

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

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Introduction

Marine plankton communities are naturally exposed to fluctuating radiation regimes in their environment. The PAR (400–700 nm) and 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, which are major components of aquatic ecosystems and play a key role in biogeochemical processes (Azam *et al.*, 1983; Cotner & Biddanda, 2002), are specially sensitive to solar radiation as they are too small for efficient protection by pigments (García-Pichel, 1994). Although the effects of UVR, and mainly UVB (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;

Abstract

The sensitivity of coastal marine bacterioplankton to natural photosynthetically active radiation (PAR, 400–700 nm) and ultraviolet radiation (UVR, 280–400 nm) was evaluated in five experiments over a seasonal cycle in the Blanes Bay, NW Mediterranean Sea. Exposure to natural solar radiation generally inhibited bulk bacterial activities or damaged membrane integrity when irradiances were high (i.e. spring and summer experiments) and, in general, UVB (280–320 nm) accounted for most of the inhibition. When assessing activity (³H-leucine uptake) at the single-cell level by microautoradiography and rRNA gene probing, seasonally varying responses and sensitivities were found among bacterial groups. While autumn and winter irradiances seemed too low to cause changes in activity, variable effects were found in spring and summer. SAR11 was consistently inhibited by UVR and PAR exposure, whereas *Gamma-proteobacteria* and *Bacteroidetes* showed higher resistance. *Roseobacter*, *Synechococcus* and the NOR5 clade were occasionally photostimulated in their activity, mainly because of PAR. Our results indicate that a component of seasonality exists in the bacterial responses to solar radiation, which vary not only depending on the irradiance and the spectral characteristics, but also on the previous light history and the taxonomic composition of the community.

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.

Given that marine bacterial communities are known to show gradual changes in their taxonomic composition throughout the year (Pinhassi & Hagström, 2000; Schauer *et al.*, 2003) and that different bacterial groups may display different sensitivities to sunlight (e.g. Joux *et al.*, 1999; Alonso-Sáez *et al.*, 2006; Kataoka *et al.*, 2009), it seems reasonable to expect seasonal changes in dominant bacterial phylotypes in response to solar radiation. 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 winter-time communities were more or less sensitive to UVR.

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 (Bailey *et al.*, 1983; Herndl *et al.*, 1993; Xenopoulos & Schindler, 2003; Agogué *et al.*, 2005; Alonso-Sáez *et al.*, 2006; Hernández *et al.*, 2007), suggesting the absence of adaptive strategies driven by differences in the light conditions. In contrast, photoadaptation in marine bacteria has been inferred from circumstantial evidences, such as an increase in the percentage of pigmented cells during UV exposure of estuarine bacteria (Thomson *et al.*, 1980), different UVB responses and recovery potential of bacterial isolates according to the irradiation levels of their native environments (Fernández-Zenoff *et al.*, 2006), higher sensitivity to UVR in deeper than surface 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).

We present here the results of five experiments conducted in different seasons designed to evaluate the short-term responses to solar radiation of different marine bacterioplankton assemblages from the Blanes Bay, both from a bulk and a single-cell perspective. Our data report for the first time seasonally varying sensitivities to UVR of *in situ* dominating bacterial groups.

Materials and methods

Study area, 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 off the shore of Blanes, Spain (41°39.90'N, 2°48.03'E). An ongoing time-series study with monthly samplings in this area provides information about a broad range of physicochemical and biological variables (Fig. 1, <http://www.icm.csic.es/bio/projects/icmicrobis/bbmo>). For the experiments, surface samples (0.5 m depth) were collected with polycarbonate carboys and held in the dark in black plastic bags during transport to the laboratory. Water was collected at dawn to avoid exposure to sunlight before the experiments. Chlorophyll *a* concentration was determined as described in the study of Yentsch & Menzel

(1963) 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 4 °C for 24 h, and fluorescence was measured with a Turner Designs 10-005 R fluorometer. Underwater PAR and UVR profiles were performed a few days before sampling with a PUV 2500 radiometer (Biospherical Instruments).

Experimental design

Experiments were carried out on 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 in aluminium foil). The transmission spectra of the two filters used are shown in Supporting information, Fig. S1. Bottles were incubated 5 cm under the surface inside a black tank (200 L) 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 because of their movement within the mixed layer developing at this 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, postexposure ³H-leucine incorporation and ectoenzyme activity analyses, and two 50-mL replicates were amended with radioactive ³H-leucine for microautoradiography combined with catalysed reporter deposition-fluorescence *in situ* hybridization (MAR-CARD-FISH) incubations. Only in the experiment Sm1, we incubated two replicates for general parameters (flow cytometry measurements and bulk activity assays) 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. The radiometer was placed

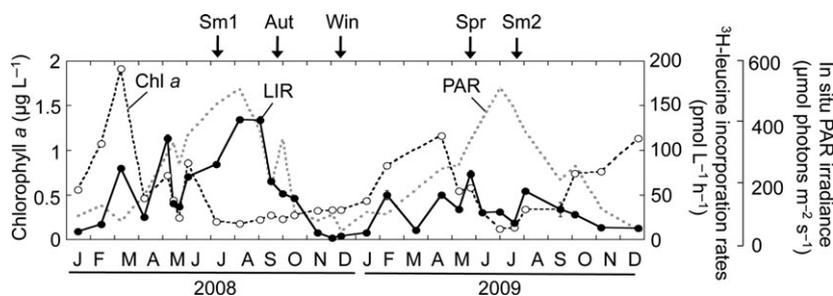


Fig. 1. Temporal dynamics of chlorophyll *a* (Chl *a*, open circles), ^3H -LIR (solid circles, average \pm standard errors) and mean PAR irradiance within the surface mixed layer (grey dashed line) during 2008 and 2009. Arrows indicate the timing of each of the experiments: Sm1, Aut, Win, Spr and Sm2.

inside the incubation tank, with the sensor covered by *c.* 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 six bands in the UVR (305, 313, 320, 340, 380 and 395 nm, in units of $\text{mW cm}^{-2}\text{ nm}^{-1}$) and one integrated band in the visible (PAR, in $\mu\text{mol photons cm}^{-2}\text{ s}^{-1}$). The mean spectral irradiance in the six UV bands was converted to mean UVB and UVA irradiance (mW cm^{-2}) by integrating over the spectrum (sum of trapezoids), between 305–320 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 UVA, and $\text{mol photons m}^{-2}$ for PAR).

The 'light history' of the sampled microbial communities, that is, 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 comparison with the doses measured during incubation. For this purpose, the maximum daily exposure was calculated by combining the maximum irradiance values (average irradiance at noon \pm 2 h) of the day prior to sampling, the 'actively mixing layer' depth (mLD), and the underwater attenuation of solar radiation. 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, <http://www.meteo.cat>). mLD was 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 $0.03\text{ }^\circ\text{C}$ was encountered relative to 1 m depth. These criteria were optimized for our particular dataset and yielded mLD estimates consistent with the vertical profiles of other variables (M. Galí, unpublished data). Diffuse attenuation coefficients of downwelling radiation ($K_{d,\lambda}$) were calculated as the slope of the linear regression between the

natural logarithm of spectral cosine irradiance ($E_{d,\lambda,z}$) and depth (z). $K_{d,320}$ and $K_{d,380}$ were chosen as representative of UVB and UVA attenuation, respectively, while PAR (and its corresponding $K_{d,\text{PAR}}$) was originally measured in one integrated band.

Abundance of prokaryotes

Samples for enumeration of bacteria were preserved with 1% paraformaldehyde and 0.05% glutaraldehyde (final concentrations) and kept frozen at $-80\text{ }^\circ\text{C}$ until analysis with a FACSCalibur flow cytometer (Becton-Dickinson). Heterotrophic prokaryotes were stained with SYBR Green I (Molecular Probes, Eugene, OR) and counted by their signature on the side scatter (SSC) vs. FL1 (green fluorescence) plot (Gasol & Del Giorgio, 2000). *Synechococcus* abundances were estimated from unstained samples, and abundances were quantified by their signature when plotting SSC vs. red fluorescence (FL3) and that one vs. orange fluorescence (FL2).

CTC labelling

Aliquots of 0.5 mL were spiked with 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, 5 mM final conc., Polysciences) and incubated for 1.5 h in the dark at *in situ* temperature for detection of actively respiring prokaryotes (Sieracki *et al.*, 1999). The samples were immediately counted with the FACSCalibur flow cytometer. CTC particles were identified by their signature when plotting SSC vs. FL3 (see Gasol & Aristegui, 2007).

Nucleic acid double staining (NADS)

SYBR Green I (Molecular Probes) and propidium iodide (PI; Sigma Chemical Co.) were used for the double staining of nucleic acids as described by Gregori *et al.* (2001) and Falcioni *et al.* (2008). Samples were stained and analysed by flow cytometry after 20 min of incubation in the

dark. Plotting red (PI) vs. green fluorescence (SYBR Green I) allowed differentiation of 'live' cells (i.e. with undamaged membranes) from those considered 'dead' (with damaged or compromised membranes).

³H-leucine incorporation rates (LIR)

Bacterial heterotrophic activity was estimated before and after exposure to solar radiation using the ³H-leucine incorporation method described by Kirchman *et al.* (1985). From each quartz bottle, three aliquots (1.2 mL) and one trichloroacetic acid (TCA)-killed control were incubated with ³H-leucine (40 nM final conc., 160 Ci mmol⁻¹) in the dark at *in situ* temperature. After 2 h, incorporation was stopped with cold TCA and samples were processed as in the study of Smith & Azam (1992).

Ectoenzyme activity

For the determination of the activities of ectoenzymes [beta-glucosidase (β glu), 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 β glu, 4-MUF-P-phosphate for APA and L-leucine-7-amido-4-methyl-coumarin for AMA. Substrates were added at saturating concentrations (100 μ M final concentration) to 0.9 mL replicate subsamples, and fluorescence was measured immediately after addition and after a 1- to 3-h incubation. Fluorescence was read on a Shimadzu spectrofluorometer RF-540 at 365 nm excitation and 446 nm emission wavelengths. Increase in 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 catalysed reporter deposition-fluorescence *in situ* hybridization

After 4 h exposure of samples amended with trace ³H-leucine (0.5 nM final conc., 160 Ci mmol⁻¹), samples were fixed overnight with PFA at 4 °C in the dark, gently filtered on 0.2- μ m polycarbonate filters (GTTP, 25 mm diameter; Millipore), and then the filters were 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). These probes were selected based on previous information on the composition of Blanes Bay bacterial communities and include most dominant taxa (Alonso-Sáez *et al.*, 2007).

Before subjecting samples to microautoradiography, smaller pieces from each hybridized section were cut and stained with 4,6-diamidino-2-phenylindole (DAPI, 1 μ g mL⁻¹) to estimate the relative abundance of each group. Between 500 and 800 DAPI-positive cells were counted manually using an Olympus BX61 epifluorescence microscope within a minimum of 10 fields.

For microautoradiography, we followed the protocol described in the study of Alonso & Pernthaler (2005) modified as in the study of Alonso-Sáez & Gasol (2007). The optimal exposure time was determined for each experiment and resulted in 3 days for experiment Sm1, 5 days for experiments Aut and Sm2, 17 days for experiment Win and 2 days for experiment Spr. Slides were developed as described previously (Alonso-Sáez & Gasol, 2007), dried in a dessicator overnight, stained with DAPI and 500–700 hybridized cells were manually counted by epifluorescence microscopy 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 the Blanes Bay (Fig. 1, Table 1), with lowest temperatures in winter (14 °C) and highest in summer (23 and 20 °C in experiments Sm1 and 2, respectively). Chlorophyll *a* concentrations ranged from 0.13 (Sm2) to 0.58 μ g L⁻¹ (Spr), and the *in situ* bulk ³H-LIR varied between 3.6 pM h⁻¹ in winter and 84.4 pM h⁻¹ in experiment Sm1. The LIR measured at the beginning of the spring experiment was the highest recorded for 2009, and it followed the spring phytoplankton bloom that was observed at the end of April (Fig. 1). Water transparency varied slightly between samplings (Table 1). The diffuse attenuation coefficients for UVR at 320 nm ($K_{d,320}$) measured a few days before the experiments 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 18 m.

Total UVR and PAR doses varied among experiments mainly because of seasonal variations in the solar angle, as 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 UVB 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.

Effects of solar radiation on prokaryote abundances

The picophytoplankton community was generally 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 *Synechococcus* abundances did not seem to be affected by sunlight exposure (Table 3), whereas their numbers were significantly reduced upon UVA exposure in experiment Aut and to a less extent in Win (23% and 5% decrease, respectively, Tukey's test, $P < 0.05$). For heterotrophic bacteria, instead, we only found a significant PAR-driven decrease of *c.* 20% in experiment Sm2, the one receiving the highest radiation dose (Table 3).

Quantification of NADS green-positive cells (a surrogate for 'live' cells, see Falcioni *et al.*, 2008) indicated that membrane integrity was consistently affected by PAR + UVR exposure compared with dark controls (12–35% decline) in all experiments but in winter, with PAR alone also causing a significant inhibition (7–20% decline, Table 3).

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 with the dark control, which seemed to be mainly caused by PAR.

In contrast, exposure to full sunlight significantly inhibited bulk ³H-LIR measured after the incubations (Tukey's test, $P < 0.05$, Fig. 2) compared with the dark treatments in autumn, winter and both summer experiments. The effect was not significant in spring, in spite of the substantial UVR doses received by those samples. The response of bacteria to the different wavelength ranges was variable. Inhibition of bacterial activity because of PAR exposure was only significant in the summer experiments when PAR doses were highest. We did not detect significant differences between PAR and PAR + UVA treatments in any of the experiments, indicating that UVB 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 UVR levels were recorded in summer, a much stronger PAR + UVR-driven inhibition was found in winter (68% decrease compared with dark control) than in summer samples (36% and 45% decrease in experiments Sm1 and Sm2, respectively).

Activities of βglu, APA and AMA were analysed immediately after exposure to the different light conditions (Fig. 3). In general, exposure to the full-sunlight spectrum caused the greatest inhibition of enzyme activities compared with PAR exposure, especially 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 *c.* 60% stimulation of this enzyme. Depending on the samples and seasons, either the PAR, UVA or UVB wavelength ranges were responsible for most of the inhibition of activities, with no clear patterns detected.

In spite of the low UVR levels in winter, the percentage of inhibition on a per photon basis of most of the aforementioned parameters was higher in winter samples (Fig. 4). When we compared the UVB doses received by winter samples with their *in situ* UVB levels (calculated as the mean UVB irradiance measured within the mixing

Table 1. Temperature, chlorophyll a concentration and bacterial heterotrophic activity (measured as ³H-LIR) in the *in situ* starting samples of each experiment, downwelling diffuse attenuation coefficients for UVR at 320 nm ($K_{d,320}$) measured a few days before sampling, and integrated doses of PAR, UVA and UVB received by the samples during the experiments

Experiment	Date	Temp (°C)	Chla (µg L ⁻¹)	LIR (pM h ⁻¹)	$K_{d,320}$ (m ⁻¹)	UVB (kJ m ⁻²)	UVA (kJ m ⁻²)	PAR (E m ⁻²)
Sm1	9 July 2008	23.0	0.20	84.4 ± 2.4	0.35	22.0	419.4	20.6
Aut	30 September 2008	20.3	0.23	51.5 ± 2.0	0.34	11.4	262.2	13.3
Win	11 December 2008	14.1	0.33	3.6 ± 0.5	0.35	4.1	135.7	8.0
Spr	26 May 2009	16.8	0.58	73.7 ± 8.2	0.34	13.7	278.1	13.5
Sm2	21 July 2009	20.3	0.13	18.5 ± 2.1	0.26	23.9	455.7	22.2

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

Exp.	Date	Bac (10 ⁵ mL ⁻¹)	Syn (10 ⁴ mL ⁻¹)	Fraction (%) of total DAPI counts						
				Eub	Gam	Bcdt	Sar11	Ros	NOR5	Syn
Sm1	9 July 2008	8.5	1.3	88 (7)	18 (4)	15 (3)	40 (5)	2 (1)	4.1 (1.7)	1 (1)
Aut	30 September 2008	7.5	1.5	67 (7)	8 (5)	12 (4)	39 (8)	5 (2)	2.4 (1.3)	4 (3)
Win	11 December 2008	9.1	0.5	94 (4)	5 (4)	11 (3)	30 (7)	3 (4)	< 1	2 (1)
Spr	26 May 2009	8.9	0.3	83 (4)	14 (4)	20 (6)	20 (3)	10 (3)	12.1 (2.6)	2 (2)
Sm2	21 July 2009	9.2	3.3	87 (7)	11 (4)	8 (5)	43 (8)	2 (2)	2.0 (1.8)	7 (3)

Eub, *Eubacteria*; Gam, *Gammaproteobacteria*; Bcdt, *Bacteroidetes*; SAR11; Ros, *Roseobacter*; NOR5; Syn, *Synechococcus*. CARD-FISH values represent means ± (standard deviations).

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

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	8.6 ± 0.01 ^a	12.0 ± 0.6
	PAR	8.7 ± 0.3	12.1 ± 0.1	8.0 ± 0.00 ^b	11.9 ± 0.02
	PAR + UVA	9.1 ± 0.3	12.8 ± 0.2	8.1 ± 0.04 ^b	10.7 ± 0.5
	PAR + UVR	8.2 ± 0.2	11.8 ± 0.4	7.5 ± 0.05 ^c	10.6 ± 0.04
Aut	DARK	7.5 ± 0.2	14.5 ± 0.2 ^a	6.3 ± 0.1 ^a	4.7 ± 0.0 ^a
	PAR	7.4 ± 0.3	14.4 ± 0.2 ^a	5.7 ± 0.1 ^b	3.0 ± 0.7 ^b
	PAR + UVA	7.1 ± 0.2	11.7 ± 0.1 ^b	5.7 ± 0.1 ^b	3.2 ± 1.4 ^b
	PAR + UVR	6.8 ± 0.2	11.1 ± 0.2 ^b	5.3 ± 0.1 ^c	2.8 ± 0.5 ^b
Win	DARK	8.8 ± 0.3	5.8 ± 0.1 ^a	7.5 ± 0.1 ^{ab}	3.6 ± 0.8
	PAR	–	–	–	–
	PAR + UVA	9.0 ± 0.6	5.5 ± 0.01 ^b	6.6 ± 0.3 ^a	4.0 ± 1.2
	PAR + UVR	10.0 ± 0.3	5.5 ± 0.02 ^b	7.7 ± 0.2 ^b	4.8 ± 0.4
Spr	DARK	14.3 ± 0.6	2.9 ± 0.01	13.4 ± 0.1 ^a	17.2 ± 11.0 ^{ab}
	PAR	12.7 ± 1.5	2.8 ± 0.1	11.2 ± 0.2 ^b	18.6 ± 1.0 ^a
	PAR + UVA	11.2 ± 1.4	2.7 ± 0.1	8.6 ± 0.3 ^c	15.4 ± 0.4 ^b
	PAR + UVR	11.4 ± 0.7	2.8 ± 0.1	8.8 ± 0.2 ^c	16.2 ± 0.9 ^{ab}
Sm2	DARK	12.1 ± 0.1 ^a	34.1 ± 0.2	9.9 ± 0.5 ^a	14.1 ± 4.2 ^a
	PAR	9.9 ± 0.1 ^b	33.4 ± 0.4	8.1 ± 0.2 ^b	8.2 ± 0.5 ^b
	PAR + UVA	9.9 ± 0.2 ^b	32.8 ± 0.5	8.1 ± 0.1 ^b	7.2 ± 0.6 ^b
	PAR + UVR	10.1 ± 0.2 ^b	34.1 ± 0.1	7.5 ± 0.1 ^b	5.1 ± 0.1 ^b

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.

layer during the 4 h of maximal irradiance of the day prior to the sampling, see Materials and 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 with 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 the full-sunlight and the dark treatments; when differences were not significant, the percentage of change compared with the dark control was assigned a value of zero (following Pakulski *et al.*, 2007).

Differential sunlight sensitivity 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 often the most abundant one, accounting for 20–43% 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 15%. The number of cells of each group active in the

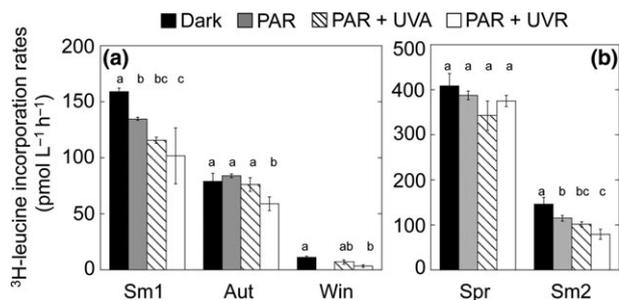


Fig. 2. Bulk bacterial activity measured as ³H-LIR after 4 h exposure to different wavebands of natural sunlight or kept in the dark in the five experiments: Sm1, Aut and Win of 2008 (a), and Spr and Sm2 of 2009 (b). Bars represent means \pm standard errors. Letters refer to results of an ANOVA followed by a *post hoc* Tukey's test ($P < 0.05$). Different letters indicate significant differences among treatments.

uptake of ³H-leucine varied among treatments and depended on the studied season. In accordance with the lower levels of bacterial heterotrophic activity measured in winter samples, most of the groups were much less active in this experiment, showing weakly labelled cells (i.e. much smaller silver grain areas).

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

No significant sunlight effects were observed for *Bacteroidetes* in any of the experiments (Fig. 5b), yet this group was often weakly labelled in the uptake of ³H-leucine. The great error bars in the autumn experiment were because of 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 photosyn-

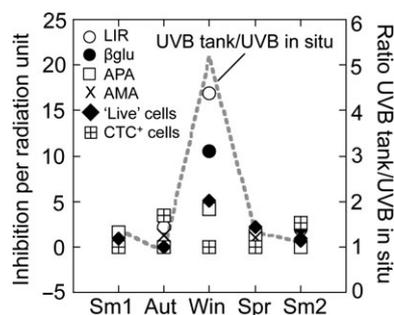
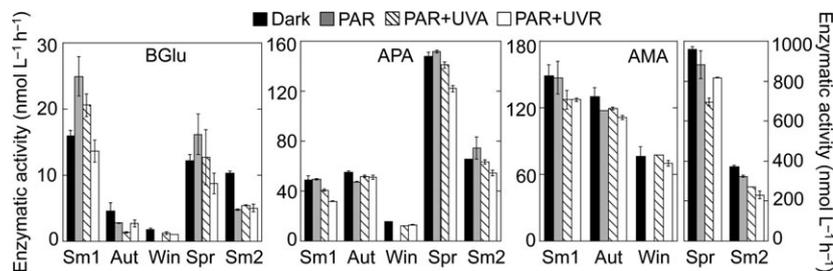


Fig. 4. Percentage of full-sunlight-driven inhibition of different parameters relative to UVB doses. (LIR) ³H-leucine incorporation rates; beta-glu, APA and AMA activities. Dashed line behind shows the ratio between the UVB doses measured during incubation and the UVB doses naturally experienced by samples within the surface mixing layer during the 4 h of maximal irradiance of the day prior to the sampling (i.e. UVB *in situ*, see Materials and methods).

thetic cyanobacterium *Synechococcus*. Members of the dominant SAR11 clade (Fig. 5c) showed a consistent strong inhibition after full-sunlight exposure compared with dark controls in the experiments with the highest doses of UVB (64%, 52% and 48% reduction in percentage of active cells in the uptake of ³H-leucine in experiments 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 UVA and PAR treatments, respectively. *Roseobacter* showed no sensitivity to UVR except for an 8% reduction in experiment Sm1 caused by UVB 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 PAR 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

Fig. 3. Activities of the ectoenzymes beta-glu, APA and AMA measured after exposure to the different light treatments or kept in the dark (average \pm standard errors).



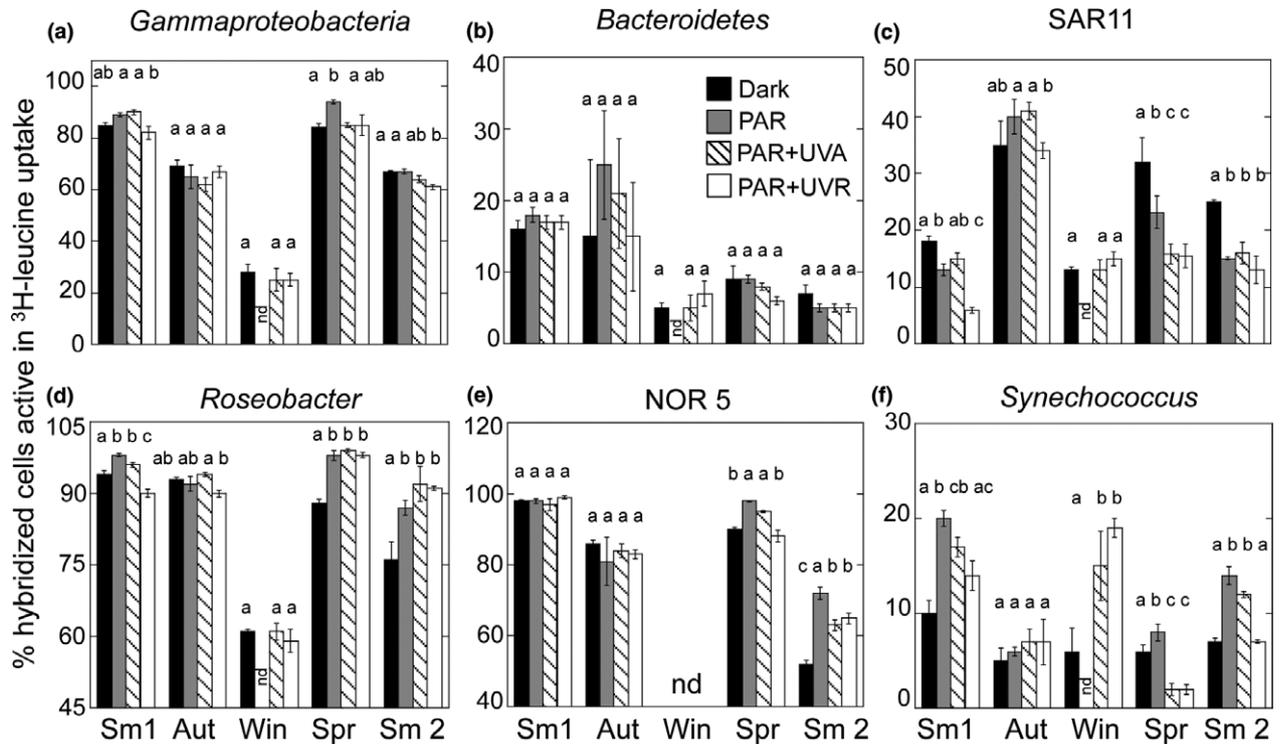


Fig. 5. Percentage of positively hybridized cells with probes for *Gammaproteobacteria* (a), *Bacteroidetes* (b), SAR11 (c), *Roseobacter* (d), NOR 5 (e) and *Synechococcus* (f) taking up ^3H -leucine (average \pm standard error of duplicates) as measured by MAR-CARD-FISH after exposure to each treatment in the five experiments. PAR treatment is missing in the winter experiment, and NOR5 cells from the winter experiment could not be counted because of low abundances ('nd' = not determined). Note that the Y-axes have different scales. Letters refer to results with a *post hoc* Tukey's test ($P < 0.05$). Different letters indicate significant differences among treatments.

spring *Gammaproteobacteria*, which also showed such a PAR-driven stimulation. Active NOR5 cells from the winter samples could not be quantified because of 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 with dark treatments in all experiments except in autumn, showing higher activity under stronger PAR doses (Pearson's $r = 0.96$, $P < 0.05$, $n = 4$). A significant reduction in activity was observed after full-sunlight exposure compared with PAR treatment in the spring and both summer experiments, showing 80%, 28% and 53% inhibition in the number of active cells, respectively. However, in experiment Spr, the decline seemed to be entirely caused by UVA radiation, as we did not find significant differences between both UVR treatments.

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 ^3H -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 ^3H -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 solar radiation 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 particularly in spring, whereas the contribution of *Bacteroidetes* and *Synechococcus* always remained below 8% and 4%, respectively. Interestingly, exposure to full sunlight significantly reduced the contribution of SAR11 in accordance with increasing UVR levels (Pearson's $r = 0.90$, $P < 0.05$, $n = 5$). As a consequence, 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 because of *Gammaproteobacteria*).

Discussion

Solar radiation effects on cell abundances and membrane integrity

Exposure to natural sunlight only caused a significant reduction in bacterial density in the summer experiment Sm2, the one where the highest radiation doses and the lowest Chl *a* concentration were recorded. Instead, we observed a consistent decline in the number of cells with intact membranes in most experiments caused both by PAR and UVR exposure, indicating that experimental light levels were indeed damaging cells. This contrasts with the results of Alonso-Sáez *et al.* (2006), who found that most bacteria maintained membrane integrity after light exposure in spring and summer and suggests that causes other than irradiance may be affecting bacterial sensitivity.

Decreased *Synechococcus* abundances after PAR + 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 carried out 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 phlotypes with greater UVR resistance. UVR has been shown to directly cause cell death among picophytoplankton communities from different ecosystems (Llabrés & Agustí, 2006; Llabrés *et al.*, 2010), and although *Synechococcus* have generally shown higher resistance than *Prochlorococcus* or picoeukaryotes, their sensitivity to light seems to vary depending on the location of

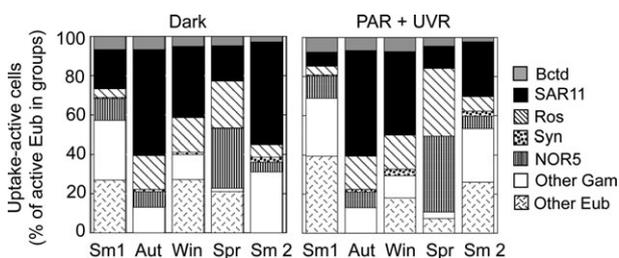


Fig. 6. Seasonal variation of the contribution of the different bacterial groups to total number of active *Eubacteria* (Eub) after dark incubation (a) or full-sunlight exposure (b). *Gammaproteobacteria* (Gam), *Bacteroidetes* (Bctd), SAR11, *Roseobacter* (Ros), NOR5 and (*Syn*) *Synechococcus*. Note 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 sunlight.

origin, depth and time of the year, maybe indicating changing phlotypes 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, unpublished data), thus pointing to physiological acclimation rather than succession towards photoresistant strains.

Effects of solar radiation on bacterial activity

Exposure to solar radiation caused a general decrease in the bulk incorporation of ^3H -leucine and in the ectoenzyme activities, whereas the number of respiring cells (CTC^+ cells) was only significantly reduced in the autumn and Sm2 experiments. Even though no significant correlation was found between solar radiation doses and the degree of inhibition of the activity measurements, higher inhibition because of UVR generally occurred in the summer experiments, the ones with the highest irradiances. The exception was ^3H -leucine incorporation, which exhibited the greatest inhibition in winter. Indeed, when we considered the absolute values of bulk measurements under full sunlight relative to the total doses of UVB received during each experiment, in most cases we found higher inhibition per unit of radiation in winter. 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 accumulated DNA damage when exposed to UVB doses $< \sim 1 \text{ kJ m}^{-2}$, so the dose received by the winter samples (4 kJ m^{-2}) was high enough to potentially damage bacteria. In our case, though, a different reason could be postulated 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 five-fold more UVB than the *in situ* levels. Water column mixing in winter likely prevents cells from being damaged (Jeffrey *et al.*, 1996). Our 4-h incubation right under the surface seemed to cause 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 considering the past light exposure history of samples for an accurate interpretation of the results.

In the rest of the experiments, inclusion of UVB generally inhibited bulk ^3H -leucine incorporation compared with dark controls except in spring, despite the substantial UVR doses received by spring samples. UVA 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, in accordance with previous findings (Aas *et al.*, 1996; Sommaruga *et al.*, 1997; Pakulski *et al.*, 2007). In any case, current information on the effects of different portions of the solar spectrum on bacterial heterotrophic activity is not conclusive. It is possible that our observations result from the combination of the distinct susceptibilities of bacterial populations to UVR (revealed by the MAR-CARD-FISH data as discussed later) and the sunlight effects on phytoplankton and DOM.

Bacterial extracellular enzymatic activity is thought to be a major agent in cleaving and processing DOM (Chróst, 1991). Of the three studied enzymes, β Glu and AMA (Chróst, 1991, 1992) are considered mainly of bacterial origin, whereas APA can also be associated with algae and zooplankton (Cembella *et al.*, 1985; Myklesstad & Sakshaug, 1983). Ecto-enzyme 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. As observed elsewhere (Herndl *et al.*, 1993; Santos *et al.*, 2010), exposure to PAR + UVR generally inhibited total ecto-enzyme activities compared with dark incubations, but some variability was measured in the responses to the different wavebands among experiments. Besides direct damage of UVR absorption on enzymes, light-driven changes in DOM bioavailability because of photosynthate release, photoalteration or even cell death (Herndl *et al.*, 1997; Pausz & Herndl, 1999; Llabrés & Agustí, 2006) might indirectly affect enzyme activities. Yet we did not find any statistical relationship with chlorophyll *a* or with UVR-driven increases in damaged cells.

Different responses to solar radiation of *in situ* dominating bacterial groups

Application of MAR-CARD-FISH revealed that the sunlight levels bacteria were exposed to in autumn and winter experiments were in general too low to decrease the numbers of cells actively taking up ^3H -leucine. Nearly all groups exhibited inhibition of activity because of UVB in the summer experiments and, unlike the bulk bacterial activity measurements, most of the phylotypes from the spring experiment clearly responded to solar radiation exposure. Using the same experimental design, Alonso-Sáez *et al.* (2006) observed a pattern of sunlight stimulation of *Roseobacter* and strong inhibition of SAR11 members (Alonso-Sáez *et al.*, 2006) which was also found here in spring and summer but not in autumn and winter. In contrast to these results, leucine incorporation by SAR11 cells from both the Delaware Bay (Straza & Kirchman,

2011) and summer Arctic waters (C. Ruiz-González, unpublished data) was often stimulated by PAR, pointing to latitudinal differences in the within-group occurrence of phylotypes or ecotypes adapted to contrasting light regimes. 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 the findings of Alonso-Sáez *et al.* (2006) 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 UVB-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 greater 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 spring than in summer (details not shown). This hints to the existence of radiative thresholds and acclimation processes: winter and autumn levels were too low to cause a response in the activity of the different groups; spring and summer UV irradiances were strong enough to cause damage, but summer assemblages were more resistant because they had acclimated to higher light.

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, up to 90% of spring *Gammaproteobacteria* hybridized with the NOR5 probe, a gammaproteobacterial subgroup that presented photostimulation of activity in experiments Spr and Sm2. A cultured representative of this lineage characterized by Fuchs *et al.* (2007) was shown to harbour bacteriochlorophyll *a*, and preliminary results indicated an enhanced cell yield under artificial PAR light. However, we cannot discard an indirect response of bacteria because of light-driven photosynthate release. In any case, it seemed that the spring enhancement in the activity of *Gammaproteobacteria* after exposure to PAR was driven by the significant 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.

Some members of the *Roseobacter* group are also known to contain bacteriochlorophyll *a* (Shiba, 1991; Allgaier *et al.*, 2003). This factor, or alternatively the pres-

ence of the widespread proteorhodopsin (Béjà *et al.*, 2000), could be the reason for the light-driven stimulation of the number of active *Roseobacter* cells. However, to date, no study has demonstrated any increase in ^3H -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. 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 PAR + UVR to percentages lower than those of the dark control. Contrary to previous reports on the resistance of *Synechococcus* to UVR in terms of cell counts, fluorescence or mortality (Sommaruga *et al.*, 2005; Llabrés & Agustí, 2006; Llabrés *et al.*, 2010), our results show that although their heterotrophic activity was largely stimulated by PAR, it was generally inhibited by UVA and UVB radiation. Uptake of amino acids by marine cyanobacteria has been shown to be stimulated by PAR light exposure (Michelou *et al.*, 2007; Mary *et al.*, 2008). Our study adds to recent evidence for UVR effects on the heterotrophic activity of this widely distributed picoplankton (Ruiz-González *et al.*, 2011)

Looking at the results altogether, decreases in the number of prokaryotic active cells were generally mostly attributable to UVB, although in some cases, UVA or even PAR was responsible for most of the inhibition (i.e. SAR11 in experiments Spr and Sm2, respectively). Either the presence of intragroup phylotypes with different sensitivities (e.g. Arrieta *et al.*, 2000; Agogue *et al.*, 2005) or prior acclimation of cells to different *in situ* proportions of PAR, UVA and UVB wavebands might explain some of the observed variability.

Group-specific responses to solar radiation as drivers of bulk patterns

The lack of UVR effects on bulk ^3H -leucine incorporation in spring was noticeable despite of the significant responses found at the single-cell level for some of the studied groups. Variations in the contribution of each phylogenetic group to the total number of ^3H -leucine assimilating cells seemed to 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 increased numbers of

Roseobacter and NOR5, resulted in a significant contribution of these latter two clades that was even greater under full-sunlight exposure. The lack of inhibition of bulk community ^3H -leucine incorporation in spring could be thus 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 behaviours 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 first exposed the samples to sunlight for 4 h and then measured the number of active cells by adding the radioisotope and further incubating them in the dark incubations for 4 h. That is, they were measuring the effects of sunlight as they hold for a while in the dark. Kaiser & Herndl (1997) had shown that 3 h of darkness is enough for bacteria to recover the activity levels previous to UVB exposure. We thus wanted to avoid this possibility by exposing the organisms together with the added ^3H -leucine. This approach allows a more realistic estimation of the *in situ* incorporation rates because irradiation and uptake processes are not separated in time. However, it also carries its own uncertainties. Although a potential photodegradation of leucine by UVR has recently been discarded (Vaughan *et al.*, 2010), addition of the radiotracer at the very beginning of the light exposure allows microorganisms to start taking it up before their activity becomes progressively inhibited by the cumulative UVR dose, so that they will appear labelled even though they may end up the sunlight exposure being severely inhibited. Should this 'early labelling' of cells occur, it will tend to reduce the magnitude of the observed detrimental effects of UVR.

In summary, our results confirm that solar radiation, 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 solar radiation on plankton with realistic manipulation experiments represents a formidable challenge.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Transmission spectra of the two UV-filters used in our experiments.

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