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# Biochemical characteristics and bacterial community structure of the sea surface microlayer in the South Pacific Ocean

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## Abstract

The chemical and biological characteristics of the surface microlayer were determined during a transect across the South Pacific Ocean in October-December 2004. Concentrations of particulate organic carbon (1.3 to 7.6-fold) and nitrogen (1.4 to 7), and POC:PON ratios were consistently higher in the surface microlayer as compared to subsurface waters (5 m). The large variability in particulate organic matter enrichment was negatively correlated to wind speed. No enhanced concentrations of dissolved organic carbon were detectable in the surface microlayer as compared to 5 m, but chromophoric dissolved organic matter was markedly enriched (by 2 to 4-fold) at all sites. Based on pigment analysis and cell counts, no consistent enrichment of any of the major components of the autotrophic and heterotrophic microbial community was detectable. CE-SSCP fingerprints and CARD FISH revealed that the bacterial communities present in the surface microlayer had close similarity (>76%) to those in subsurface waters. By contrast, bacterial heterotrophic production (3H-leucine incorporation) was consistently lower in the surface microlayer than in subsurface waters. By applying CARD-FISH and microautoradiography, we observed that *Bacteroidetes* and *Gammaproteobacteria* dominated leucine uptake in the surface microlayer, while in subsurface waters *Bacteroidetes* and *Alphaproteobacteria* were the major groups accounting for leucine incorporation. Our results demonstrate that the microbial community in the surface microlayer closely resembles that of the surface waters of the open ocean. However, even short time periods in the surface microlayer result in differences in bacterial groups accounting for leucine incorporation, probably as a response to the differences in the physical and chemical nature of the two layers.

## 1 Introduction

The air-water interface is a site of dynamic exchange processes of gaseous, liquid and particulate matter between the atmosphere and aquatic environments (Liss et al.,

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1997). The aquatic boundary layer, comprised of a series of sub-layers that are generally summarized as the sea surface microlayer (1–1000  $\mu\text{m}$ ), plays a pivotal role in these transfer processes. The enrichment of the surface microlayer in organic and inorganic matter affects physical features of the air-sea interface such as the surface tension, and thus air-sea fluxes (Frew, 1997). The potential contribution of biological activity in the surface microlayer to air-water exchange processes is far less known. The partial pressure of  $\text{CO}_2$  in the top layer (2 cm) of the ocean was recently reported to be linked to microbial community metabolism (Calleja et al., 2005). The consistently enhanced rates of respiration in samples collected from the surface microlayer in different coastal and offshore marine environments could support this notion (Garabétian, 1990; Obernosterer et al., 2005; Reinthaler et al., 2007). The biogenic gases dimethylsulfoxide (DMSO) and dimethylsulfoniopropionate (DMSP) were markedly enriched in the surface microlayer as compared to subsurface waters during calm wind conditions (Zemmelink et al., 2005). These observations could indicate that important biological processes at the air-water interface differ from those in subsurface waters, however, their impact on air-sea fluxes remains to be determined.

Whether organisms inhabiting the surface microlayer act as sources or sinks for various components will strongly depend on the abundance and activity of the different members of the microbial community. Reports on these biological features display large variability among studies, with enrichments, depletions or no differences observed (Agogué et al., 2004, and references therein). This is most likely due to the spatial and temporal variability in the development of the surface microlayer, but the utilization of different sampling devices that collect layers of varying thickness could also account for the differences observed. Another important issue to be addressed is the specificity of the autotrophic and heterotrophic community inhabiting the surface microlayer. Phytoneuston has long been viewed as a unique group of organisms inhabiting the surface film of aquatic environments (see review by Zaitsev, 1997), but this idea has recently been challenged. Based on the tight relationship between the abundance of different groups of the plankton community in subsurface waters and that in

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the surface microlayer, the upward transport of organisms by physical processes rather than the in situ development has been suggested the source for their presence in the surface microlayer (Joux et al., 2006).

Only recently have molecular tools allowed to addressing the question whether heterotrophic bacteria inhabiting the surface microlayer display a specific community structure. The only two studies available up to date reveal contrasting conclusions (Agogué et al., 2004; Franklin et al., 2005). Based on different sampling techniques and molecular approaches, the bacterial community structure associated with the surface microlayer was found to be similar (Agogué et al., 2004) and distinctly different (Franklin et al., 2005) to that from subsurface waters.

The main objective of the present study was to evaluate by two complementary molecular-based techniques whether the top layer of the ocean is inhabited by a distinct bacterioneuston community. Our aim was to describe the bacterial community structure in relation to major biological and chemical features that characterized the surface microlayer in contrasting marine environments across the South Pacific Ocean.

## 2 Material and methods

### 2.1 Study sites

The BIOSOPE (Biogeochemistry and Optics South Pacific Experiment) cruise track crossed the South Pacific Ocean from the Marquise Islands to Chile (Claustre et al., 2007<sup>1</sup>). The surface microlayer was sampled at 6 stations ranging from oligotrophic to ultraoligotrophic in the center of the South Pacific Gyre (Fig. 1). The sampled sites were located in the vicinity of the Marquise Islands (Station MAR), in High-Nutrient-Low-Chlorophyll waters east of the Marquise Islands (Station HNL), in the center of

<sup>1</sup>Claustre, H., Sciandra, A., and Vaultot, D.: Introduction to the special section: bio-optical and biogeochemical conditions in the South East Pacific in late 2004 – the BIOSOPE cruise, Biogeosciences Discuss., in preparation, 2007.

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the South Pacific Gyre (Station GYR), in the eastern part of the South Pacific Gyre (Station EGY), and in the western (STB3) and eastern (STB17) part of the South Pacific Ocean (Fig. 1, Table 1). Wind speed was recorded every 10 s. Sea surface microlayer collection was done during calm wind conditions ( $<3 \text{ m s}^{-1}$ ) and the mean wind speed 6h prior to sampling varied between 0.5 and  $2.7 \text{ m s}^{-1}$  at the different stations.

## 2.2 Surface microlayer sampling

We used a metal screen (Garrett, 1965) to collect the surface microlayer (250–400  $\mu\text{m}$ ) as described previously (Obernosterer et al., 2005). Sampling was done from a dinghy, roughly 0.5 nautical miles upwind from the research vessel. To prevent the collection of water adhering to the frame, the first 100 ml of water draining from the metal screen were rejected. Prior to collection, the metal screen was rinsed several times with the respective sample water. Subsurface water was collected at 5 m using a 5-l Niskin bottle. Samples collected in the surface microlayer and in subsurface waters were transferred to 9-l polycarbonate (PC) carboys. Prior to sampling, the PC carboys were rinsed with 1 N HCl, Milli-Q water and 3 times with the respective water samples.

## 2.3 Particulate organic carbon (POC) and nitrogen (PON) and dissolved organic carbon (DOC)

For POC-analysis, duplicate 700–1000-ml samples were filtered onto combusted (450°C for 5h) Whatman GF/F filters. The filters were rinsed with ~2 ml of Milli-Q water to remove salts, then stored frozen ( $-20^\circ\text{C}$ ) until analysis. POC and PON measurements were performed on a 2400 Perkin Elmer CHN analyzer. The error for duplicate samples of POC and PON was, on average, 4% and 12%, respectively.

For DOC-analysis, GF/F filtered samples were preserved by adding 100  $\mu\text{l}$   $\text{H}_3\text{PO}_4$  (35%) to 20 ml subsamples. The samples were stored in combusted glass vials with Teflon-lined screw caps in the dark at  $4^\circ\text{C}$  until analyzed. DOC measurements were performed on a Shimadzu TOC-V-CSH (Benner and Strom, 1993). Prior to injection,

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DOC samples were sparged with CO<sub>2</sub>-free air for 6 min to remove inorganic carbon. Hundred  $\mu$ l of sample were injected in triplicate and the analytical precision was ~2%. Standards were prepared with acetanilid. The error for duplicate DOC samples was, on average, 1%.

## 5 2.4 Chromophoric Dissolved Organic Matter (cDOM)

The absorption coefficients of cDOM were measured throughout the ultraviolet-visible domain (280–735 nm) using the multiple pathlength, liquid core waveguide system UltraPath (WPI Inc.), with a pathlength of 2 m. Samples were filtered immediately after collection, in dim light, using 0.2  $\mu$ m Millipore filters pre-rinsed with Milli-Q water. Filtered samples were then placed in the automatic sampler (maintained in the dark), and pumped into the sample cell of the Ultrath instrument. Absorbance spectra were measured with reference to a salt solution (35 PSU), prepared with High Pressure Liquid Chromatographie (HPLC)-quality water and granular NaCl, to match the salinity and refractive index of samples. Between measurements, the cell was flushed successively with diluted detergent, high reagent grade MeOH, 2 M HCl, and Milli-Q water, and the cleanliness of the tube was controlled using a reference value for the transmittance of the reference water. As the absorption coefficients of pure water vary with temperature (especially in the infra-red), we minimized the temperature differences between the reference and the sample. The presence of microbubbles in the sample cell was also avoided by using a peristaltic pump and a debubbler.

## 2.5 Pigment analysis

Three to six liters of seawater were filtered onto GF/F filters (25 mm diameter) and the filters were stored in liquid nitrogen until HPLC-analysis according to Van Heukelem and Thomas (2001). A detailed description of the pigment extraction and analysis is

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given in Ras et al. (2007)<sup>2</sup>.

## 2.6 Enumeration of heterotrophic bacteria, autotrophic prokaryotic and eukaryotic cells by flow cytometry

Three-ml subsamples were fixed with 2% formaldehyde (final conc.), stored for 30 min at 4°C, then frozen in liquid nitrogen and stored at -80°C until analysis. For flow cytometric analysis of heterotrophic bacteria, samples were stained with the nucleic acid dye SYBR Green-I (Molecular Probes) at 0.025% (vol/vol) final concentration (Lebaron et al., 2001). Counts were performed on a FACS-Calibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488 nm wavelength, 15 mW Argon laser. Stained bacteria were excited at 488 nm wavelength and discriminated according to their right angle-light scatter (SSC, related to cell size) and green fluorescence at 530±15 nm wavelength. Based on a plot of green versus red fluorescence we distinguished photosynthetic from non-photosynthetic prokaryotes. Enumeration of autotrophic cells was performed according to Marie et al. (2000). *Synechococcus* spp. was discriminated by its strong orange fluorescence (585±21 nm) and pico- and nanoeukaryotes were discriminated by their scatter signals of the red fluorescence (>670 nm). The coefficient of variation among replicate samples is generally <5% (Agogué et al., 2004). To convert bacterial abundance to bacterial biomass we applied a conversion factor of 12.4 fg C cell<sup>-1</sup> (Fukuda et al., 1998).

## 2.7 Enumeration of nanoflagellates

Plastidic and heterotrophic nanoflagellates were determined after staining with Primulin according to Sherr et al. (1993). Twenty to one hundred ml of raw seawater were fixed with 2% formaldehyde (final conc.) and filtered onto 0.8 µm black PC membrane filters

<sup>2</sup>Ras, J., Claustre, H., and Uitz, J.: Spatial variability of phytoplankton pigment distributions in the Subtropical South Pacific Ocean: comparison between in situ and modelled data, Biogeosciences Discuss., in preparation, 2007.

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(25 mm filter diameter). The filter was subsequently rinsed with two 1-ml aliquots of 0.1 M Trizma HCl (pH 4.0). The filter was covered with Primulin (250 g ml<sup>-1</sup>, 0.1 M Trizma-HCl) and incubated in the dark for 15 min. After removing the Primulin solution by filtration, the filter was rinsed again with 0.1 M Trizma HCl, before being mounted  
5 onto a glass slide. Heterotrophic and autotrophic nanoflagellates were subsequently visualized using UV and blue light excitation, respectively, using an epifluorescence microscope (x 1000, Zeiss, Axiovert Mot plus).

## 2.8 Bacterial heterotrophic production

Bacterial production was determined by [<sup>3</sup>H] leucine incorporation applying the cen-  
10 trifugation method (Smith and Azam, 1992). Briefly, 1.5 ml samples were incubated with a mixture of [4,5-<sup>3</sup>H]leucine (Amersham, specific activity 160 Ci mmol<sup>-1</sup>) and non-radioactive leucine at final concentrations of 7 and 13 nM, respectively. Samples were incubated in the dark at the respective in situ temperatures for 2–3 h. Linearity of leucine incorporation over this time period was tested at the three stations. Incubations  
15 were terminated by the addition of trichloroacetic acid (TCA, Sigma) to a final concentration of 5%. To facilitate the precipitation of proteins, bovine serum albumine (BSA, Sigma, 100 mg l<sup>-1</sup> final concentration) was added prior to centrifugation at 16 000 g for 10 min (Van Wambeke et al., 2002). After discarding the supernatant, 1.5 ml of 5% TCA were added and the samples were subsequently vigorously shaken on a vortex  
20 and centrifuged again. The supernatant was discarded, 1.5 ml of PCS liquid scintillation cocktail (Amersham) added and the radioactivity incorporated into bacterial cells was counted aboard with a Packard LS 1600 Liquid Scintillation Counter. The error associated with the variability between replicate measurements (i.e. difference between two replicates/2) was on average 10%.

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## 2.9 Catalyzed reporter deposition – fluorescence in situ hybridization and microautoradiography (MICRO-CARD-FISH)

At three stations (MAR, GYR and STB17) the bacterial community structure and the activity of the major bacterial groups was determined applying MICRO-CARD-FISH.

5 Raw seawater samples (50 ml) were incubated with [4,5-<sup>3</sup>H]leucine (Amersham, specific activity 160 Ci mmol<sup>-1</sup>) at a final concentration of 20 nM in the dark at in situ temperature for 2–4 h. The incubation was terminated by adding paraformaldehyde (PFA, 2% final concentration), and controls were fixed with PFA (2% final concentration) prior to incubation with <sup>3</sup>H-leucine. Samples were stored at 4°C in the dark for  
10 12h before filtration onto 0.2 μm PC filters (25 mm filter diameter, Nuclepore). The filters were stored at –20°C until treated by CARD-FISH using the protocols described in Pernthaler et al. (2002) and Sekar et al. (2003). Filters were embedded in low-melting-point agarose (0.2% final concentration), dried, dehydrated (96% EtOH, 1 min) and treated with lysozyme (Fluka, 10 mg ml<sup>-1</sup>, 100 mM Tris [pH 8], 50 mM EDTA) for 1h  
15 at 37 °C to allow cell wall permeabilization. The filters were subsequently washed in Milli-Q water and dehydrated in ethanol (96%) for 1 min.

We determined the relative contribution of the major bacterial groups using the probes ALF968 (5'-GGT AAG GTT CTG CGC GTT-3') for *Alphaproteobacteria*, GAM42a (5'-GCC TTC CCA CAT CGT TT-3') for *Gammaproteobacteria*, and CFB319a  
20 (5'-TGG TCC GTG TCT CAG TAC-3') for *Bacteroidetes*, and the probe EUB338 (5'-GCT GCC TCC CGT AGG AGT-3') for the identification of *Bacteria*. The negative control (NON338, 5'-ACT CCT ACG GGA GGC AGC-3') was used to determine non-specific binding. Probe working solution was added at a final concentration of 2.5 ng μl<sup>-1</sup> and the hybridization was done at 35°C for 2 h.

25 For the microautoradiographic development, we followed the protocol described by Cottrell and Kirchman (2000). Briefly, the previously hybridized filter sections were placed onto slides coated with photographic emulsion (type NTB-2; Kodak, diluted 1:1 with Milli-Q water). The photographic emulsion was heated at 43°C for 30–60 min be-

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fore utilization. The slides were then dried (for 15 min) on an ice-cold aluminum plate and kept in a dark box during exposure at 4°C. The slides were developed for 2 min (Dektol developer [1:1 dilution with Milli-Q water], Kodak), rinsed with Milli-Q water for 10 s, fixed for 6 min (Fixer Tmax, Kodak) and again rinsed for 6 min in Milli-Q water.

5 After having dried the slides in a desiccator for 12 h, filter sections were peeled off and the cells were counterstained with a DAPI mix (4 parts Citifluor, 1 part Vectashield (Vector Laboratories) with DAPI at a final concentration of  $0.5 \mu\text{g ml}^{-1}$ ). To determine the appropriate exposure time for samples collected in the surface microlayer and sub-surface waters, slides were developed after 12 h, 24 h, 36 h, 48 h, 72 h and 96 h at  
10 Stations GYR and STB17. At both stations, the percent of DAPI-stained cells associated with silver grains increased during the first 48 h and remained stable thereafter. We subsequently used an exposure time of 48 h. The slides were examined under a Olympus AX70 epifluorescence microscope and an image analysis system as described in Cottrell and Kirchman (2003). For each slide, 20 fields were enumerated,  
15 resulting in a minimum of 500 DAPI-stained cell counts. Mean values and the standard errors among the cell counts of 20 fields are presented. To determine the contribution of different bacterial groups to leucine incorporation, the number of probe-positive cells associated with silver grains was divided by the number of DAPI-stained cells associated with silver grains.

## 20 2.10 Capillary Electrophoresis – Single Strand Conformation Polymorphism (CE-SSCP)

At all stations CE-SSCP was performed on DNA extracts to obtain molecular fingerprints of the surface microlayer and subsurface waters. Seawater samples ( $<3 \mu\text{m}$  size fraction, 0.5 l) were filtered onto  $0.2 \mu\text{m}$  PC filters (47 mm, Nuclepore) and filters  
25 were kept frozen ( $-80^\circ\text{C}$ ) until nucleic acid extraction. Nucleic Acid extraction was performed as described previously (Ghiglione et al., 2005). Primers used for polymeric chain reaction (PCR) were the specific bacterial primers w49dir (5' –A CGG TCC AGA CTC CTA CGG G– 3'; Delbès et al., 2000) and w34rev (5' –TTA CCG CGG CTG CTG

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GCA C– 3'; Lee et al., 1996) synthesized commercially (Eurogentec). Primer w34 was fluorescently 5'-labelled with phosphoramidite (TET, Eurogentec). Primers were designed to target a short sequence (around 200 pb), allowing a good resolution of the CE-SSCP signal (V3 region of 16SrDNA, *Escherichia coli* positions 329–533, Brosius et al., 1981). Amplifications were performed in 50  $\mu$ l, with 1  $\mu$ l of DNA, 0.3  $\mu$ M of each primer, 0.8 mM dNTPs, and 1X buffer (Promega), before adding 1.0 U of *pfu* polymerase (Promega). Samples were amplified (Robocycler 96, Stratagene) with the following program: 94°C (3 min) for denaturation, 25 cycles at 94°C (denaturation, 30 s), 61°C (annealing, 25 s), 72°C (extension, 30 s), before a final extension at 72°C (10 min). The amplicon size was checked on agarose gel (2%), and the PCR products were purified using a PCR purification kit (Qiagen).

### 2.10.1 CE-SSCP electrophoresis

CE-SSCP was performed according to a previously described protocol (Ghiglione et al., 2005) on the Genetic Analyser ABI310 (Applied Biosystems). Briefly, each sample was diluted between 2- and 40-fold in sterile Tris-borate-EDTA (TBE)(10 mM Tris, 1 mM EDTA) to obtain 10 ng  $\mu$ l<sup>-1</sup> of PCR product. From this resulting dilution, 1  $\mu$ l of PCR product was mixed with 0.1  $\mu$ l of an internal size standard (GeneScan-400 Rox, Applied Biosystems) and 18.9  $\mu$ l of deionised formamide (Applera), before heating (94°C, 10 min) and cooling in a water/ice bath (10 min). Samples were electrokinetically injected (5 s, 12 kV) into a capillary (47 cm×50  $\mu$ m) filled with 5.6% GeneScan polymer (Applied Biosystems) gel containing 10% autoclaved glycerol in sterile TBE buffer (90 mM Tris-borate, 2 mM EDTA [pH 8.0]). Samples migrated for 30 min (15 kV, 30°C). Phosphoramidite (TET)-labelled fragments were detected by a laser with a virtual filter C (detection wavelengths 532, 537 and 584 nm). Data collection was performed with the ABI Prism 310 collection software (Applied Biosystems).

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## 2.10.2 CE-SSCP fingerprints analysis

Electrophoregrams were aligned with the GeneScan analysis software by fixing positions of the internal standard (GeneScan-400 Rox, Applied Biosystems) with a second-order least-square curve (i.e. linear regression). Peak detection on environmental fingerprints was realized using the first derivative of a polynomial curve fitted to the data within a window that was centred on each data point (GeneScan analysis software). Peaks overlapping were observable on the obtained fingerprints, to avoid artefacts in peak enumeration or in peak area estimation we used a high polynomial degree (10) to increase peak sensitivity. The peak amplitude threshold was fixed at 50 and applied for both Rox and TET fluorescent dyes.

## 3 Results

### 3.1 Particulate and dissolved organic matter

The surface microlayer was markedly enriched in particulate organic matter, by factors varying between 1.3 and 7.6 for POC, and between 1.4 and 7 for PON (Table 2). The variability in particulate organic matter enrichment was largely explained by wind history. The ratio of POC in the surface microlayer to that of subsurface waters (i.e. the enrichment factor) was negatively correlated to the mean wind speed 6 h prior to sampling ( $r^2=0.97$ ,  $n=6$ , Fig. 2). This was also the case for the PON enrichment factor ( $r^2=0.93$ ,  $n=6$ ). No relationship between the concentration of particulate organic matter in the surface microlayer and in subsurface waters was detectable. C/N ratios of particulate organic matter were by 1.1 to 1.6-fold higher in the surface microlayer than at 5 m, indicating preferential accumulation of carbon-rich organic matter. Concentrations of dissolved organic carbon did not display any particulate pattern (Table 2). By contrast, cDOM was enriched in the surface microlayer by 2-fold at Stations MAR, HNL, STB3 and GYR, and by 4-fold at Station EGY (Table 2).

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## 3.2 Autotrophic and heterotrophic components of the sea surface microlayer

The components of the microbial community determined in the present study did not exhibit any consistent pattern in terms of biomass or abundance in the surface microlayer as compared to subsurface waters (Fig. 3). Concentrations of chlorophyll *a* in the surface microlayer exceeded those at 5 m at Stations MAR and EGY by factors of 1.2 and 1.5, respectively. At Stations HNL, GYR and STB17 concentrations of chlorophyll *a* in the surface microlayer were similar or depleted compared to those at 5 m (Table 3). The group specific pigments chlorophyll *b* (Chlorophytes) and chlorophyll *c* (Chromophytes) revealed a similar trend as chlorophyll *a* with pronounced enrichments only at Station EGY (by 2.8- and 1.9-fold, respectively). Concentrations of divinyl chlorophyll *a* indicated an enrichment of *Prochlorococcus* in the surface microlayer at Stations MAR (by 1.8-fold) and EGY (by 1.3-fold), similar to that of *Synechococcus* based on cell abundances (by 1.2- and 1.4-fold, respectively)(Tables 3 and 4). Pico- and nanoeukaryotes displayed similar abundances in the surface microlayer and in subsurface waters. Plastidic and heterotrophic nanoflagellates were more abundant (by 1.2- to 4-fold) in the surface microlayer in 3 out of 5 surface microlayer samplings. Phaeopigments were below the limit of detection in the surface microlayer and in subsurface waters of all stations. No marked enrichment of heterotrophic bacterial abundances was detectable in the surface microlayer (mean 1.04, range 1.01–1.08), except for Station EGY where abundances exceeded those at 5 m by 1.3-fold (Table 4).

## 3.3 Bacterial community structure

The CE-SSCP fingerprints revealed overall close similarity (>76%) of the bacterial community structure between the surface microlayer and subsurface waters (Fig. 4). The Euclidian distance dendrogram is based on the comparison of the presence or absence of peaks (thereafter referred to as ribotype), and the relative peak height among the CE-SSCP fingerprints. Minor, but noticeable differences between the surface microlayer and subsurface waters were detectable at Stations EGY and STB17, owing to

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the presence of two distinct ribotypes in the surface microlayer. At Station HNL, a few minor ribotypes distinguished the CE-SSCP profile from the surface microlayer to that of subsurface waters.

At Stations MAR, GYR and STB17 we compared the relative contribution of major bacterial groups in the surface microlayer and subsurface waters, using in situ hybridization. The percent DAPI-stained cells identified by the general probe EUB varied between 19–54% and 45–65% in the surface microlayer and in subsurface waters, respectively (Fig. 5). The generally lower percentage of DAPI-stained cells identified as *Eubacteria* in the surface microlayer, most pronounced at Station MAR, probably reflects the lower heterotrophic bacterial activity in this layer as compared to subsurface waters (see below). The sum of the relative contributions of *Alpha*-, and *Gammaproteobacteria* and *Bacteroidetes* roughly equaled (mean±SD, 99±14%) the percent EUB positive cells. Overall, no differences in the relative contribution of the major bacterial groups were detectable between the surface microlayer and subsurface waters (Fig. 5). At Station MAR, *Alpha*-, and *Gammaproteobacteria* and *Bacteroidetes* had similar relative contributions in both layers. At Station GYR *Bacteroidetes* were the dominant phylogenetic group (33 to 39% of DAPI-stained cells), and at STB17 *Bacteroidetes* (16 to 19% of DAPI-stained cells) and *Gammaproteobacteria* (20 to 22% of DAPI-stained cells) dominated in terms of abundance the heterotrophic bacterial community.

### 3.4 Contribution of major bacterial groups to bulk leucine incorporation

By contrast to the heterotrophic bacterial abundance, distinct differences in bacterial heterotrophic production, as determined by <sup>3</sup>H-leucine incorporation, were detectable between the two layers. Bacterial leucine incorporation in the surface microlayer accounted for 5% to 80% (n=6) of that at 5 m and the inhibition of bacterial heterotrophic production in the surface microlayer was most pronounced at low wind speeds (Fig. 6). An exception to this pattern was Station HNL, where leucine incorporation in the surface microlayer was enhanced by 20% as compared to subsurface waters. Only a small fraction of DAPI-stained cells incorporated leucine in the surface microlayer (4–13%)

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in comparison to subsurface waters (19–33%), further indicating the inhibition of the bacterial activity in the surface microlayer.

Different bacterial groups were responsible for bulk leucine incorporation in the surface microlayer as compared to subsurface waters at Stations GYR and STB17 (Fig. 7).

5 At Station GYR, *Bacteroidetes* clearly dominated leucine uptake in the surface microlayer (43% of DAPI-stained cells associated with silver grains), while in subsurface waters both *Alphaproteobacteria* and *Bacteroidetes* (26% and 36%, respectively, of DAPI-stained cells associated with silver grains) had similar contributions to leucine uptake. At Station STB17, *Gammaproteobacteria* and *Bacteroidetes* dominated leucine  
10 uptake in the surface microlayer (28% and 25% respectively, of DAPI-stained cells associated with silver grains), while the three bacterial groups studied contributed equally to leucine uptake in subsurface waters (13% to 16% of DAPI-stained cells associated with silver grains)(Fig. 7).

## 4 Discussion

15 The thickness of the surface microlayer sampled, and thus its definition for a given study, depends on the sampling device applied. The metal screen we used in the present study collects the upper 250–440  $\mu\text{m}$  water layer (Garrett 1965), while the layers collected by the glass plate (Harvey and Burzell 1972) and the rotating drum (Harvey 1966) are thinner (60–100  $\mu\text{m}$ ). For specific microbiological studies membranes  
20 are used to collect the upper 1 to 40  $\mu\text{m}$  (Kjelleberg et al., 1979). These differences are mainly due to the physical mechanisms to sample the uppermost layer of aquatic systems, with some devices being more selective than others. Based on a large data set of chemical (Momzikoff et al., 2004) and biological (Agogu e et al., 2004) parameters collected both with the metal screen and the glass plate in the coastal Mediterranean  
25 Sea, no significant differences in the enrichment factors between the two samplers were observed. Kuznetsova et al. (2004) report a more efficient collection of surface microlayer dissolved and particulate material by the screen than by the drum, despite

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the thinner layer sampled by the latter. These studies demonstrate that the collection of the surface microlayer to study the parameters considered herein are most likely not biased due to dilution with subsurface waters, despite the larger thickness of the layer sampled. Our choice of the screen sampler is driven by the efficient, non-selective sampling of a relatively large water volume for chemical and biological analyses.

The marked accumulation of particulate organic matter at the sea surface observed in the present study was not accompanied by any pronounced enrichment in photo- or heterotrophic organisms. This suggests that detrital particles, including colloidal aggregates, transparent exopolymeric particles (TEP), and submicron particles most likely constitute an important fraction of the surface microlayer biofilm. The higher C:N ratios of particulate organic matter observed in the present and previous studies (Nishizawa, 1971; Taguchi and Nakajima, 1971) further support this notion. The enrichment in particulate organic matter was strongly controlled by wind history, but independent of particulate organic matter concentrations in subsurface waters, suggesting that physical processes, such as turbulent mixing, transport by rising bubbles or buoyant particles were important for the surface microlayer formation. The most pronounced accumulation of particulate organic matter was observed at Station EGY where calm wind conditions ( $\leq 2 \text{ m s}^{-1}$ ) prevailed over roughly 18 h prior to surface microlayer collection. It was interesting to note that at this site the C:N ratio of particulate organic matter in the surface microlayer was similar to that in subsurface waters (Table 2). Station EGY was the only site where a pronounced enrichment in cell abundance and biomass of autotrophic and heterotrophic organisms was observed (Tables 3 and 4). These observations could indicate the transport of fresh material to the ocean surface, but they could also reflect photo- and heterotrophic biomass production in the surface microlayer when calm wind conditions exist over an extended time period. The latter idea is, however, not supported by results from the present study, as the inhibition in bacterial heterotrophic production in the surface microlayer was most pronounced at calm sea conditions (Fig. 6). This suggests that wind-induced physical processes determine not only the amount, but to some extent also the characteristics of the particulate organic

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matter that accumulates at the air-water interface.

The chemical characteristics of surface microlayer dissolved organic matter (DOM) appear to contrast the observations on particulate organic matter. Recent studies performed in the open Atlantic and the western Mediterranean Sea report a pronounced enrichment in dissolved free amino acids and a substantially lower C:N ratio in the DOM from the surface microlayer as compared to that from subsurface waters (Kuznetsova et al., 2004; Reinthaler et al., 2007<sup>??</sup>). Interestingly, Reinthaler et al. (2007<sup>??</sup>) concluded that the bioreactivity of the surface microlayer DOM was low, despite the high contribution of amino acids to DOC and DON. Selective accumulation, and also in situ production were both suggested to account for the enrichment in amino acids in the surface microlayer (Kuznetsova et al., 2004; Reinthaler et al., 2007<sup>??</sup>). The consistently higher concentrations of chromophoric DOM (cDOM) in the surface microlayer observed in the present and previous studies (Carlson, 1983) are clearly indicative for the selective accumulation of DOM at the air-water interface. Removal of cDOM by photomineralization and photobleaching is most likely rapid in the surface microlayer, the observation that cDOM is consistently enriched strongly suggests the continuous supply from bulk seawater by selective scavenging.

In the present study, bacterial heterotrophic production was clearly reduced in the surface microlayer as compared to subsurface waters. Previous studies support (Sieburth et al., 1976; Carlucci et al., 1986) or contrast (Dietz et al., 1976; Bell and Albright, 1982; Bailey et al., 1983; Williams et al., 1986; Agogué et al., 2004; Reinthaler et al., 2007<sup>??</sup>) this observation. A more consistent pattern among studies is observed on rates of bacterial (<0.8  $\mu\text{m}$  fraction) or microbial community respiration in the surface microlayer, generally exceeding those in subsurface waters by several-fold (Garabétian, 1990; Obernosterer et al., 2005; Reinthaler et al., 2007<sup>??</sup>). These contrasting responses most likely reflect the different characteristics of the surface microlayer. The enrichment in organic and inorganic matter renders the surface microlayer a potentially favorable habitat for heterotrophic bacteria, at the same time it is exposed

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to drastic changes in temperature, pH, salinity and also to high intensities of solar radiation, in particular ultraviolet radiation. Based on the short incubation time, bacterial heterotrophic production provides an instantaneous response to the conditions in the surface microlayer. By contrast, respiration measurements require 12h-24h incubations in the dark and they more likely reflect the potential of organic matter originating from the surface microlayer in sustaining bacterial metabolism.

Bacterial growth rates could further indicate the activity of the bacterial community in the surface microlayer. Bacterial growth rates are commonly calculated on the basis of total bacterial cell abundance even though several independent approaches have shown that only a fraction of the bacterial community can be considered active (Gasol et al., 1999; Lebaron et al., 2001; Zubkov et al., 2001). The microautoradiographic observations performed in the present study allowed us to estimate the fraction of active cells, accounting for roughly 10% and 25% of DAPI-stained cells in the surface microlayer and subsurface waters, respectively. Assuming that this percentage represents the active part of the bacterial community, growth rates in the surface microlayer ( $0.6 \text{ d}^{-1}$ ) exceeded those in subsurface waters ( $0.3 \text{ d}^{-1}$ ) by a factor of two. Could this indicate that only a few bacterial groups are active in the surface microlayer accounting for most of the bacterial heterotrophic production in this particular environment?

Our results from the fingerprints and the in situ hybridization show that the surface microlayer is not inhabited by a particular bacterioneuston community. The use of the two complementary approaches, a PCR-based (i.e. CE-SSCP) and a PCR-independent method (i.e. CARD-FISH), strongly support this conclusion. Given the low bacterial growth rates determined in the present study, and the relatively short time period over which the surface microlayer persists, an in situ development of a specific bacterioneuston community is unlikely. Physical processes are mainly responsible for the formation of the surface microlayer, with upward transport of particulate material being a predominant process. The minor differences that we observed in the fingerprints between the two layers is most likely owing to the selective enrichment of the surface microlayer by specific ribotypes. Likely candidates are bacteria attached to

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particles that are transported to the air-sea interface. The structure of the bacterial community attached to particles in the water column differs from that of free-living bacteria (Acinas et al., 1999; Riemann and Winding, 2001; Ghiglione et al., 2007) and *Bacteroidetes* were found to have an important contribution to the bacterial communities associated with aggregates (DeLong et al., 1993; Ploug et al., 1999; Simon et al., 2002). Marine aerosol particles have also been observed to be enriched in bacteria, and their transport in the atmosphere has been suggested a potentially important dispersal mechanism (Aller et al., 2005).

Our conclusion on the close similarity between the bacterial community structure in the surface microlayer and subsurface waters is supported by results from samples collected in the same way from the coastal Mediterranean Sea (Agogué et al., 2004), but they contrast the conclusion of a study conducted in the coastal North Sea (Franklin et al., 2005). These authors utilized hydrophilic membranes to collect surface microlayer bacteria, and based on 16S rRNA clone libraries they observed a lower bacterial diversity in the surface microlayer as compared to subsurface waters (Franklin et al., 2005). The potentially selective adsorption of bacteria onto hydrophilic membranes as discussed in Agogué et al. (2004), could at least partly account for the different conclusions obtained by these studies.

In contrast to the similarity between the two layers in terms of community structure and abundance of major bacterial groups, differences in the composition of the active community were detectable. This was particularly pronounced at Station STB17 where *Bacteroidetes* and *Gammaproteobacteria* dominated the active community in the surface microlayer, while the three bacterial groups investigated contributed equally to the active community in subsurface waters (Fig. 7). Even though these bacterial groups contain each a diverse assemblage of sub-groups, they have been attributed specific characteristics, based on different experimental approaches. There is for instance increasing evidence that members of the *Bacteroidetes* group play an important role in the degradation of complex polymeric substances (Cottrell and Kirchman, 2000; Kirchman, 2002; Cottrell et al., 2005). *Gammaproteobacteria* are suggested

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“opportunistic” due to the ability of several members belonging to this group to rapidly respond to nutrient enrichment (Pinhassi and Berman. 2003). Pronounced growth of *Gammaproteobacteria* was detectable in nitrogen-amended incubations at Station GYR (Van Wambeke et al., 2007). The dominance of *Bacteroidetes* and *Gammaproteobacteria* in the active community in the surface microlayer could reflect the rapid response of members of these bacterial groups to changes in the growth conditions, such as the enrichment and composition of organic and inorganic matter. These results suggest that even short time periods in the surface microlayer result in differences in bacterial groups accounting for leucine incorporation, probably as a response to the differences in the physical and chemical nature of the two layers.

In the open ocean, the organic matter accumulating at the air-sea interface originates predominantly from phytoplankton primary production. The vertical flux of phytoplankton-derived organic carbon to the ocean surface has, to the best of our knowledge, never been evaluated. The potential importance of an upward flux of organic matter has been illustrated by studies on buoyant particles. TEP-rich microaggregates, for example, have been reported to ascend at velocities as fast as  $0.1\text{--}1\text{ m d}^{-1}$  to the ocean surface (Azetsu-Scott and Passow, 2004). The accumulation of organic matter at the sea surface impacts its physical structure, thus the gas transfer rates. The biological and photochemical mineralization of surface microlayer organic matter could have further important consequences for the air-sea gas exchange (Calleja et al., 2005; Obernosterer et al., 2005). To better understand and determine these latter processes, clearly novel techniques allowing in situ measurements of biological and photochemical fluxes at the air-water interface are required.

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**Table 1.** Date and local time of the day of the surface microlayer collection and general characteristics of the study sites. Phytoplankton biomass (chl *a*) is integrated over the euphotic zone. Mean wind speed for 6 h prior to the surface microlayer collection is given. *Ze* – depth of the euphotic zone.

Station	Date	Time of the Day	<i>Ze</i> (m)	chl <i>a</i> (mg m <sup>-2</sup> ) <sup>1</sup>	Wind speed (m s <sup>-1</sup> )
MAR	29/10/2004	10:00–11:00	78	21	2.6
HNL	01/11/2004	13:30–14:30	90	17	2.6
STB3	05/11/2004	15:00–16:00	134	16	1.9
GYR-2	12/11/2004	12:00–13:00	160	11	1.9
GYR-4	14/11/2004	13:30–14:30	142	7	2.0
EGY	28/11/2004	09:00–10:30	94	15	0.5
STB17	01/12/2004	13:30–14:30	96	15	1.4

<sup>1</sup> Data are from Ras et al. (2007)<sup>2</sup>.

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**Table 2.** Concentration of particulate organic carbon (POC) and nitrogen (PON), dissolved organic carbon (DOC) and chromophoric dissolved organic matter (cDOM) in the sea surface microlayer (SML) and in sub-surface waters (SSW). n.d.- not determined.

		MAR	HNL	STB3	GYR-2	EGY	STB17
POC ( $\mu\text{M}$ )	SML	10.3	11.5	14.8	7.3	25.7	23.0
	SSW	5.4	8.8	4.0	2.8	3.4	4.7
PON ( $\mu\text{M}$ )	SML	1.0	1.0	1.2	0.5	4.2	2.8
	SSW	0.7	1.0	0.5	0.3	0.6	0.8
POC:PON	SML	10.8	11.5	12.7	14.6	6.2	8.3
	SSW	7.6	8.5	7.9	8.4	5.2	6.0
DOC ( $\mu\text{M}$ )	SML	80	85	94	81	105	88
	SSW	85	92	88	78	99	88
cDOM ( $\text{Abs}_{350\text{nm}} \text{m}^{-1}$ )	SML	0.072	0.097	0.051	0.036	0.073	n.d.
	SSW	0.037	0.050	0.027	0.016	0.014	n.d.

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**Table 3.** Concentrations of chlorophyll *a* (chl *a*), *b* (chl *b*), *c* (chl *c*) and Divinyl chl *a* in the sea surface microlayer (SML) and subsurface waters (SSW). BD – below limit of detection.

		MAR	HNL	GYR-4	EGY	STB17
chl <i>a</i> ( $\mu\text{g l}^{-1}$ )	SML	0.201	0.075	0.013	0.095	0.12
	SSW	0.168	0.128	0.021	0.064	0.11
chl <i>b</i> ( $\mu\text{g l}^{-1}$ )	SML	0.017	0.011	0.001	0.011	0.124
	SSW	0.017	0.020	0.001	0.004	0.110
chl <i>c</i> ( $\mu\text{g l}^{-1}$ )	SML	0.047	0.003	0.001	0.015	0.024
	SSW	0.033	0.016	0.002	0.008	0.023
Divinyl chl <i>a</i> ( $\mu\text{g l}^{-1}$ )	SML	0.048	0.042	0.003	0.010	BD
	SSW	0.027	0.049	0.004	0.008	BD

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**Table 4.** Abundance of the major components of the microbial community in the sea surface microlayer (SML) and subsurface waters (SSW). n.d. – not determined; b.d. – below limit of detection. \* – data are based on flow cytometric analysis; \*\* – data are based on microscopic observations.

		MAR	HNL	STB3	GYR-2	GYR-4	EGY	STB17
<i>Synechococcus</i> spp.* ( $\times 10^7 \text{ l}^{-1}$ )	SML	5.8	1.2	0.3	0.1	0.02	0.7	0.2
	SSW	4.9	1.4	0.3	0.1	0.04	0.5	0.2
Pico/Nanoeukaryotes* ( $\times 10^6 \text{ l}^{-1}$ )	SML	3.9	5.1	0.8	0.5	0.4	3.0	3.9
	SSW	3.6	5.7	1.4	0.6	0.4	2.6	3.7
Plastidic Nanoflagellates** ( $\times 10^5 \text{ l}^{-1}$ )	SML	19.9	21.5	5.3	0.4	0.4	n.d.	n.d.
	SSW	16.6	22.2	3.5	0.1	0.6	n.d.	n.d.
Heterotrophic bacteria* ( $\times 10^8 \text{ l}^{-1}$ )	SML	9.6	8.9	6.5	3.8	3.6	9.0	8.4
	SSW	9.4	8.7	6.1	3.7	3.5	7.0	7.8
Heterotrophic nanoflagellates** ( $\times 10^5 \text{ l}^{-1}$ )	SML	7.6	5.4	1.1	1.8	2.4	n.d.	n.d.
	SSW	4.2	2.7	1.7	B.D.	2.4	n.d.	n.d.

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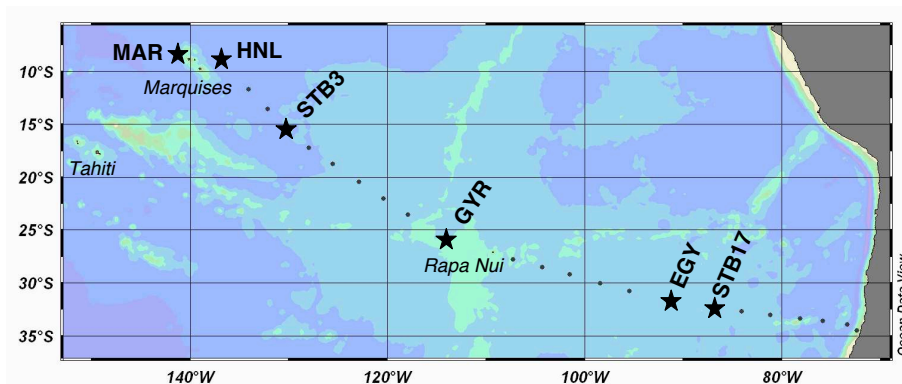
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**Fig. 1.** Cruise track and sampled stations of the BIOSOPE (Biogeochemistry and Optics South Pacific Ocean Experiment) project. Stations where the sea surface microlayer was collected are indicated by stars.

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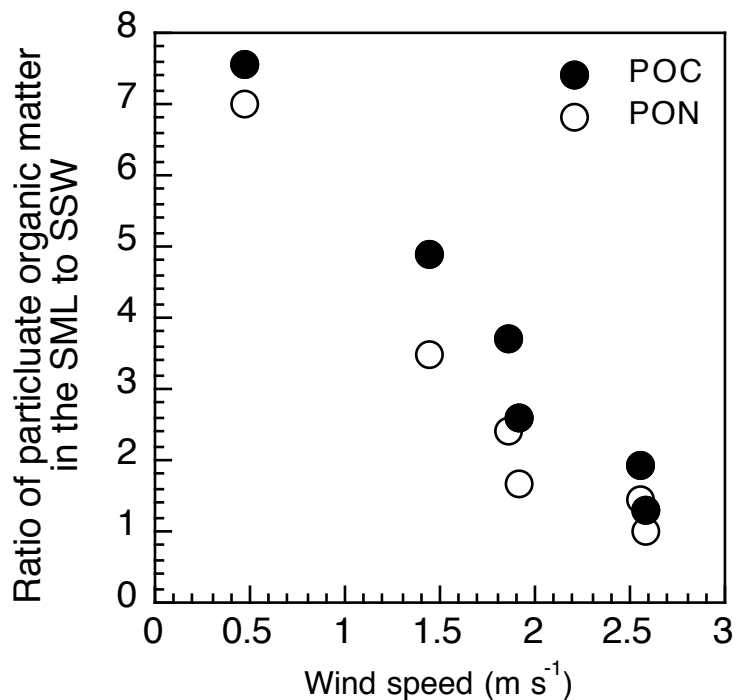
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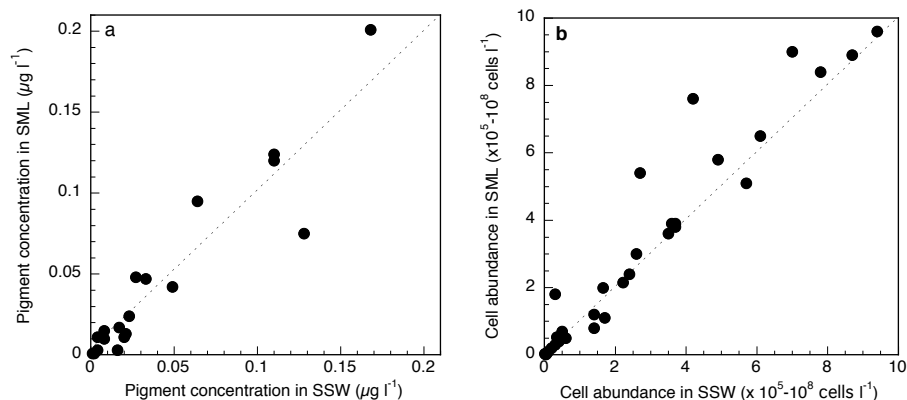
**Fig. 2.** Relation between the ratio of particulate organic carbon (POC) and nitrogen (PON) in the surface microlayer (SML) to that in subsurface water (SSW) and the mean wind speed 6 h prior to sampling.

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**Fig. 3.** Relation between (a) pigment concentration and (b) the abundance of autotrophic and heterotrophic cells in the surface microlayer (SML) and in subsurface waters (SSW). Data of all pigment analyses and all cell counts are pooled. See Tables 3 and 4 for individual pigment analysis and cell abundances, respectively. Dashed line indicates the 1:1 line.

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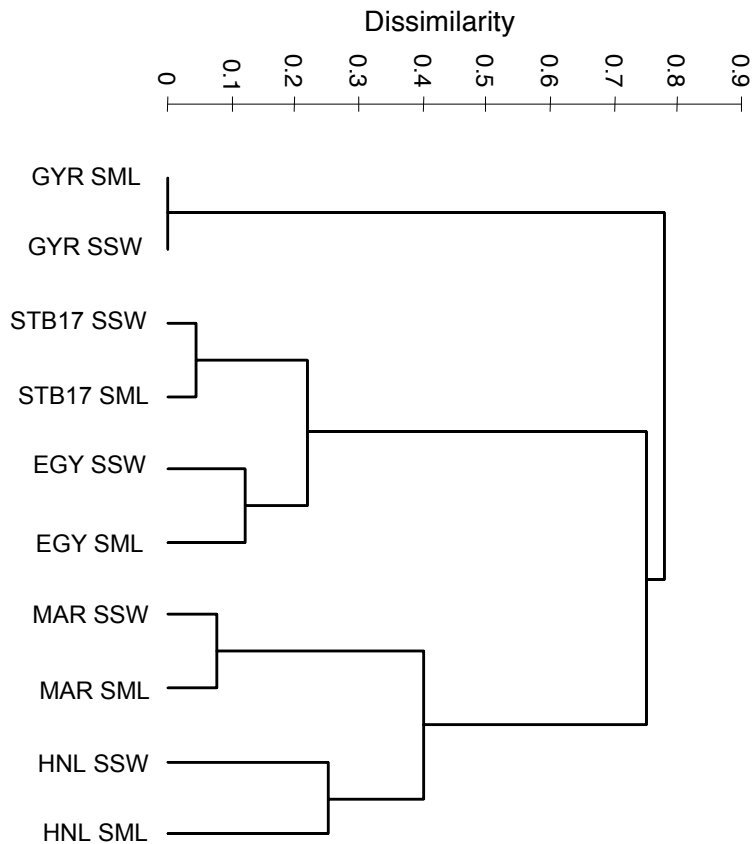
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**Fig. 4.** UPGMA Euclidian distance dendrogram generated from the CE-SSCP profiles from the surface microlayer (SML) and subsurface waters (SSW). Scale bar indicates the Euclidean distance.

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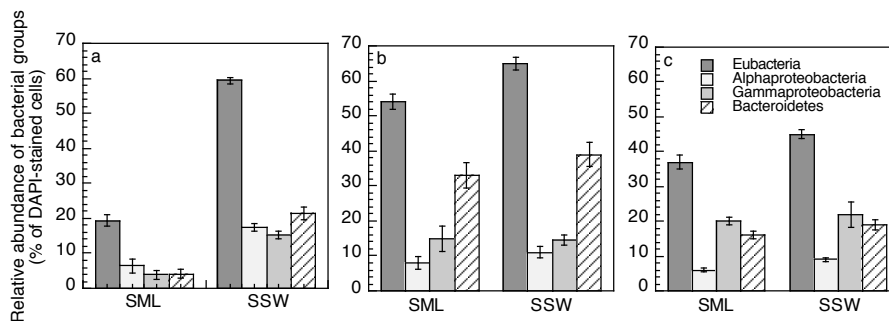
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**Fig. 5.** Relative contribution of *Eubacteria* and major bacterial groups to total DAPI-stained cells in the surface microlayer (SML) and in subsurface waters (SSW) at Stations MAR **(a)**, GYR **(b)** and STB17 **(c)**. The control probe NON was on average 2% of DAPI-stained cells. Mean values  $\pm$  SE are given.

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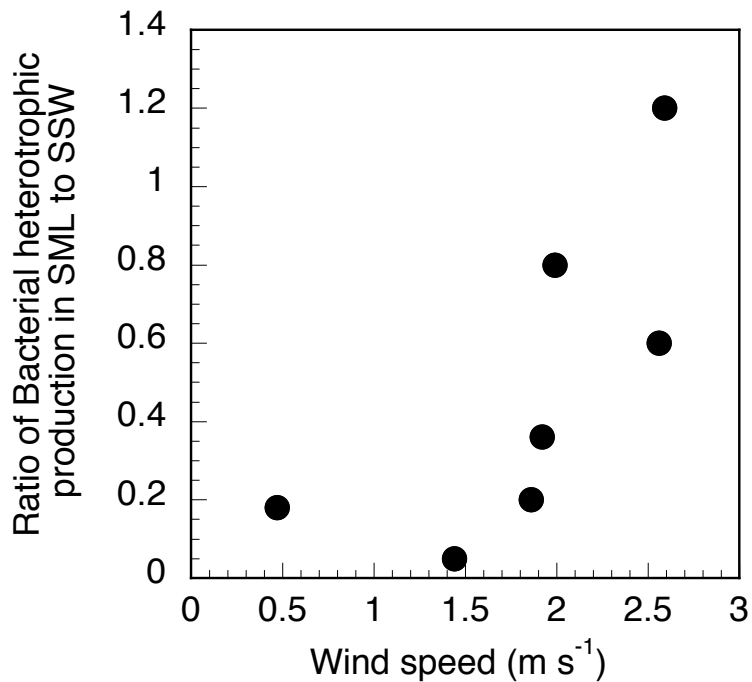
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**Fig. 6.** Relation between the ratio of bacterial heterotrophic production in the surface microlayer (SML) to subsurface waters (SSW) and the mean wind speed 6 h prior to sampling.

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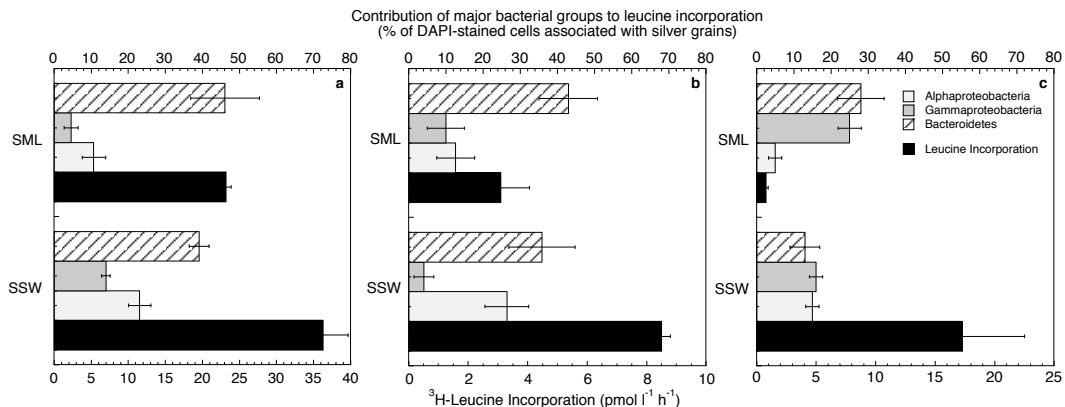
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**Fig. 7.** Bulk  $^3\text{H}$ -leucine incorporation and relative contribution of bacterial groups to leucine-incorporation in the surface microlayer (SML) and in subsurface waters (SSW) at Stations MAR (a), GYR (b) and STB17 (c). For  $^3\text{H}$ -leucine incorporation, mean values  $\pm$  variation of duplicate incubations, and for the relative contribution of bacterial groups mean values  $\pm$  SE are given.

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