). Estos autores encontraron valores de LIR inusualmente altos

coincidiendo con un drástico cambio en la composición de la comunidad, que pasó a estar dominada por miembros de las *Gammaproteobacteria* y específicamente, por el grupo *Alteromonas* (Alonso-Sáez et al. 2007). Algunos miembros pertenecientes al grupo de las *Gammaproteobacteria* son oportunistas capaces de reaccionar con prontitud ante cambios en su ambiente (Eilers et al. 2000; Pernthaler et al. 2001), por lo que parece probable que los patrones que observamos fueran causados por su rápida respuesta tanto a la recuperación del ciclo diario de liberación de DOM (**Capítulo 1**) como frente a un repentino aumento en la disponibilidad de luz o de fotosintato (**Capítulos 2.1** y **2.2**).

Por otra parte, a pesar de que tanto la LIR como las asimilaciones totales medidas en las muestras del Ártico se inhibían significativamente en algunos casos, no se encontraron respuestas claras entre grupos bacterianos (**Capítulo 3**). Esto podría deberse de nuevo a la naturaleza no cuantitativa de esta técnica: dado que la actividad se analizó en términos de presencia/ausencia (% de células activas) y no como asimilación por célula (midiendo las áreas de gránulos de plata), algunos efectos podrían verse enmascarados. Únicamente en las estaciones antárticas, los tiempos de incubación ligeramente más cortos parecieron permitir la detección de cambios importantes en el número de células activas en algunos de los grupos. Concretamente en la estación AN1, las respuestas globales parecían estar controladas por el grupo dominante *Bacteroidetes,* que, al igual que las muestras de LIR y de asimilación fraccionada de ^{3}H-leucina, se inhibió significativamente por PAR+UVA y UVB.

Variabilidad estacional y espacial entre grandes grupos bacterianos

Cuando se emplean sondas de CARD-FISH para grandes grupos bacterianos como los considerados en esta tesis, hay que tener en cuenta que pueden albergar una gran variedad de taxones dentro de ellos, a su vez variables tanto espacialmente (p. ej. Field et al. 1997) como temporalmente (Schauer et al. 2003) provocando diferencias en las respuestas observadas y probablemente ocultando algunos efectos. Por ejemplo, cuando Alonso-Sáez et al. (2006) analizaron las respuestas del grupo *Alphaproteobacteria* a la UVR, observaron que éste se inhibía mayoritariamente por UVA. Sin embargo, cuando aumentaron la resolución y examinaron las respuestas de los subgrupos alfaproteobacterianos *Roseobacter* y SAR11, se detectaron comportamientos opuestos entre ellos (estimulación de la actividad por PAR en el primero e inhibición en el segundo). Por tanto, aunque da la impresión de que podríamos encontrar comportamientos diferentes cada vez que nos concentremos en niveles filogenéticos más concretos, también es posible

identificar algunos patrones comunes dentro de estos grandes grupos bacterianos, como por ejemplo los incrementos repetitivos en la actividad de las *Gammaproteobacteria* causados por la luz (**Capítulo 2.1**). La figura 5 muestra la magnitud de los cambios debidos a la radiación en el número de células activas de los grupos considerados. Aunque que en general no se observaron diferencias significativas entre comportamientos de grupos de los distintos ecosistemas, se pudieron apreciar algunos patrones comunes: mientras que las SAR11 del Ártico exhibieron un ligero incremento debido a la PAR en el número de células activas en la toma de ^{3}H-leucina, sus homólogos mediterráneos mostraron una generalizada fotoinhibición de su actividad, aunque dependiente de las condiciones de luz (Fig. 5a). Por el contrario, mientras que las *Gammaproteobacteria* de la Bahía de Blanes se estimulaban en la toma de ^{35}S-DMSP por la exposición a la PAR (Fig. 5b), este mismo grupo no parecía responder a la luz ni en el Ártico ni en la Antártida. Todo esto apunta hacia diferencias latitudinales en la composición intra-grupo de los filotipos, o hacia ecotipos adaptados a estos distintos regimenes de radiación, dificultando por tanto cualquier tipo de clasificación general de estos grandes grupos bacterianos en relación a sus repuestas a la luz.

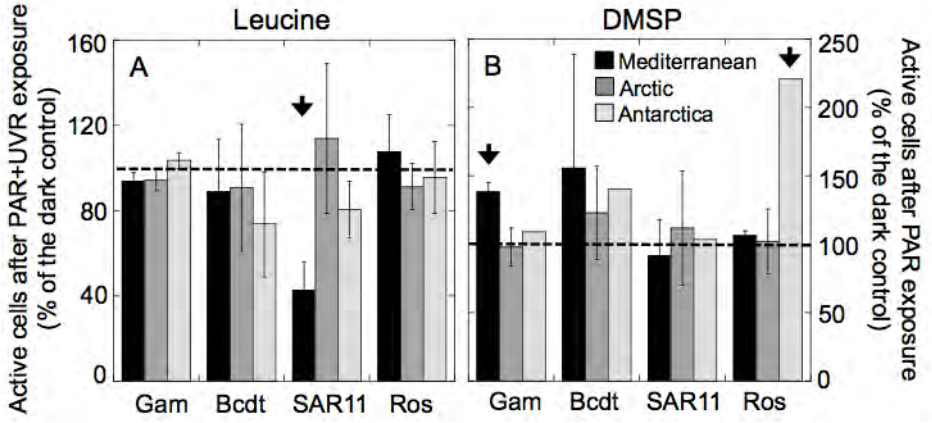

Fig. 5. Comparación de las respuestas a la radiación de los grandes grupos bacterianos de los distintos ecosistemas en las muestras de verano. A) Incorporación de leucina. Los valores por encima o bajo la línea del 100% indican estimulación o inhibición del número de células marcadas a causa de la exposición a la radiación total en comparación con los tratamientos oscuros. Las columnas y las barras de error corresponden a las medias y desviaciones estándar, respectivamente, de entre 2 y 5 experimentos distintos. B) Incorporación de DMSP (se incluyen dos experimentos adicionales realizados en el Mediterráneo –no mostrados en esta tesis- para su comparación con las muestras polares). Los valores por encima o por debajo de la línea del 100% indican tanto estimulación o inhibición del número de células activas debido a la exposición a la radiación total en comparación con el control oscuro. Las flechas señalan respuestas significativamente distintas entre filotipos de ecosistemas diferentes ($p < 0.05$).

Sin embargo, la ausencia de una respuesta sistemática de estos grupos en relación a las dosis de radiación sugiere una gran variabilidad incluso a escalas temporales y locales. Únicamente en el caso de las SAR11 mediterráneas y polares (Fig. 6a) y en el de los *Roseobacter* del Ártico y la Antártida (Fig. 6b) se pudo detectar una correlación significativa entre inhibición e irradiancia, mostrando ambos grupos menos células activas cuanto mayores eran las dosis de UVB recibidas. Por tanto, a pesar de que la utilización de estos grandes grupos bacterianos proporciona un primer paso esencial en el estudio de las comunidades bacterianas y sus respuestas generales, es innegable que niveles de aproximación más concretos revelarán interesantes aspectos necesarios para entender algunas de las pautas observadas. Así, el análisis del grupo gammaproteobacteriano NOR5 desveló que, en primavera, donde representaban más de un 90% de las *Gammaproteobacteria*, ellas eran las responsables del aumento en actividad debido al PAR observable entre las *Gammaproteobacteria*.

Por otra parte, esta mayor inhibición en la actividad de SAR11 y *Roseobacter* causada por las dosis más elevadas de UVR (Fig. 6) parecía descartar la hipótesis de la fotoaclimatación o de selección de taxones fotoresistentes, como han sugerido algunos autores (Alonso-Sáez et al. 2006). Tampoco ningún grupo en Blanes parecía más sensible a la UVR en invierno u otoño que en verano (**Capítulo 2.2**); sin embargo, cuando consideramos estos cambios debidos a la luz en relación a las dosis recibidas, la mayoría de los grupos mostró una inhibición menor por unidad de radiación en las muestras de verano (ver texto en **Capítulo 2.2**), apuntando hacia algún tipo de adaptación fisiológica o cambios en la comunidad hacia nuevas especies más fotoresistentes en concordancia con irradiancias más altas.

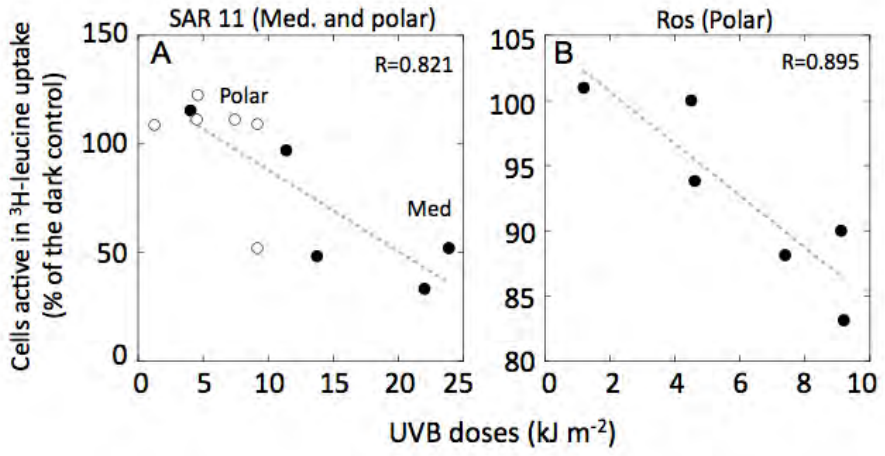

Fig. 6. Relación entre la reducción en el número de células marcadas para ^{3}H-leucina causada por exposición a la radiación solar en comparación con los controles medidos en oscuridad (es decir, expresada como % de los controles oscuros) y las dosis de UVB recibidas por las muestras. (A) SAR11 de muestras mediterráneas (círculos negros) y polares (círculos blancos); (B) *Roseobacter* del Ártico y de la Antártida. Únicamente estos dos grupos mostraron respuestas significativas ($p < 0.05$) con relación a la intensidad de la luz, aunque para el caso de SAR11 las diferencias no fueron significativas en los ecosistemas por separado.

Fotoheterotrofía en bacterias

Lamentablemente, nuestros experimentos no fueron diseñados para garantizar inequívocamente la presencia de metabolismos fotoheterotróficos como los de bacterias con BChl*a* o con PRs (Béjà et al. 2000; Kolber et al. 2000). Aún así, hubo varias ocasiones en que detectamos incrementos en la actividad de las bacterias causados por la luz (fundamentalmente PAR), como en las *Gammaproteobacteria* del **Capítulo 2.1**, en *Roseobacter* y NOR5 del **Capítulo 2.2** o en SAR11 del **Capítulo 3**. Entre las posibilidades que descartan una fotoheterotrofía se encuentran una posible estimulación indirecta de las bacterias causada bien por un aumento del fotosintato o por un incremento en la biodisponibilidad de la DOM, así como otros posibles impactos sobre las interacciones con virus u organismos bacterívoros. Sin embargo, el hecho de que las abundancias de las *Gammaproteobacteria* de Blanes estuvieran significativamente correlacionadas con condiciones de irradiancia en el agua pero no con ningún otro parámetro como temperatura, Chl *a* o datos de producción primaria (**Capítulo 2.1**) podría indicar una cierta preferencia de este grupo por ambientes iluminados. Hasta la fecha, ningún estudio ha desvelado las ventajas reales que confiere la posesión de Bchl *a* o PRs a las bacterias en condiciones naturales, aunque experimentos recientes con cultivos marinos comienzan a

revelar algunos de los secretos acerca de la naturaleza y la función de estos fotosistemas (Gómez-Consarnau et al. 2010; Gómez-Consarnau et al. 2007). De cualquier modo, aún es necesario profundizar en su estudio para llegar a entender y cuantificar la relevancia ecológica de la fotoheterotrofía en los ecosistemas marinos.

Efectos de la radiación solar en el destino microbiano del DMSP

Al contrario que las medidas de incorporación de leucina, que a menudo parecían inhibirse por la radiación total, los resultados de las muestras inoculadas con DMSP evidenciaban una tendencia común de estimulación por la luz, principalmente en el caso de organismos fototróficos; por el contrario, las bacterias heterotróficas mostraban patrones menos definidos (**Capítulos 3, 4** y **5**) según los cuales, a pesar de las condiciones de luz, la asimilación bacteriana de DMSP se estimulaba, inhibía o no mostraba efectos (**Capítulos 3** y **5**). Esto podría explicarse parcialmente por un efecto diferencial de la UVR sobre distintos sistemas de transporte (Herndl et al. 1997), pero seguramente también a causa de los muchos procesos influidos por la luz que controlan la disponibilidad de DMSP para otros microorganismos (Hefu y Kirst 1997; Sakka et al. 1997; Slezak et al. 2001; Slezak y Herndl 2003; Slezak et al. 2007; Sunda et al. 2002). En cambio, tanto el fitoplancton procariota como eucariota parecía estimularse a menudo por la luz en la incorporación de ^{35}S-DMSP. Este hecho era especialmente aparente en los resultados de las asimilaciones fraccionadas, aunque los efectos variaban cuando se consideraban organismos individuales (**Capítulos 4** y **5**). La generalizada capacidad de tomar DMSP por el fitoplancton polar, particularmente las diatomeas del Ártico (**Capítulo 4**), fue un descubrimiento inesperado aparentemente asociado a las elevadas concentraciones de DMSP en las regiones polares medidas aquel verano en el Ártico (Galí y Simó 2010), que probablemente conformaban un escenario favorable para grupos de bacterias y fitoplancton capaces de aprovechar este sustrato.

Por otra parte, en la Bahía de Blanes, donde a diferencia de las regiones polares, el picofitoplancton es el productor dominante, distintos experimentos mostraban que la exposición a la luz podría incrementar la competición del picofitoplancton frente a la de las bacterias por la toma de DMSP. Esto sería especialmente significativo en las aguas altamente irradiadas del verano cuando *Synechococcus* domina y además se dan las concentraciones más altas de DMSP disuelto (Mura et al. 1996; Schauer et al. 2003; Vila-Costa et al. 2008). Sin embargo, compilando todos los resultados de asimilaciones fraccionadas no observamos tendencias claras, y no siempre la exposición a la luz provocaba

un aumento en la contribución de la fracción de los fotótrofos frente a las de las bacterias (en comparación a los controles medidos en oscuridad, Fig. 7); del mismo modo, tampoco estos efectos parecían depender directamente de los niveles de radiación. Parece, por tanto, que dependiendo de la identidad de los organismos implicados y probablemente también de otros factores ambientales, las respuestas observadas variarán sustancialmente, si bien es cierto que las fracciones consideradas como fotótrofos y bacterias pueden incluir otros organismos que estén contribuyendo a los porcentajes medidos.

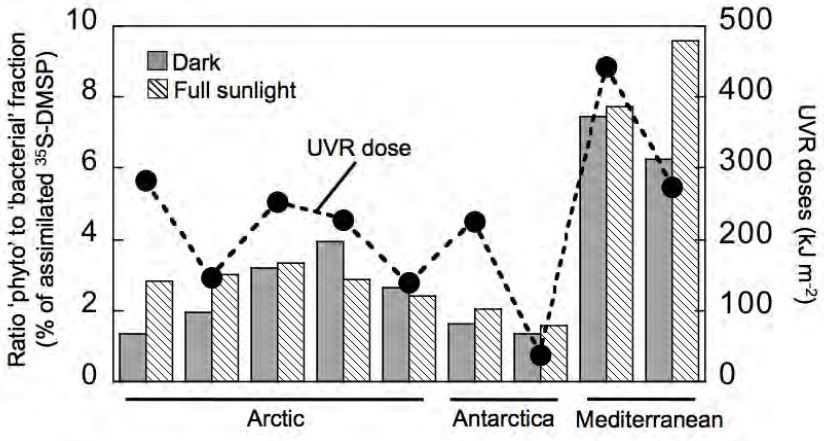

Fig. 7. Contribución relativa de la fracción de los "fotótrofos" (> 5 μm en las muestras polares, > 0.65 μm en las muestras de Blanes) frente a la de la fracción "bacteriana" (0.22-0.65/5 μm) al total de ^{35}S-DMSP asimilado medido tras la incubación en la oscuridad o expuesto a la luz total. Las barras representan el ratio entre ambas fracciones ("fotótrofos" versus "bacteria"), y la línea discontinua señala las dosis de UVR recibidas por las muestras durante cada experimento. Se incluyen además los resultados de dos experimentos de asimilación fraccionada de Blanes no mostrados en esta tesis para su comparación con las muestras polares. Esta figura ilustra que no siempre se da una mayor contribución de los fotótrofos a la incorporación de ^{35}S-DMSP medida a la luz, y que no siempre parece depender de las dosis de radiación; en cambio, a la vista de nuestros datos a nivel de células individuales, la composición taxonómica parece jugar un papel fundamental.

Evidencias empíricas indican que tanto la profundidad de la capa de mezcla como las dosis de UVR actúan conjuntamente provocando una producción de DMSP y DMS mayor que su consumo en los meses de verano, debido a un incremento del fitoplancton productor de DMSP y a la inhibición del uso del DMSP y DMS por las bacterias (Simó y Pedrós-Alió 1999; Toole y Siegel 2004). Nuestros resultados confirman este papel fundamental de la UVR en las dinámicas del DMSP en aguas oceánicas, pero añadiendo nueva vía a través de la cual, dependiendo de la comunidad microbiana presente, los niveles de radiación podrían favorecer su incorporación por una fracción del componente

autotrófico, que podría influir, en última instancia, en el flujo de DMS a la atmósfera. Parece por tanto que este desconocido papel del fitoplancton como sumidero de DMSP merecería ser explorado en mayor profundidad.

La importancia de la simulación de las condiciones de radiación naturales

Uno de los puntos claves de nuestra investigación fue la consideración de la historia lumínica previa, frecuentemente ignorada en la mayoría de estudios. En vista de nuestros resultados, parece que el conocimiento de las condiciones previas de exposición es esencial para una interpretación adecuada de las respuestas obtenidas, o incluso para simular con la mayor exactitud posible los niveles de radiación recibidos naturalmente por los microorganismos. Esta `historia lumínica' de las muestras se analizó únicamente en el **Capítulo 2,** donde ésta parecía influir consistentemente los patrones observados. Por una parte, la contribución relativa de UVB y UVA a la inhibición de la LIR (ver Fig. 2) parecía depender del ratio UVB/UVA previamente experimentado por las muestras, de forma que las bacterias naturalmente aclimatadas a niveles bajos de UVB se inhibían más por estas longitudes de onda durante nuestras incubaciones (**Capítulo 2.1**). Del mismo modo, la LIR anual medida bajo condiciones de luz (sólo PAR) controladas y constantes se estimulaba más cuando las diferencias entre esta radiación y los niveles *in situ* eran mayores, mientras que, cuando ambos niveles de radiación eran similares, apenas se detectaban efectos o incluso en algún caso se observó inhibición. Todo esto sugiere que las respuestas de las bacterias estarán parcialmente influidas por cuánto difieran las condiciones de nuestras incubaciones de los niveles de radiación naturales previamente experimentados. Por último, al analizar el efecto de la calidad de la luz sobre algunos procesos bacterianos a lo largo de las estaciones (**Capítulo 2.2**), los efectos mayores observados en invierno parecían explicarse por el elevado ratio entre las dosis de UVB medidas en el incubador frente a las UVB dosis *in situ*; es decir, nuestro diseño experimental provocó una sobreexposición a la región UVB de las células que habrían estado de otra forma sometidas a la profunda mezcla vertical que se da en Blanes en invierno, recibiendo por tanto menores dosis de UVB. Por otra parte, tampoco las mallas neutras simulan con exactitud la atenuación espectral del agua de mar, enriqueciendo el espectro incidente en longitudes de onda más cortas con respecto a UVA y PAR; es primordial mantener ratios realistas entre UVB, UVA y PAR ya que, entre otros aspectos, el balance entre el daño y la reparación depende de estas proporciones (p. ej. Kaiser y Herndl 1997). Sin embargo y al igual que nosotros, aún hoy muchos investigadores exponen las muestras ignorando los efectos de la mezcla vertical, la cual, junto con las irradiancias diarias, determina las

dosis a las que los organismos están expuestos naturalmente. Así, cuanto más similares sean las condiciones de incubación y las ambientales, más realistas serán los resultados obtenidas.

La importancia de las técnicas de resolución individual

La complejidad inherente que caracteriza las redes tróficas planctónicas es sin duda un desafío para los estudios sobre efectos ambientales. Debido a las respuestas específicas de cada especie y a las interacciones entre ellas y otros factores, los patrones pueden ser a menudo muy variables y difíciles de predecir. Nuestros resultados corroboran la importancia de conocer la identidad de los organismos presentes en la comunidad y sus respuestas específicas a la radiación para una correcta comprensión de las dinámicas del ecosistema.

Tanto la asimilación fraccionada como las medidas de la LIR pueden incluir algunos sesgos: nuestros datos de asimilación fraccionada de ^{3}H-leucina (**Capítulo 4**), técnica que no siempre separa completamente las algas de agregados de bacterias o detritus, incluían una importante fracción de bacterias asimilando ^{3}H-leucina en la fracción > 5 μm; de la misma manera, ya que se ha observado que tanto el fitoplancton procariota como el eucariota pueden tomar ^{3}H-leucina por osmotrofía (**Capítulos 2** y **5**), dependiendo de sus abundancias y su contribución relativa a la incorporación del aminoácido estos organismos podrían causar sobreestimas de las medidas de producción bacteriana; de hecho, teniendo en cuenta que la incorporación de ^{3}H-leucina por las algas es a menudo mayor en oscuridad que a la luz (**Capítulo 4**), es posible que estas incubaciones oscuras promuevan un aumento en la contribución de ciertos fotótrofos a la toma de ^{3}H-leucina. Por tanto, ya que en vista de nuestros resultados parece que las medidas totales dependen en gran parte de las respuestas específicas de algunos componentes particulares del ecosistema, no será posible lograr una interpretación certera de los resultados sin disponer de un adecuado conocimiento de los organismos implicados.

¿Son adecuadas las medidas en oscuridad?

La mayoría de las medidas de actividad bacteriana se realizan en ausencia de luz para evitar la estimulación de las algas y los problemas antes mencionados de reproducir los niveles de radiación ambientales (p. ej. Kirchman et al. 1985; Smith y Azam 1992).

Sin embargo, debido a que las comunidades planctónicas marinas están naturalmente expuestas a condiciones de radiación fluctuantes, parece que sería más adecuado realizar incubaciones simultáneas en oscuridad y luz para conseguir estimas realistas de las variaciones en los flujos de carbono. Además, aunque la exposición a la luz puede provocar indirectamente aumentos en la producción bacteriana debido a una estimulación de las bacterias por excreción de fotosintato, fotolisis de la DOM o la presencia de metabolismos fotoheterotróficos, estas incubaciones en la oscuridad también podrían provocar incrementos en las tasas de incorporación de sustratos: Morán et al. (2001) proponían que la inhibición aparente por PAR en muestras de este área era el resultado de la estimulación del crecimiento bacteriano en la oscuridad, aunque estos autores no pudieron identificar tales mecanismos. Podría ocurrir también que al exponerse las muestras a la luz se libere leucina como un producto de la fotosíntesis (p. ej. Braven et al. 1995) diluyendo por tanto el trazador y reduciendo la señal radioactiva.

Por otra parte, el hecho de incubar las muestras de LIR del **Capítulo 1** en la oscuridad impidió determinar si nuestros resultados eran realistas o si estábamos subestimando o sobreestimando la LIR diurna ya que no consideramos ni los posibles efectos negativos de la UVR, ni una potencial fotoestimulación de la actividad durante las horas de luz. Además, las incubaciones oscuras podrían alejarse mucho de la realidad, como por ejemplo durante los meses de verano Ártico o Antártico, donde los microorganismos están continuamente expuestos a la radiación solar y las incubaciones en ausencia de luz podrían estar causando sobreestimas en la incorporación de ^{3}H-leucina o subestimas en la de ^{35}S-DMSP (**Capítulos 3** y **4**). Nuestros resultados y los de la literatura existente sugieren que sólo será posible cuantificar con precisión los flujos de DOM a través de las redes tróficas microbianas si éstos se miden en condiciones de radiación realistas. Aún así, un control medido en ausencia de luz parece inevitable ya que (1) facilitará cualquier comparación entre datos de distintos estudios y (2) una simulación inexacta de las condiciones de luz podría afectar la medida de los procesos y llevar a conclusiones erróneas.

Consecuencias del incremento de la UVR y nexos con el cambio climático

En vista de los resultados obtenidos parece evidente que no será fácil predecir las respuestas del ecosistema ante cambios en el régimen lumínico. Además de la reducción en la concentración de ozono, otros factores como los cambios en las nubes, aerosoles, polución del aire, reflexión de la superficie o atenuación en el agua también pueden influir

en la cantidad de UVR que penetra en el océano, procesos que a su vez pueden verse afectados por el cambio climático (Kerr et al., 2003; Mckenzie et al. 2007).

Algunos experimentos en sistemas lacustres han mostrado que la sequía derivada de temperaturas más cálidas provocaba una reducción en la escorrentía o una acidificación que se traducía en aguas más transparentes a la UVR (Schindler et al. 1996a; Schindler et al. 1996b; Yan et al. 1996). Por otro lado, hay ciertas evidencias de que la tendencia hacia temperaturas superficiales más cálidas en el océano (Hansen et al. 2005; Willis et al. 2004) podría influir en el ritmo y la intensidad de la estratificación (Young y Holt 2007); por tanto, si este proceso llevara a una estratificación más superficial en algunas áreas, los organismos atrapados en esa zona estarían expuestos a niveles de radiación más intensos.

Aparte de los cambios en las condiciones de luz derivados del cambio climático, cualquier alteración en la composición de las comunidades en respuesta a la luz (Santos et al. 2010; Belzile et al. 2006; Roy et al. 2006) podrían traducirse en alteraciones de los ciclos de la DOM y los nutrientes en los sistemas marinos.

Nuestra capacidad para predecir las dinámicas de la comunidad en relación a las variaciones en la radiación parece, por tanto, bastante limitada; la interpretación de experimentos individuales es complicada y es necesario considerar las interacciones entre la UVR y otros factores ambientales antes de que podamos extrapolar desde nuestros experimentos a pequeña escala hasta los complejos sistemas naturales y dar algunas predicciones fidedignas.

Conclusiones de la tesis

Conclusiones específicas

1. Durante el período invernal de mayor producción primaria en la Bahía de Blanes, todos los grandes grupos bacterianos estudiados siguieron ciclos diarios en sus actividades específicas, mostrando mayores números de células activas durante la noche que por el día. Estas tendencias se reflejaron en las medidas de actividad total como las tasas de incorporación de ^{3}H-leucina y ^{3}H-timidina.

2. A lo largo de un ciclo anual, la radiación solar ambiental afectó negativamente a la actividad bacteriana en la Bahía de Blanes, aunque la contribución relativa a la inhibición de la región UVB parecía estar influenciada por el diseño experimental, que llevó a una sobreexposición de las muestras a la UVB. Cuando la actividad bacteriana se midió bajo una luz (sólo PAR) artificial constante, los consistentes aumentos observados en las muestras expuestas se explicaron por una estimulación importante del grupo de las *Gammaproteobacteria*, probablemente debido a su capacidad de responder rápido a cambios ambientales o a la potencial fotoheterotrofía de algunos subgrupos como NOR5.

3. Los grupos bacterianos mayoritarios en la Bahía de Blanes mostraron distintas sensibilidades a las PAR y UVR ambientales, como se observó a nivel de célula individual. Tanto los grupos *Gammaproteobacteria* como *Bacteroidetes* mostraron una elevada resistencia a la radiación, mientras la exposición a niveles naturales de PAR y UVR a menudo inhibían la actividad de SAR11. *Roseobacter, Synechococcus* y el grupo NOR5 se estimulaban con frecuencia particularmente ante la PAR. Los cambios absolutos en el número de células activas de cada grupo debidos a la luz fueron generalmente más pronunciados durante los periodos de irradiancias más altas, pero la inhibición relativa por unidad de radiación fue generalmente más baja durante el verano, lo que sugiere un cierto grado de fotoadaptación o fotoaclimatación por parte de las bacterias.

4. En las aguas de verano del Ártico y la Antártida se detectaron elevados porcentajes de células activas en la toma de ^{3}H-leucina y ^{35}S-DMSP de todos los grandes grupos de bacterias estudiados. En las muestras del Ártico, SAR11 dominaba tanto numéricamente como los porcentajes de células asimilando ambos

sustratos, mientras en que las estaciones de la Antártida, tanto este grupo como las *Gammaproteobacteria* fueron los contribuidores mayoritarios al total de células activas. El uso bacteriano de la leucina parecía ser más sensible a la exposición a la UVR que el uso del azufre del DMSP, si bien dicha sensibilidad fue más aparente en términos de tasas de asimilación que en los números de células activas.

5. El análisis microautoradiográfico de muestras del Ártico y la Antártida en verano reveló una importante y extendida capacidad del fitoplancton eucariota de incorporar los sustratos radiactivos ^{3}H-leucina y ^{35}S-DMSP, incluyendo diatomeas pennadas y centrales, dinoflagelados autotróficos y flagelados, cuyas tasas conjuntas de asimilación eran comparables o incluso a veces superiores a las de las bacterias. Distintos grupos de algas mostraron distintas sensibilidades a la UVR, y, mientras que la variabilidad en la toma de ^{35}S-DMSP no mostró patrón claro en relación a las condiciones de luz, la incorporación de ^{3}H-leucina fue en general menor en las muestras expuestas que en las oscuras.

6. El análisis de muestras de Blanes con las técnicas de resolución individual *cell sorting* por citometría de flujo y microautoradiografía mostró que la contribución relativa de los *Synechococcus* a la incorporación de ^{35}S-DMSP se estimulaba significativamente en presencia de radiación total en comparación con la de las bacterias heterotróficas, fundamentalmente debido a una mayor inhibición en la actividad de las últimas. Además, experimentos de asimilación fraccionada por tamaños revelaron una mayor asimilación relativa de las cyanobacterias durante el día que por la noche. Estos resultados indican un importante papel de la radiación solar como regulador de la competición entre el picoplancton auto- y heterotrófico por la utilización del azufre del DMSP.

Conclusiones generales

7. La luz modula los flujos de sustratos orgánicos a través de la red trófica planctónica a diferentes escalas, pero los efectos de conjunto variarán dependiendo en gran medida de los organismos implicados y de sus respuestas específicas a la luz. Las distintas afinidades encontradas para los sustratos estudiados así como los comportamientos variables de los grupos en relación a las condiciones de luz indican que estas interacciones distan mucho de ser sencillas.

8. El grado de similitud entre la 'historia lumínica' previa de los organismos y las condiciones experimentales causó parte de la variabilidad observada de los efectos de la UVR en la comunidades naturales de la Bahía de Blanes, señalando algunos problemas asociados con este tipo incubaciones estáticas.

9. El grupo filogenético *Gammaproteobacteria* de la Bahía de Blanes parecía ser un contribuidor mayoritario a la LIR medida causando la mayoría de los patrones observados a distintas escalas, probablemente a causa de su capacidad de responder rápido a cambios en el ambiente o a la potencial fotoheterotrofía de algunos subgrupos como el clado NOR5.

10. La fotoheterotrofía del fitoplancton puede ser en ocasiones relevante en los flujos de DOM, especialmente cuando consideramos DMSP. La presencia de elevadas concentraciones de DMSP disuelto en el Ártico y en menor medida en la Antártida durante los meses de verano parecían favorecer a muchos grupos de algas capaces de tomar este sustrato del medio, fundamentalmente diatomeas. Los resultados del Mediterráneo sugieren que la radiación solar podría causar en ocasiones una mayor competición de los fotótrofos por la toma de DMSP frente a las bacterias heterotróficas, influyendo por tanto el ciclo del azufre orgánico en la superficie del océano.

11. Las medidas de incorporación de DOM realizadas en oscuridad pueden ser más fáciles y prácticas dada la dificultad de simular con precisión las condiciones naturales de luz. Sin embargo, como en vista de nuestros resultados la luz es un modulador esencial de los flujos de DOM a través de las bacterias y el fitoplancton, procesos claves como la producción bacteriana deberían realizarse en incubaciones paralelas en oscuridad y condiciones realistas de luz para poder estimar con cierta fiabilidad flujos de carbono a través de las redes tróficas microbianas.

Perspectivas de futuro y preguntas abiertas

La preocupación por los efectos de la radiación sobre los ecosistemas marinos ha ido incrementándose a lo largo de las últimas décadas en paralelo con el desarrollo de instrumentos de medida de la UVR bajo el agua (ver ejemplos en Tedetti y Sempéré 2006) y con el creciente conocimiento acerca de estos efectos sobre distintos organismos y ecosistemas (Häder et al. 2007). Sin embargo, existe aún una carencia remarcable de datos en lo referente a los efectos de la UVR sobre grupos particulares de las comunidades naturales. En nuestro estudio hemos intentado evaluar algunos de estos efectos sobre la actividad de organismos particulares del Mediterráneo y de las aguas polares; sin embargo, somos conscientes de que esta tesis abre muchas preguntas para futuros trabajos, y esperamos que nuestros hallazgos estimulen nuevas investigaciones en este campo. Técnicas en desarrollo como los métodos moleculares brindan la oportunidad de responder preguntas más detalladas en relación a los efectos de la UVR sobre la estructura de la comunidad, o cómo influye la radiación en la expresión diferencial de algunos genes, por ejemplo. La consideración de algunos de los aspectos que se enumeran a continuación podrían ayudarnos en nuestra comprensión del papel de la luz sobre los ecosistemas marinos:

1. Una buena caracterización de los factores responsables de la atenuación de la UVR en los sistemas estudiados y su estacionalidad favorecerán un mayor conocimiento de las dinámicas relacionadas con la luz de los organismos planctónicos. Dicho estudio se está llevando a cabo en el área de Blanes (Galí y Simó, in prep.), hecho que además aumentará nuestra capacidad de simular con precisión las condiciones de exposición a la luz con el fin de evitar, por ejemplo, una sobreexposición de las muestras.

2. El desarrollo de sistemas para la simulación de la mezcla vertical en el océano permitiría la incubación de las muestras bajo condiciones realistas de luz evitando por tanto los problemas derivados de las incubaciones estáticas.

3. El diseño de sondas nuevas y más específicas para CARD-FISH posibilitaría un estudio más detallado de las respuestas de organismos particulares a la UVR (sin la necesidad de usar aislados bacterianos) que permitiera explicar la variabilidad intra-grupal observada tanto a escalas espaciales como temporales.

4. Es preciso un estudio detallado del papel de los organismos fototróficos en el uso de la DOM en condiciones de luz a fin de corregir algunos modelos de flujos de DOM donde las bacterias son protagonistas indiscutibles; es posible que en algunas ocasiones la proporción de la DOM fluyendo a través de las bacterias decaiga en favor de una estimulación del papel de los fotoheterótrofos causada por la luz.

5. Del mismo modo, aún es necesario un examen más exhaustivo del papel de otros metabolismos fotoheterotróficos en la naturaleza que permita evaluar las ventajas que confiere a los organismos el hecho de pertenecer a alguno de estos grupos. Por ejemplo, la combinación de este tipo de técnicas de resolución individual con métodos para identificar fotoheterotrofía de bacterias (p. ej. microscopía con cámara de infrarrojos para la detección de BChl*a* o análisis de la expresión de genes de la proteorodopsina) podrían revelar si esta capacidad de utilizar la luz puede traducirse en un aumento de la incorporación de algún sustrato concreto, por ejemplo, en organismos de las comunidades naturales.

6. En vista de que, según el sustrato analizado, las respuestas parecen variar, un conocimiento detallado sobre la regulación por la luz de los distintos sistemas de transporte permitiría una identificación más precisa de los procesos que tienen lugar.

7. Por último, un análisis integral de los múltiples efectos de la radiación sobre los distintos componentes de las redes tróficas posibilitará la comprensión de interacciones ecológicas y distintos efectos sinérgicos y antagónicos. El estudio simultáneo de todos los compartimentos de las redes planctónicas requiere una aproximación multidisciplinar, pero sin duda facilitaría una interpretación más realista de la dinámica de los ecosistemas.

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