

Effect of Natural Sunlight on Bacterial Activity and Differential Sensitivity of Natural Bacterioplankton Groups in Northwestern Mediterranean Coastal Waters

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Received 13 March 2006/Accepted 19 June 2006

We studied the effects of natural sunlight on heterotrophic marine bacterioplankton in short-term experiments. We used a single-cell level approach involving flow cytometry combined with physiological probes and microautoradiography to determine sunlight effects on the activity and integrity of the cells. After 4 h of sunlight exposure, most bacterial cells maintained membrane integrity and viability as assessed by the simultaneous staining with propidium iodide and SYBR green I. In contrast, a significant inhibition of heterotrophic bacterial activity was detected, measured by 5-cyano-2,3 ditolyl tetrazolium chloride reduction and leucine incorporation. We applied microautoradiography combined with catalyzed reporter deposition-fluorescence in situ hybridization to test the sensitivity of the different bacterial groups naturally occurring in the Northwestern Mediterranean to sunlight. Members of the *Gammaproteobacteria* and *Bacteroidetes* groups appeared to be highly resistant to solar radiation, with small changes in activity after exposure. On the contrary, *Alphaproteobacteria* bacteria were more sensitive to radiation as measured by the cell-specific incorporation of labeled amino acids, leucine, and ATP. Within *Alphaproteobacteria*, bacteria belonging to the *Roseobacter* group showed higher resistance than members of the SAR11 cluster. The activity of *Roseobacter* was stimulated by exposure to photosynthetic available radiation compared to the dark treatment. Our results suggest that UV radiation can significantly affect the in situ single-cell activity of bacterioplankton and that naturally dominating phylogenetic bacterial groups have different sensitivity to natural levels of incident solar radiation.

Aquatic bacterioplankton has been shown to be sensitive to sunlight radiation, especially to the shortest-wavelength fraction of UV radiation (for examples, see references 22, 27, 52, and 53). Solar UV radiation (UVR, 290 to 400 nm) causes cellular damage on different cell targets, including nucleic acids, proteins, and lipids, which may end up in mutations, cell inactivation, and death. Because bacteria are considered to be too small to develop efficient photoprotection against UVR (16) and their genetic material comprises a significant portion of their cellular volume (25), bacteria are probably among the most susceptible group to photodamage within the plankton.

A relatively large number of studies have assessed the effect of UVR on bulk metabolic activities of natural bacterioplankton assemblages (see compilation in reference 24). Results from field studies on marine bacteria indicate that exposure to natural solar UVR results in a decrease in total bacterial abundance (38, 41), amino acid uptake (7), exoenzymatic activities (38), and oxygen consumption (41) and a significant inhibition of protein and DNA synthesis (22, 52). However, the effects of UVR on bacterioplankton have seldom been studied at the single-cell level (but see reference 33). Thus, we do not know whether the negative effects of UVR on cellular components or activity are uniformly distributed within the bacterioplankton assemblage.

Furthermore, there is little information about the impact of

UVR on bacterioplankton community composition. Previous studies have focused on isolates originated from marine bacterioplankton, bacterioneuston (bacteria isolated from the sea surface microlayer), and from other environments, such as marine snow and sediments. The results concur to show interspecific variability in sensitivity to UVR and also in the subsequent recovery potential of the isolates (2, 6, 26). Other significant results are that pigmentation seems not to be directly related to resistance of marine isolates and that bacteria isolated from the surface microlayer do not show higher resistance to UVR than isolates from subsurface waters (2). However, since isolates poorly represent natural communities (5), it is uncertain whether the conclusions of these studies can be extrapolated to natural bacterial assemblages. For example, little is known about the response to UVR of SAR11, the potentially more important oceanic bacteria in terms of abundance (37).

We present here results from an experimental study designed to assess the effect of natural sunlight on marine bacterioplankton assemblages at the single-cell level. The study was carried out in a highly UV transparent area of the Northwestern Mediterranean Sea. Microautoradiography was combined with catalyzed reporter deposition-fluorescence in situ hybridization (MAR-CARD-FISH), to report the first evidence of different sensitivities of in situ dominating bacterial groups.

MATERIALS AND METHODS

Study area and sample collection. Sampling was carried out in a shallow (20-m depth) oligotrophic coastal station in the Mediterranean Sea, located ~800 m off the shore of Blanes, Spain (41°39.90'N, 2°48.03'E, the Blanes Bay Microbial

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Observatory). Water samples were collected with a Niskin Go-flow bottle (5 liters), prefiltered through a large 200- μm -mesh-size net, and transported under dim light to the lab. On 4 August 2003, we measured vertical profiles of photosynthetic available radiation (PAR) and UV radiation to characterize the underwater radiation climate (51). Experiments were done with surface samples (0.5-m depth) on 5 and 11 August 2003 (experiments 1 and 3, respectively) and subsurface water (5-m depth) on 7 August 2003 (experiment 2). Water was collected at 0800 h in the morning to avoid exposure of the bacteria to sunlight before the experiments.

Underwater profiles of PAR and UVR in the sampling site and the surface incident UVR during the experiments were measured with a multichannel filter radiometer (PUV-501; Biospherical Instruments Inc., San Diego, CA) equipped with a sensitive pressure transducer and a temperature sensor. The integrated incident UV radiation during the experimental period was estimated using the trapezoidal integration rule and compared to the daily integral light value.

During the spring experiment (13 April 2005, experiment 4), when only the bacterial intergroup sensitivity was assessed, we estimated the UV cumulative exposure from the incident UV radiation measured at one station placed on the coast, about 20 km away (Malgrat de Mar). For this experiment, water was collected at around 1000 h and further processed in the same way as for the summer experiments.

Experimental design. The experimental design to test the effect of different wavebands was similar to that used in references 51 and 52. Briefly, within 3 to 4 h after sampling, 100-ml spherical quartz glass bottles were filled with the sample. Bottles were exposed for 4 h, centered at solar noon, to the full sunlight spectrum, the full spectrum minus UVR (i.e., PAR only), or the full spectrum minus UVB radiation (i.e., PAR plus UVA) or kept in the dark. Four replicates were used for each treatment. Quartz bottles with no additional spectral filters were used for full-spectrum treatments. Bottles kept in the dark were wrapped with 3 layers of aluminum foil and exposed apart from the others inside a black plastic bag to avoid reflection. Bottles were wrapped with one layer of a vinyl chloride foil (50% transmittance at 405 nm; CI Kasei Co., Tokyo, Japan) for PAR-only treatment or one layer of Mylar-D (150 μm thickness, 50% transmission at 325 nm) for PAR plus UVA treatment. In the spring experiment (experiment 4), Courtguard foils (50% transmittance at 383 nm) were used instead of vinyl chloride for PAR-only treatments. The transmittance of the different foils was regularly checked in a double-beam spectrophotometer (Uvikon 923).

Bottles were incubated 4 cm under the surface inside a large water bath (200 liters) with walls painted in black and circulating seawater to maintain in situ temperatures within 2°C. After sunlight exposure, subsamples were taken to immediately measure different parameters of bacterial activity.

During experiment 3, we assessed the effect of alga removal on bacterioplankton activity and the potential for bacterial recovery after radiation. To separate eukaryotic phytoplankton and cyanobacteria from bacterioplankton, we filtered the sample through glass fiber filters with a 142-mm diameter (AP15-14250; Millipore) with a peristaltic pump. The efficiency of phytoplankton removal after the filtration was checked by flow cytometry. We found that, on average, 91% of bacteria crossed the AP15 filters, while only 10% of *Synechococcus* cells, 37% of *Prochlorococcus* cells, and 20% of phototrophic picoeukaryotes went through the filter.

To assess bacterial recovery after exposure, samples from the different radiation treatments in experiment 3 were placed in the darkness for 24 h, and different parameters, such as bacterial abundance, CTC reduction, and the abundance of high-nucleic-acid (HNA)-content cells (see below), were again measured after the dark period.

Bacterial abundance and heterotrophic production. Samples for bacterial abundance determinations (1.6 ml) were preserved with 1% paraformaldehyde and 0.05% glutaraldehyde (final concentrations), deep frozen in liquid nitrogen, and stored at -80°C. Cell counts were done with a FACSCalibur flow cytometer (Becton Dickinson) after staining with SYTO13 (17). Regions were established on the SSC versus FL1 (green fluorescence) plot to discriminate cells with HNA content from cells with low nucleic acid content, and cell abundance was determined for each subgroup.

Bacterial heterotrophic production was determined using the [³H]leucine incorporation method (28), modified as described by Smith and Azam (50). We used four replicates for each treatment in experiments 1 and 2 and triplicates in experiment 3. For each sample, three aliquots (1.2 ml) plus one trichloroacetic acid-killed control were incubated with [³H]leucine (40 nM final concentration) for 1.5 h at in situ temperature.

CTC. Two 0.5-ml sample aliquots were spiked with CTC (5 cyano-2,3 dithiol tetrazolium chloride, 5 mM final concentration; Polysciences) and incubated for 90 min in the dark at in situ temperature. The samples were immediately run through the FACSCalibur flow cytometer. An additional 0.5-ml aliquot fixed with

paraformaldehyde was used as a background control of CTC fluorescence on dead samples. CTC-formazan is excited by wavelengths between 460 and 530 nm and has bright red fluorescence. CTC⁺ particles were those that showed red fluorescence (above 630 nm, FL3 in our instrument) higher than the background fluorescence level (for examples, see references 12 and 48). A dual plot of 90° light scatter and red fluorescence was used to separate CTC⁺ cells from background noise. The FL2 (orange fluorescence) versus FL3 (red fluorescence) plot was used to differentiate the populations of photosynthetic microbes (*Synechococcus*, *Prochlorococcus*, and picoeukaryotes) from the CTC⁺ particles.

Nucleic acid double staining (NADS). SYBR green I (Molecular Probes, Eugene, Ore.) and propidium iodide (PI; Sigma Chemical Co.) were used for the double staining of nucleic acids as described elsewhere (21). Samples were stained with 1:10,000 (vol/vol) SYBR green I and 10 $\mu\text{g ml}^{-1}$ PI commercial solutions. Flow cytometric analysis was done after 20 min of incubation in the dark. SYBR green I and PI fluoresce green (maximum at 521 nm) and red (maximum at 617 nm), respectively, when associated to nucleic acids and excited with a 488-nm argon laser. A plot of red versus green fluorescence allowed differentiation of cells considered "live" (i.e., with undamaged membranes) from those considered "dead" (with damaged or compromised membranes). We tested whether this protocol was sensitive enough to detect membrane-level damage as induced by UVR, and found that after a 15-min exposure of a coastal sample, initially having 90% "live" cells, at 20 cm from a UVC light source (PHILIPS TUV30W/G30 T8), most cells (95%) were detected as "dead."

CARD-FISH. We basically followed the protocol described by Pernthaler et al. (42). Several horseradish peroxidase probes were used to characterize the composition of the microbial community in the original water samples: EUB 338-II-III (target most *Eubacteria*) (4, 11), ALF968 (targets most *Alphaproteobacteria*) (39), GAM42a (targets most *Gammaproteobacteria*) (33), CF319 (targets many groups belonging to the *Bacteroidetes* group) (32), ROS537 (targets members of the *Roseobacter-Sulfitobacter-Silicibacter* group) (14), and SAR11-441R (targets the SAR11 cluster) (37). The EUB antisense probe NON338 (54) was used as a negative control. All probes were purchased from Biomers.net (Ulm, Germany). Hybridizations were carried out at 35°C; overnight and specific hybridization conditions were established by addition of formamide to the hybridization buffers (20% formamide for NON338 probe, 45% formamide for ALF968 and SAR11-441R probes, and 55% for the other probes). Counterstaining of CARD-FISH preparations was done with 4,6-diamidino-2-phenylindole (DAPI, 1 $\mu\text{g ml}^{-1}$). Between 500 and 1,000 DAPI-positive-cells were counted in a minimum of 10 fields.

MAR-CARD-FISH. We followed the protocol described by Alonso and Pernthaler (3). Briefly, after sunlight exposure, 20 ml of water from duplicate treatments was incubated for 4 h at in situ temperature with the following tritiated substrates (0.5 nM final concentration): ³H-labeled mixture of amino acids (Amersham TRK440) for the summer experiment (experiment 1); [³H]leucine (Amersham TRK754) and [³H]ATP (Amersham TRK747) for the spring experiment (experiment 4). Single samples (for each compound and treatment) were killed with formaldehyde before the addition of the tritiated compounds and were used as controls. After the incubation, samples were fixed overnight with formaldehyde (1.8%) at 4°C and gently filtered on 0.2- μm polycarbonate filters (GTTP, 25-mm diameter; Millipore). The filters were then hybridized following the CARD-FISH protocol and subsequently glued onto glass slides with an epoxy adhesive (UHU plus; UHU GmbH, Bühl, Germany). For microautoradiography, the slides were embedded in 46°C tempered photographic emulsion (KODAK NTB-2) containing 0.1% agarose (gel strength, 1%; >1 kg cm⁻²) in a dark room. Slides were placed on an ice-cold metal bar for about 5 min to allow the emulsion to solidify and subsequently placed inside black boxes at 4°C until development. The optimal exposure time was determined for each experiment and compound and resulted in 7 days for the mixture of amino acids (experiment 1), 4 days for ATP, and 10 days for leucine (both in experiment 4). For development, we submerged the exposed slides for 3 min in the developer (KODAK D19), rinsed them for 30 s with distilled water, and placed them in fixer for 3 min (KODAK Tmax), followed by 5 min of washing with tap water. Slides were then dried in a desiccator overnight, stained with DAPI (1 $\mu\text{g ml}^{-1}$), and counted in an Olympus BX61 epifluorescence microscope.

RESULTS

Effects of sunlight on bulk and single-cell bacterial activity.

We performed three experiments to assess the impact of natural sunlight on bulk bacterial heterotrophic production (BHP) and single-cell bacterial activity during August 2003

TABLE 1. Physical data, bacterial abundance, leucine incorporation rates, and bacterial assemblage structure described as percentages of hybridized cells with specific probes by CARD-FISH in the in situ starting samples of each experiment^a

Expt no.	Date (day/mo/yr)	Depth (m)	Temp (°C)	ΔTemp (°C)	UV cum. (KJ m ⁻²)	BA (cells ml ⁻¹)	LIR (pM Leu h ⁻¹)	% of probe positive with respect to DAPI					
								Eub	Alph	Ros	SAR11	Gam	CFB
1	5/08/03	0.5	24	1	9.5 ^b	9.2 × 10 ⁵	41.3	79	25	2	30	10	10
2	7/08/03	5	23.6	5	9 ^b	1.3 × 10 ⁶	14.9	84	27	11	24	17	9
3	11/08/03	0.5	25.4	9	9.2 ^b	1.1 × 10 ⁶	38.2	86	28	11	24	26	20
4	13/04/05	0.5	13	0	5.4 ^c	7.3 × 10 ⁵	39.0	84	39	10	22	10	35

^a ΔTemp is the difference between the temperature at the surface (the depth sampled) and the bottom of the water column (20 m) and is used to describe the magnitude of water column stratification. UV cum., cumulative exposure during the experiment; BA, bacterial abundance; LIR, leucine incorporation rate; Eub, Eubacteria (EUB338-II-III); Alph, *Alphaproteobacteria* (Alf968); Ros, *Roseobacter* (Ros537); SAR11, SAR11 cluster (SAR11-441R); Gam, *Gammaproteobacteria* (Gam42a); CFB, *Bacteroidetes* (CF319a).

^b Integrated values during the experiments were determined with the multichannel filter radiometer.

^c Estimated from the irradiance values collected at the Malgrat de Mar meteorological station (see Materials and Methods).

(Table 1). During the course of the 6 days from experiment 1 to experiment 3, a cold water mass entered the system, resulting in a strong water column stratification. This intrusion was followed by changes in the in situ BHP and bacterial assemblage composition in surface waters (Table 1).

Exposure to sunlight generally inhibited bulk BHP compared to the dark treatment in all the experiments. However, we measured some variability in the response of bacteria to the different wavebands (Table 2). As an example, BHP inhibition after PAR plus UVA exposure was less marked compared to the other treatments in experiment 2 (i.e., PAR-only or full sunlight), although the difference was not significant (Tukey's test, $P > 0.05$). Remarkably, BHP was unaffected by full sunlight exposure in experiment 3 (whole-water fraction) (Table 2), although it was significantly inhibited by PAR plus UVA treatment.

When assessed at the single-cell level, UVR consistently had a significant negative effect on the percentage of actively respiring cells (CTC⁺ cells) (Fig. 1). The percentage of HNA-containing cells (% HNA) was also negatively affected by UVR in experiments 1 and 2, but we did not find significant differences in experiment 3 (Tukey's test, $P > 0.05$) (Fig. 1). The abundance of CTC⁺ and HNA cells after sunlight exposure decreased by an average (\pm standard error) of 54% \pm 10% and 25% \pm 4%, respectively, compared to the dark treatment, while the total bacterial abundance only decreased by 18% \pm 4% (Table 3).

TABLE 2. Bacterial heterotrophic production measured as leucine incorporation rates in three experiments and in each treatment

Treatment	Leucine incorporation rate on indicated date ^a			
	5 August 2003 (whole water)	7 August 2003 (whole water)	11 August 2003	
			Whole water	Filtered water
Dark	100*	100*	100*	100*
PAR	88 \pm 14*	17 \pm 7†	61 \pm 12*†	58 \pm 5†
PAR + UVA	66 \pm 12*	40 \pm 4†	46 \pm 11†	45 \pm 9†
PAR + UVR	52 \pm 11*	21 \pm 11†	106 \pm 4*	79 \pm 3‡

^a Values are expressed as percentages of the dark treatment (\pm standard error). Symbols refer to results with a post hoc Tukey's test ($P < 0.05$). Different symbols indicate significantly different treatment effects. The "filtered water" in the last column refers to water filtered through AP15 (bacterial fraction, see Materials and Methods).

Effects of sunlight on cell membrane integrity and recovery experiments. The effect of UVR on cell membrane integrity was assessed by the NADS protocol. We did not detect significant changes in the percentage of cells with intact membranes ("live" cells) in all treatments (Fig. 1).

The potential recovery of cells after exposure was assessed in experiment 3. We found that total, CTC, and HNA cell abundance recovered after 24 h in the darkness, reaching values in the samples that had been exposed to UVR similar to those from samples kept in the dark (Table 3).

Effects of primary producers on bacterial sensitivity to UVR. We tested whether the removal of phytoplankton was a relevant factor influencing bacterial sensitivity to UVR in experiment 3. The percentage of inhibition of BHP was similar in both samples (with and without primary producers) (Table 2). Similarly, the general effect of different solar wavebands on the percentage of "live" (as measured by the NADS protocol) and "active" (HNA and CTC⁺) cells was unaffected by the removal of phytoplankton (Fig. 1).

Differential sensitivity of dominant bacterial groups. Two experiments were conducted in different seasons to study the sensitivity of different phylogenetic groups of bacteria under relatively higher and lower levels of radiation (experiment 1 in summer and experiment 4 in spring, respectively). In the summer experiment, we found a significant reduction in activity of the broad phylogenetic group *Alphaproteobacteria* (70% inhibition in percentage of active cells in the uptake of amino acids) after sunlight exposure, while no significant reduction was observed in the activity of *Gammaproteobacteria* or *Bacteroidetes* (Fig. 2). However, the results with the *Bacteroidetes* group were not conclusive, since members of this group did not significantly take up the amino acid mixture.

In the spring experiment, we tested the effect of solar radiation (including UVA and UVB separately) on the uptake of two different substrates (leucine and ATP) (Fig. 3). The activity of the *Alphaproteobacteria* was significantly inhibited by both UVA and UVB wavebands (55% and 44% inhibition in percentage of active cells taking up leucine and ATP, respectively, after full sunlight exposure). *Gammaproteobacteria* showed higher inhibition at this time than in the summer experiment, but the inhibition was only attributable to the UVB waveband (30% and 46% inhibition in percentage of active cells in the uptake of leucine and ATP, respectively, after full sunlight exposure). The *Bacteroidetes*

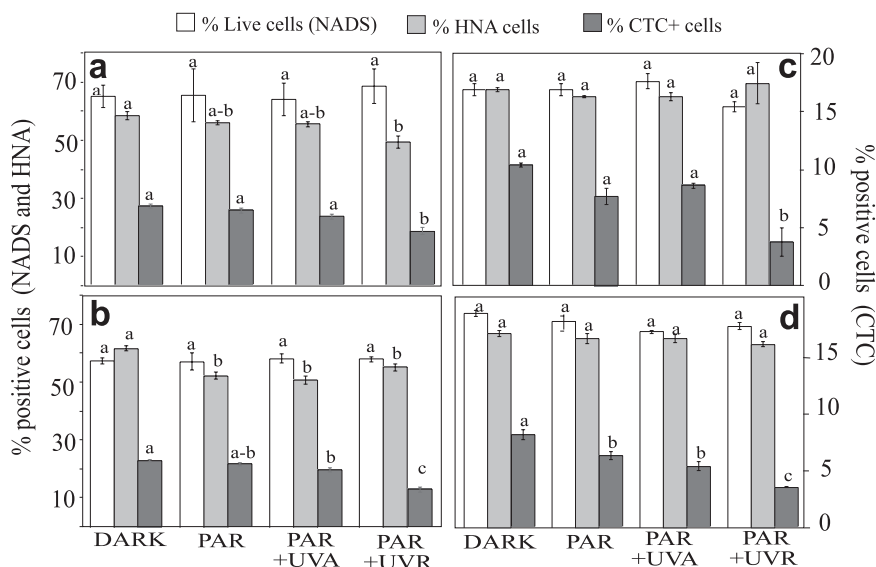


FIG. 1. Percentage of active cells measured as NADS green-positive cells (“live” cells), HNA content cells (HNA), and CTC-positive cells (cells actively respiring) in experiment 1 (5 August 2003) (a), experiment 2 (7 August 2003) (b), experiment 3 (11 August 2003) with unfiltered water (c), and experiment 3 with filtered water containing mostly the heterotrophic bacterial fraction (d).

showed high resistance to UVB radiation, being only partially affected by solar radiation for ATP uptake (Fig. 3a).

During the spring experiment, we also assessed the sensitivity of two more specific groups: SAR11 and *Roseobacter*. SAR11 were highly inhibited after full sunlight exposure (48% and 67% inhibition in percentage of active cells taking up leucine and ATP, respectively) although the response to the different wavebands was very different with the two different substrates assessed. Exposure to PAR inhibited the percentage of active cells in leucine uptake, while ATP uptake was only inhibited after full sunlight exposure.

Roseobacter showed lower sensitivity to UVR than the SAR11, and was only significantly inhibited after full sunlight exposure (34% and 49% inhibition in percentage of active cells in the uptake of leucine and ATP, respectively). Remarkably, we found that PAR significantly stimulated the activity of this group compared to the dark treatment (Fig. 3b).

DISCUSSION

Solar UVR has the potential to negatively affect bacterio-plankton in environments such as the surface layers of the

TABLE 3. Abundance of total bacteria, HNA bacteria, and CTC+ bacteria after 4-h exposure to the different treatments in the three experiments performed in August 2003^a

Expt date and treatment group	Abundance ± SE (10 ⁶ cells ml ⁻¹) of cell type:					
	Total		HNA		CTC ⁺	
	Treatment	Recovery	Treatment	Recovery	Treatment	Recovery
5 August 2003						
Dark	1.49 ± 0.02		0.87 ± 0.01		0.10 ± 0.003	
PAR	1.40 ± 0.01		0.78 ± 0.02		0.09 ± 0.002	
PAR + UVA	1.39 ± 0.005		0.77 ± 0.01		0.08 ± 0.003	
PAR + UVR	1.32 ± 0.03		0.65 ± 0.03		0.06 ± 0.004	
7 August 2003						
Dark	1.89 ± 0.05		1.18 ± 0.04		0.11 ± 0.004	
PAR	1.58 ± 0.02		0.83 ± 0.02		0.09 ± 0.001	
PAR + UVA	1.56 ± 0.02		0.80 ± 0.02		0.08 ± 0.002	
PAR + UVR	1.47 ± 0.01		0.82 ± 0.02		0.05 ± 0.004	
11 August 2003						
Dark	2.44 ± 0.03	3.28 ± 0.13	1.66 ± 0.04	2.49 ± 0.14	0.26 ± 0.005	0.44 ± 0.03
PAR	2.21 ± 0.03	3.03 ± 0.85	1.45 ± 0.02	2.19 ± 0.07	0.17 ± 0.01	0.37 ± 0.02
PAR + UVA	2.16 ± 0.02	3.43 ± 0.13	1.42 ± 0.04	2.60 ± 0.14	0.19 ± 0.005	0.53 ± 0.05
PAR + UVR	1.94 ± 0.17	4.14 ± 0.55	1.34 ± 0.02	3.29 ± 0.64	0.08 ± 0.03	0.44 ± 0.06

^a The values corresponding to the recovery experiment (i.e., 24-h incubation in the dark), carried out with water exposed to the different treatments from experiment 3 (11 August 2003), are also included.

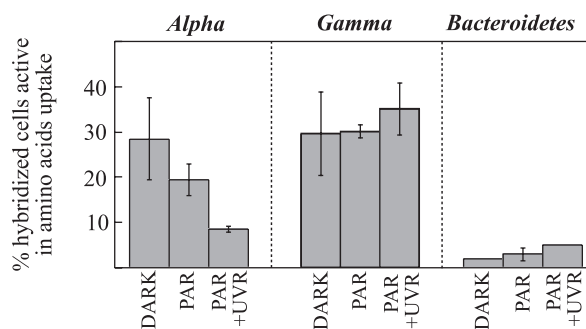


FIG. 2. Percentage of positively hybridized cells with probes for *Alphaproteobacteria* (Alpha, ALF968), *Gammaproteobacteria* (Gamma, GAM42a), and *Bacteroidetes* (CF319) taking up a mixture of ^3H -labeled amino acids (average \pm standard deviation) as measured by MAR-CARD-FISH during experiment 1 (summer experiment).

open ocean, where diurnal stratification of the water column is common (13), or in highly transparent shallow coastal areas, as our sampling site (Blanes Bay). At the time of the summer experiments, UVR penetrated the whole water column with 1% of incident UVR at 320 nm reaching the bottom (51).

We used an approach different from that used in previous studies elsewhere by focusing on the effects of sunlight at the single-cell level, including the differential sensitivity of the bacterial groups that dominate in situ. The most remarkable results found are that the effect of 4 h of exposure to UVR did not gravely damage the integrity of the cells but inhibited most markedly the physiologically active cells and that the inhibitory

effect was not the same for all bacterial groups. For example, *Alphaproteobacteria* (and the SAR11 cluster within this group) showed higher sensitivity than *Gammaproteobacteria* and *Bacteroidetes*. Considering that *Alphaproteobacteria* dominate the bacterial assemblage in Blanes Bay all year round (L. Alonso-Sáez, V. Balagué, E. L. Sà, O. Sánchez, J. M. González, J. Pinhassi, R. Massana, J. Pernthaler, C. Pedrós-Alió, and J. M. Gasol, submitted for publication), solar radiation could have a strong effect on C-cycling in this environment.

Effects of UVR on bulk bacterial heterotrophic production.

Similar to previous studies in other marine areas, our experiments provided variable results regarding the extent to which bulk BHP was affected by different wavebands of the solar spectrum. The main inhibitory effect on BHP has been assigned to UVA radiation (52) or UVB radiation (1, 38), but PAR has also been shown to significantly affect BHP (1, 52).

Differences in spectral sensitivity of bacteria, between and within experiments, have been assigned to environmental factors, such as nutrient status, dissolved organic matter concentration, presence and composition of the primary producers, and levels of incident solar radiation (24). We specifically tested the effect of excluding most primary producers from our samples, and in contrast to other studies (for an example, see reference 1), we did not find a clear change in bacterial sensitivity at either the bulk or single-cell level. The effect of algae on bacterioplankton activity could be related to the composition and sensitivity of the phytoplankton assemblage. High sensitivity to UVR can induce cell lysis (31), which would produce a short-term increase in dissolved organic carbon and stimulate the bacterial assemblage. In our summer experi-

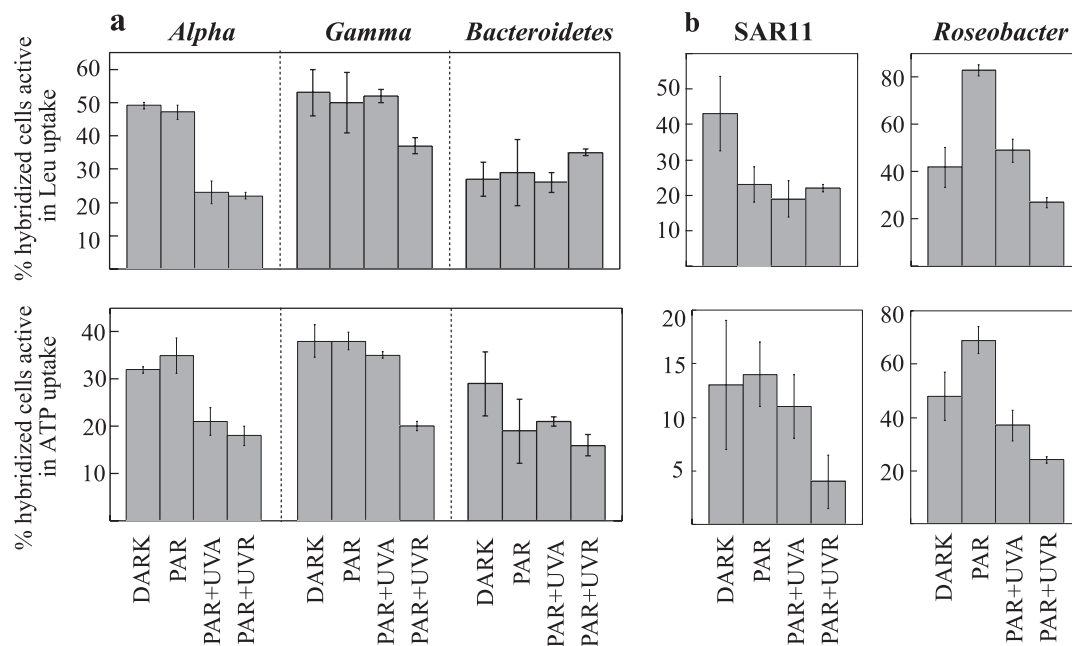


FIG. 3. Percentage of positively hybridized cells with probes for *Alphaproteobacteria* (Alpha, ALF968), *Gammaproteobacteria* (Gamma, GAM42a), and *Bacteroidetes* (CF319) groups (a) and *Roseobacter* (ROSS537) and SAR11 (SAR11-441R) groups (b) taking up ^3H leucine (upper panels) or ^3H ATP (lower panels) (averages \pm standard deviations), as measured by MAR-CARD-FISH during experiment 4 (spring experiment).

ments, the total phytoplankton assemblage was dominated by picophytoplankton (78% of total chlorophyll crossed a 3- μ m-pore-size filter), mainly by *Synechococcus*. This population has been shown to be resistant to UVR (31, 51), in agreement with the lack of effect of phytoplankton on bacterioplankton.

Since the methodology used for the three experiments was the same, the variability that we observed was probably due to environmental changes. Indeed, we observed the development of a strong stratification of the water column between the first and the last summer experiment, probably related to an intrusion of offshore deep water from a nearby submarine canyon. Water from the canyon periodically enters this site (35, 40) and can be enriched with particulate organic matter, since this submarine structure can act as a trap for suspended particles (20). Unfortunately, we did not sample particulate or dissolved organic matter (DOM) during these experiments, but changes in DOM supply can affect the response of the bacterial assemblage to different wavebands. For example, UVB radiation has been shown to increase the lability of DOM, particularly of organic matter originating from deep waters (9). This could be related to the significant increase in leucine uptake after full sunlight exposure in experiment 3. The shift in bacterial assemblage composition after stratification of the water column was also probably related to the different BHP sensitivity, since changes in assemblage structure can determine the sensitivity of a given community to solar radiation.

Effects of sunlight on the bacterial assemblage at the single-cell level. In contrast to bulk measurements, UVR showed a consistent negative effect on single-cell activity measurements. These measurements assessed changes in (i) CTC⁺ cells, which are usually regarded as highly active cells (45), and (ii) HNA-containing cells, which are regarded as active cells, at least in coastal environments (18, 30, 44). If we assume that the HNA and CTC-positive cells represent the "medium-to-high" active cell fractions, then UVR mainly affected the physiologically active cells. This was evident from the higher loss in the abundance of HNA and CTC-positive cells after full sunlight exposure than in the total bacterial counts (Table 3). In agreement with this idea, Arrieta et al. (6), working with isolates, found that the cells that were dividing faster (those taking up thymidine), were also those most inhibited by solar radiation.

We further assessed the effect of UVR on cell membrane integrity using the nucleic acid double-staining protocol, which uses a combination of propidium iodide and SYBR green I (21). Propidium iodide stains nucleic acids in red but only enters cells with compromised membranes and, thus, is thought to be an indicator of "dead" cells (55). We found that most bacteria maintained membrane integrity after exposure to natural sunlight (Fig. 1). Maranger et al. (34) used another exclusion stain (TOPRO-1) with lake samples and found a significant increase in the number of damaged cells after UVR exposure, but this was after several days of exposure.

The lack of structural damage after UVB exposure could be related to the subsequent ability of the cells to recover (Table 3). Similarly, rapid recovery of the cells after exposure to UVB radiation has been shown for marine natural assemblages (27) as well as for bacterial isolates (6).

Differential sensitivity of in situ dominating groups. Previous studies have reported large differences in the sensitivity to UVB radiation and in the mechanisms of recovery from pre-

vious UV stress for different marine isolates. Clear differences in the inhibition of thymidine and leucine incorporation have been shown among five isolates of the genus *Vibrio* (approximately 40%) compared to two gram-positive isolates (>80%) (6). More recently, large interspecific differences were found among 90 marine isolates from the same oceanographic area of our study (2). However, the results from these studies may not be representative of what occurs in situ, since bacterial isolates do not usually resemble natural bacterial communities (5).

Winter et al. (56) used a different approach to test the effect of UVR on the bacterial assemblage at the DNA and RNA levels (to discriminate the metabolically active cells) using denaturing gradient gel electrophoresis. These authors performed dilution cultures, preincubated in the dark for 20 to 30 h before the start of the experiment, and compared the bacterioplankton community prior to and after UVR exposure (up to 2 days). They concluded that UVR had little effect on bacterial assemblage structure in the North Sea. However, similar to the isolation techniques, dilution cultures can also introduce a bias in the resulting composition of the bacterial community (36) and may not necessarily represent the dominant bacterial groups in situ.

We used the MAR-CARD-FISH method to assess the differential sensitivity of bacterial groups as they occur in situ. We found some variability between seasons (spring and summer), probably due to seasonal changes in species composition of these broad phylogenetic groups. Selection for photoresistant species could occur in the periods of higher solar intensity, as seemed to be the case in the *Gammaproteobacteria* group, which showed lower sensitivity to UVR in summer (Fig. 2 and 3a).

We also found some differences in the response of the groups analyzed by the inhibition in the uptake of different tracers (leucine versus ATP). Solar UVR can mediate the destruction of the ectoenzymes and uptake systems (23), and the differences we found were probably related to differential damage of the uptake systems for the different substrates. Despite these differences, the general pattern (higher sensitivity to UVR of *Alpha*- than *Gammaproteobacteria* and *Bacteroidetes*) is in agreement with the results of Agogué et al. (2), working with bacterioneuston isolates from the Mediterranean Sea. These authors found that 43% of *Gammaproteobacteria* isolates showed high resistance to UV, while only 14% of *Alphaproteobacteria* were highly resistant isolates. However, opposite to our results, a low percentage of isolates belonging to the *Bacteroidetes* group were UV resistant in their study. The higher resistance of the *Gammaproteobacteria* group is also in agreement with results on the bacterial composition of the sea surface microlayer, which is dominated by two groups of *Gammaproteobacteria* (*Vibrio* and *Pseudoalteromonas* spp.) (15). This suggests that members of this group could be resistant to the high incident levels of solar UVR typically found in this habitat.

We also found differential sensitivities at a higher level of phylogenetic resolution (SAR11 and *Roseobacter* clusters). Both groups are dominant subgroups of *Alphaproteobacteria* in this environment, accounting for 22% of total DAPI-positive cells (annual average) (Alonso-Sáez et al., submitted). The higher sensitivity of SAR11 to UVR could be related to the high A+T content (69%) reported for the genome of a rep-

representative member of this group (*Pelagibacter ubique*) (19). However, it is remarkable that Agogué et al. (2) did not find a correlation between the degree of resistance of different bacterial isolates and their G+C content, as had been postulated by Singer and Ames (49).

Whereas the shortest wavelength of solar radiation (UVR) inhibits bacterial activity, natural sunlight can also act as an energy source for some bacteria. The remarkable enhancement in the activity of the *Roseobacter* group after exposure to PAR, compared to the dark (Fig. 3b), could be related with the ability of these bacteria to derive energy from light with the use of bacteriochlorophyll *a* (46, 47). It is well known that some cultured members of the *Roseobacter* group are aerobic anoxygenic phototrophs. Recent reports of abundant aerobic anoxygenic phototrophs (29) and the discovery of proteorhodopsin (8) suggest that non-chlorophyll *a*-dependent phototrophy may be widespread among marine bacteria. However, Schwalbach et al. (43) performed light manipulation experiments with bacterial communities from the California coast and found that members of broad phylogenetic groups typical of marine waters, including *Roseobacter*, did not respond to light exclusion. These authors used a fingerprint technique (automated ribosomal intergenic spacer analysis) to determine changes in bacterial assemblage structure after 10 days of incubation under different light regimens. Clearly, this approach is significantly different from our *in situ* activity measurements (MAR-CARD-FISH) and could explain the differences between both studies.

The large differences in sensitivity found, even among broad phylogenetic groups (as *Alpha*- and *Gammaproteobacteria* and *Bacteroidetes*), can act as a significant determinant of bacterial assemblage structure at high UVR doses. During our summer experiments, the rapid stratification of the water column (within days) was followed by significant changes in bacterial assemblage structure. The groups *Gammaproteobacteria* and *Bacteroidetes* significantly increased their proportions in surface waters after stratification (Table 1). Interestingly, these two groups had shown high resistance to UVR in the experiments, suggesting that a selection for UV-resistant groups could occur.

The differential sensitivity to UVR of *in situ* dominating bacterial groups could be a relevant factor determining their relative biogeochemical role in UV-sensitive oceanic regions. Different groups of bacteria have shown remarkably different activities and patterns of DOM utilization (10). *Alphaproteobacteria* seem to be responsible for a large part of low-molecular-weight DOM uptake, while *Bacteroidetes* seem to be more specialized in high-molecular-weight DOM uptake (10). The reported higher UVR inactivation of *Alphaproteobacteria* could lead to temporary shifts in the dominant pathways of DOM use by bacteria as well as decreases in total DOM uptake and processing, given the dominance of *Alphaproteobacteria*, and SAR11 specifically, in oceanic waters (37). However, several other aspects not assessed in our study, such as the effects of UVR on DOM quality or on the release of DOM by primary producers (for an example, see reference 23), must also be considered to draw a comprehensive picture of the role of UVR on bacterial carbon processing.

ACKNOWLEDGMENTS

We thank Maria Vila, Rafel Simó, and Doris Slezak, who participated in the summer UV experiments, and Clara Cardelús, who helped with organization. We particularly thank Fabien Joux for giving us the Mylar and Courtguard foils used in the spring experiment, Françoise Auger for helping with the MAR-FISH analysis, and José M. González for valuable comments that helped improve the manuscript.

This is a contribution of the Microbial Observatory of Blanes Bay. Research was supported by Spanish research grants GEN μ MAR (CTM2004-02586/MAR) and MODIVUS (CTM2005-04795/MAR), EU project BASICS (EVK3-CT-2002-00078), the EU Network of Excellence MARBEF (FP6-2002-Global-1) to J.M.G., Austrian Science Fund (FWF) project 141513, and ÖAD project no. 17 to R.S. Financial support was also provided by a Ph.D. fellowship from the Spanish government to L.A.-S.

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