RESEARCH LETTER

Diversity of total and active free-living vs. particle-attached bacteria in the euphotic zone of the NW Mediterranean Sea

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Abstract

The structure of the total and metabolically active communities of attached and free-living bacteria were analysed in the euphotic zone in the NW Mediterranean Sea with the use of DNA- and RNA-derived capillary electrophoresis single-strand conformation polymorphism fingerprinting. More than half (between 52% and 69%) of the DNA-derived operational taxonomic units (OTUs) were common in both attached and free-living fractions in the euphotic layer, suggesting an exchange or co-occurrence between them. However, analysis targeting 16S rRNA showed that only some of them were found in the dominant active bacterial pool. Especially at the deep chlorophyll maximum, less than half of the attached bacterial populations were found to be active, with regard to the high proportion of OTUs present at the DNA level, but not at the RNA level. These results suggest that even if colonization on and detachment of particles appear to be ubiquitous, most of the particulate organic carbon remineralization appeared to be mediated by a rather low number of dominant active OTUs specialized in exploiting such specific microenvironment.

Introduction

Bacterioplankton play a central role in the biological cycle of carbon through the microbial loop (Azam *et al.*, 1983). In the upper ocean, bacteria are abundant, achieving densities of around 10^6 mL^{-1} and they consume on an average 20–50% of the daily primary production (PP) (Ducklow *et al.*, 1999). Even if heterotrophic bacterioplankton mostly use dissolved organic carbon (DOC) as direct carbon supply, a significant part of the microbial activity may occur on or in the vicinity of particles. Particulate organic carbon (POC) and, more precisely, 'phycospheres' (Bell *et al.*, 1974) and 'detritospheres' (Biddanda & Pomeroy, 1988) represent micropatches of concentrated substrates that may be used as 'microniches' or hot-spots for pelagic bacterial processes. Differences were found in cell abundance, morphology, and metabolic activity between free-living and particle-attached bacteria. In most pelagic environments, attached bacteria are less abundant than free-living bacteria (Alldredge & Gotschalk, 1990; Turley & Stutt, 2000). They are generally bigger and more active on a per-cell basis than free-living bacteria, presumably due to the more favourable nutritive conditions than those in the surrounding water (reviewed by Simon *et al.*, 2002).

The contribution of the attached fraction to the total bacterial activity is highly variable and depends mainly on the concentration of attached bacteria and on the quantity and the quality of the suspended particles. In the NW Mediterranean Sea, the percentage of attached to total bacterial activity is usually $< 30\%$ during summer stratification and could be much higher under some conditions, reaching $> 80\%$ at the deep chlorophyll maximum (DCM) under mesotrophic conditions (Ghiglione *et al.*, 2007). Hydrolytic enzyme activities of attached bacteria may help liberate DOC from the particles, thereby attracting other chemotactic bacteria (Long & Azam, 2001). Because different bacteria express different arrays of hydrolytic enzymatic activities (Martinez *et al.*, 1996), changes in the bacterial species composition may alter the bacterial hydrolytic activity on organic particles. Contrasting results were found when comparing bacterial diversity of attached and free-living communities. Comparison of attached and free-living bacterial 16S rDNA gene clone libraries showed distinct phylogenetic differences between the attached and free-living bacteria in estuarine (Crump *et al.*, 1999), coastal (DeLong *et al.*, 1993) and offshore areas (Acinas *et al.*, 1999). On the other hand, most of the attached operational

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taxonomic units (OTUs) were also found in the free-living fraction by Hollibaugh *et al.* (2000) in San Francisco Bay and by Ghiglione *et al.* (2007) in NW Mediterranean Sea. Moeseneder *et al.* (2001) also reported an overlap of 21–47% between attached and free-living OTUs in the Aegean Sea (Eastern Mediterranean Sea).

Metabolically active bacteria contain more rRNA than resting or starved cells and 16S rRNA-based fingerprinting has been suggested as an indicator of the metabolically active bacterial community structure (Kemp *et al.*, 1993; Poulsen *et al.*, 1993). In the present study, we used an integrated approach combining DNA- and RNA-derived capillary electrophoresis single-strand conformation polymorphism (CE-SSCP) in order to obtain insights into the structure of the total and metabolically active communities of attached and free-living bacteria in the productive layer (0–150 m) during summer stratification in the NW Mediterranean Sea. The main objectives of this study were (1) to determine whether the diversity (by means of OTU) of total vs. metabolically active attached and free-living bacteria in NW Mediterranean Sea were substantially different, and (2) to compare these communities at different depths in the productive layer during summer stratification.

Materials and methods

Study site and sampling

Seawater was sampled from the NW Mediterranean Sea in July 2004 at the Microbial Observatory of Laboratoire Arago (MOLA) (42°28′300N, 03°15′500E, maximum depth of 1000 m). Samples were collected with a rosette of 5-L Niskin bottles attached to a Sea-Bird conductivity–temperature–-depth profiler deployed from the oceanographic *RV Tethys II* at depths between surface and 150 m (i.e. 10, 30, 50, 80, 100, and 150 m) according to the water mass stratification and the fluorescence profile.

Nutrients, chlorophyll *a*, PP, POC and DOC

Samples for nutrients were collected into polyethylene flasks in duplicate, immediately poisoned with $HgCl_2$ ($10\,\mu g L^{-1}$) and stored at 4 °C in the dark until further analyses on a Bran Luebbe III autoanalyser according to Tréguer & LeCorre (1975) Measurement accuracy was ± 0.05, ± 0.05, ± 0.02, and $\pm 0.02\,\mu M$ for nitrates, silicates, ammonium, nitrites and phosphates, respectively.

PP was determined using the ^{14}C-tracer technique (Steemann-Nielsen, 1952) modified by Fitzwater *et al.* (1982). Samples were incubated under simulated *in situ* conditions using nickel screen for 24 h in a deck incubator cooled by surface seawater. For each simulated depth, measurements were made in triplicate and an additional sample was incubated in dark in order to estimate dark carbon fixation. Total carbonate concentration was calculated according to Parsons *et al.* (1992) and carbon assimilation rates were calculated according to Platt & Sathyendranath (1993).

For chlorophyll *a* and POC measurement, samples (600 mL) were filtered on precombusted glass fibre filters (Whatman GF/F, 25 mm, 450 °C, 12 h) and frozen (liquid nitrogen) for chlorophyll and dried at 60 °C and stored in a dessicator for POC until further analysis. POC was determined by dry combustion on a CHN 2400 Perkin Elmer analyser and chlorophyll *a* was estimated in triplicate according to Yentsch & Menzel (1963) with analytical accuracy of $\pm 0.03\,\mu g\,L^{-1}$ for chlorophyll *a*.

For the DOC measurement, duplicate filtered samples (two Whatman GF/F, 25 mm, precombusted at 450 °C for 12 h) were collected in 20-mL precombusted glass tubes (450 °C, 12 h) with a screw cap and a Teflon liner (thoroughly washed in HCl 0.5 N and rinsed with milliQ water). Samples were poisoned with orthophosphoric acid (H_3PO_4) and stored at room temperature until analysis by high-temperature catalytic oxidation (Sugimura & Suzuki, 1988; Cauwet, 1994) on a Shimadzu TOC-V analyser. Typical analytical precision is ± 0.1–0.5 (SD) or 0.2–1% (CV). Deep Sargasso Sea reference water ($47\,mol\,L^{-1}$ C ± 0.5 SE; http://www.rsmas.miami.edu/groups/biogeochem/CRM.html) was injected every 10 samples to insure stable operating conditions.

Bacterial abundance

Triplicate 2-mL samples of prefiltered seawater onto 0.8-μm-pore-size filters (47 mm, ATTP, polycarbonate nuclepore) (free-living fraction) and without prefiltration (total fraction) were fixed with 2% formaldehyde for at least 1 h at 4 °C before quick freezing in liquid nitrogen and stored at −80 °C until analysis (within 2 weeks). The samples were later thawed at room temperature, stained with SYBR Green I [final concentration 0.01% (v/v) of the commercial solution; Molecular Probes Inc.] for at least 15 min at 20 °C in the dark. For total bacterial count, the subsamples were sonicated (306 μm amplitude, 50% duty cycle, 2 min, cooling in water bath) with a Sonifier 250 (Branson Ultrasonics Corp., Danburry, CO) before SYBR Green I staining in order to disperse the bacteria from the particles (Velji & Albright, 1993). Sonication procedure followed by flow cytometry analysis has been previously shown to be as efficient as microscopy approach to estimate the total number of bacteria, including the particle-attached fraction (Riemann & Winding, 2001; Worm *et al.*, 2001; Mével *et al.*, 2008). Under conditions similar to this study, microscopic observations showed that free-living bacteria retained on the 0.8-μm filters represented $< 5\%$ of the > 0.8-μm fraction, suggesting that overestimation of attached bacteria was very low (Mével *et al.*, 2008). Bacterial counts were determined using an FACS Calibur flow cytometer equipped with a 488 nm, 15 mW argon laser

(Becton Dickinson, San Jose, CA), as described previously (Lebaron *et al.*, 1998). Stained cells were enumerated according to their side-angle-scattered light (SSC) and green cell fluorescence (FL1) collected through a 530 ± 30-nm bandpass filter. Fluorescent beads (1.0 μm; Polysciences Inc., Warrington, PA) were added to each sample analysed to normalize SSC and green fluorescence. The calibrated flow rate was controlled daily by measuring weights before and after 5 min of analysis at the same flow rate settled for the samples, but with 1 mL of milliQ water. Attached bacterial abundance was calculated by subtracting the free-living bacterial counts (with 0.8-μm-pore-size filtration) from the total bacterial counts (sonication and without prefiltration). In the same cytograms, two main bacterioplankton groups can be discriminated: bacteria with high nucleic acid (HNA) content (with high FL1 value) and bacteria with low nucleic acid (LNA) content (with low FL1 value). The SSC/FL1 cytogram for each sample was individually inspected, and the HNA and LNA regions manually moved to conform to the appropriate areas of the bacterial dot plot. HNA bacterial abundance was expressed as the percentage of total bacteria population, as described previously (Lebaron *et al.*, 2001).

Bacterial production (BP)

BP was measured by ^{3}H-leucine incorporation (Kirchman, 1993) as modified by Smith & Azam (1992). Samples (2.5 mL in triplicate) were added to a sterile polystyrene snap cap tube (5 mL), containing 8 nM ^{3}H-leucine (specific activity 117 Ci mmole^{-1}, Perkin Elmer) and 32 nM of unlabelled leucine. One killed control was prepared for each assay by adding 250 μL of 50% trichloroacetic acid (TCA), 15 min before the addition of leucine. Tubes were incubated in the dark at the *in situ* temperature for 1 h on an orbital shaker to avoid the settling of aggregates at the bottom of the tube (Ploug & Grossart, 1999). The reaction was terminated by transferring replicate 1-mL samples from each tube in microcentrifuge tubes containing 100 μL of 50% TCA. Samples were stored for at least 1 h at 4 °C and then centrifuged for 15 min at 12 000 ***g***. The precipitate was rinsed once with 5% TCA and once with 70% ethanol. The precipitates were resuspended in 1.0 mL of liquid scintillation cocktail (FilterCount, Perkin Elmer) and radioactivity determined by liquid scintillation counter (LS 5000CE Beckman). An additional set of samples was used at all depths and treated as above, except that samples (2.5 mL in triplicate) were collected after prefiltration through 0.8-μm-pore-size filters (47 mm, ATTP, polycarbonate nuclepore) for free-living bacterial production measurement. Leucine incorporation rates were converted into carbon production using the conversion factor of 1.55 kg C produced per mole of leucine incorporated (Kirchman, 1993).

DNA and RNA coextraction, PCR and reverse transcriptase (RT)-PCR

A fractionation procedure was performed onboard in one step using 2 L of seawater using a peristaltic pump applying a low vacuum pressure (< 100 mbar). The 'particle-attached' fraction was concentrated on 0.8-μm-pore-size filters (47 mm, ATTP, polycarbonate nuclepore) and the 'free-living' fraction on 0.2-μm-pore-size filters (47 mm, polycarbonate nuclepore). Filters from attached and free-living fractions were stored in sterile 2-mL RNAse-free Eppendorf tubes at −20 °C until nucleic acid extraction (< 3 weeks). To evaluate the influence of sampling size on the molecular characterization of attached and free-living bacterial communities, samples of 100, 500 mL, 2 and 5 L seawater were filtered using 0.8- and 0.2-μm-pore-size filters, stored and analysed using the same procedure. Frozen filters were cut with sterilized scissors into small strips and vortexed briefly in 840 μL of alkaline lysis buffer. Cell lysis was accomplished by an initial incubation for 45 min at 37 °C after adding 50 μL of freshly prepared lysozyme solution (20 mg mL^{-1}), and a second incubation at 55 °C for 1 h after adding 100 μL of 10% sodium dodecyl sulphate and 10 μL of proteinase K (20 mg mL^{-1}).

Six hundred microlitres of lysate was treated with 10 μL of a 100 mg mL^{-1} RNAse A solution (Qiagen) before DNA extraction using the DNeasy Tissue kit (Qiagen). DNA was used as a template for PCR amplification with primers w49 (5′-ACG GTC CAG ACT CCT ACG GG-3′) (Delbès *et al.*, 1998) and w34 (5′-TTA CCG CGG CTG CTG GCA C-3′) (Lee *et al.*, 1996), which amplify the variable V3 region of the 16S rDNA (*Escherichia coli* positions 329–533) (Brosius *et al.*, 1981). The primer w34 was fluorescently labelled at the 5′-end position with phosphoramidite (TET, Applied Biosystems). Both primers were obtained commercially (Eurogentec). Each 50-μL reaction mixture contained 50 μM of each primer, 1 × Pfu reaction buffer, 20 mM dNTPs, 1.0 U of Pfu DNA polymerase (Promega) and 0.1 μg of template DNA. PCR amplification was performed using a Robocycler (Stratagene) under the following conditions: an initial denaturation step of 94 °C for 2 min, followed by 25 cycles of denaturation at 94 °C for 30 s, annealing at 61 °C for 30 s, extension at 72 °C for 30 s, and a final elongation step at 72 °C for 10 min. The size (*c.* 200 bp length) and the amount of PCR products were determined by agarose gel electrophoresis (2%) with a DNA size standard (Low DNA Mass Ladder, GIBCO BRL).

Another 400 μL of lysate was used for RNA extraction with SV Total RNA Isolation kit (Promega) according to the manufacturer's protocol, with the exception that we used 10 U of RNAse-free DNAse I (incubation for 15 min at 20–25 °C and stopped by adding SV DNAse stop solution, Promega). The efficiency of the genomic DNA removal from

RNA was checked by amplifying each RNA extract by PCR (conditions were as indicated above), and PCR reactions that did not give a product were used for cDNA synthesis. Transcription of 16S rDNA into cDNA synthesis was performed just after total RNA extraction by first incubating 13 μL of RNA with 0.5 μL of w34 and w49 primers (50 μM each) at 90 °C for 5 min before cooling in a water/ice bath. Two hundred units of M-MLV reverse transcriptase (Promega) were added together with its buffer and 50 mM dNTPs and incubated for 1 h at 42 °C. PCR amplification of cDNA was performed immediately with the same conditions as for DNA.

CE-SSCP

The TET-labelled PCR or RT-PCR products were quantified by visualization in ethidium bromide-stained agarose gels (2%) and diluted in sterile TE (10 mM Tris, 1 mM EDTA) in order to obtain *c.* 10 ng μL^{-1} of PCR product. From the resulting dilution, 1 μL of PCR product was mixed with 18 μL of formamide (Applera) and 1 μL of an internal size standard GeneScan-400 Rox (Applied Biosystems). The mixture was then denatured for 5 min at 94 °C and immediately cooled on ice for at least 5 min. CE-SSCP electrophoresis was performed in a temperature-controlled room (around 20 °C) on an ABI Prism 310 genetic analyser (Applied Biosystems) equipped with a heat plate that kept the capillary at 30 °C during electrophoresis.

Samples were electrokinetically injected (5 s, 12 kV) into the capillary (47 cm × 50 μm) filled with a nondenaturing 5.6% GeneScan polymer (part no. 401885, Applied Biosystems) gel containing 10% autoclaved glycerol in sterile TBE buffer (part no. 402824, Applied Biosystems). Electrophoresis was carried out at 15 kV and 30 °C for 30 min for each sample, and data were collected with ABI Prism 310 collection software (Applied Biosystems). Peak detection and retention time were obtained with the software GENESCAN analysis (Applied Biosystems) that first normalized the mobility of all electropherograms with the internal size standard comigrating with each sample as reference. A peak was then defined by the local maxima of the electropherogram, by computing the first derivative of a polynomial curve fitted to the data within a window that was centred on each data point in the analysis range. Because many overlapping peaks were present in our profiles, we used a high polynomial degree value of 19 in order to increase peak detection sensitivity. All peaks were checked manually for correct retention time and shape, and samples were first compared according to the presence and absence of peaks relative to retention times (i.e. 1 OTU, ±6 retention time AU).

The similarity of the CE-SSCP profiles was assessed using the software SAFUM (Zemb *et al.*, 2007), which normalized the total area of the profiles and mobilities between different runs using the internal standard. SAFUM renders a profile of fluorescence intensity as a function of retention time per sample, thus taking into account the presence and intensity of each individual signal. Data from SAFUM were then transferred to the PRIMER 5 software (PRIMER-E Ltd, UK) for further statistical comparative analysis of CE-SSCP fingerprints. First, ordination of similarities was performed with principal component analysis (PCA). To test the null hypothesis that there was no difference between attached and free-living bacterial communities in our samples, we conducted an analysis of similarities with the subroutine ANOSIM of PRIMER. ANOSIM first calculates the R statistic that displays the degree of separation between groups. Complete separation is indicated by $R = 1$, whereas $R = 0$ suggests no separation. Having determined R, ANOSIM randomly assigns samples to different groups to generate a null distribution of R (the Monte Carlo test) to test whether within-group samples are more closely related to each other than would be expected by chance. Second, a hierarchical clustering was performed with unweighted pair group method with arithmetic averages (UPGMA) based on Euclidean distance.

Results

Site characteristics

Sampling was performed during a thermal stratification on 14 July 2004, with a temperature gradient ranging from 20.0 °C at the surface to 15.3 °C at 50 m and with a fairly constant temperature below (13.6 ± 0.3 °C) (Table 1). A strong fluorescence peak was observed between 70 and 90 m with a DCM at 80 m (0.54 μg L^{-1} chlorophyll *a*), and a maximum PP of 2.32 mg C m^{-3} day^{-1} at the same depth. POC concentrations presented also a peak at 80 m (5.9 μM). DOC concentrations were less related to the DCM, being maximal at 10 m (72.5 μM), presenting a steep gradient from 30 m (68.6 μM) to 50 m (59.8 μM), and then a moderate gradient down to 150 m (54.5 μM). Nitrates were undetectable (< 0.04 μM) in the first 50 m, increased up to 0.66 μM at 80 m and up to 2.7 μM at 150 m. Phosphates were undetectable (< 0.02 μM) in the first 100 m, and reach 0.1 μM at 150 m (data not shown).

Bacterial abundance and bacterial activity of particle-attached and free-living fractions

A marked peak of bacterial abundance was observed at 50 m depth for the free-living fraction (30.3×10^5 cells mL^{-1}), whereas the abundance of the particle-attached fraction was maximal at the DCM level (3.4×10^5 cells mL^{-1} at 80 m depth). The abundance of free-living bacteria was always higher than attached bacteria. The percentage of the attached fraction was generally < 11%, except at the DCM, where it reached 14% of the total number of bacteria (Table 1). The percentage of HNA cells varied between 65% and 77% of the attached bacterial count (mean = 70.9, SD = 4.8, $n = 6$) and

Table 1. Selected physical, chemical and biological depth profiles at MOLA

Depth (m)	Temperature (°C)	DOC (μM)	POC (μM)	Chl *a* (μg L^{-1})	PP (mg C m^{-3} day^{-1})	BA free (10^5 mL^{-1})	BA att. (10^5 mL^{-1})	%Att. BA	%Att. HNA	%Free HNA	BP free (μg C L^{-1} day^{-1})	BP att. (μg C L^{-1} day^{-1})	%Att. BP	BP cell^{-1} att./free
10	20.0	72.5 (1.5)	4.7 (0.2)	0.18 (0.05)	0.04 (0.06)	16.8 (0.3)	1.7 (0.1)	9.1	75.3	56.8	0.69 (0.03)	0.08 (0.01)	9.9	1.1
30	19.9	68.6 (3.1)	5.4 (0.2)	0.19 (0.02)	0.38 (0.05)	17.6 (0.3)	1.7 (0.1)	8.9	77.9	47.5	0.63 (0.07)	0.08 (0.01)	10.5	1.2
50	15.3	59.8 (2.8)	5.8 (0.1)	0.25 (0.02)	1.19 (0.25)	30.3 (0.3)	3.1 (0.1)	9.4	69.5	50.2	0.37 (0.02)	0.06 (0.01)	14.6	1.7
80	13.9	57.6 (3.6)	5.9 (0.3)	0.54 (0.07)	2.32 (0.20)	20.6 (0.2)	3.4 (0.1)	14.0	68.2	51.7	0.17 (0.01)	0.08 (0.01)	32.8	3.0
100	13.7	56.6 (1.2)	3.7 (0.4)	0.20 (0.03)	0.41 (0.12)	14.1 (0.1)	1.8 (0.1)	11.1	70.0	47.6	0.09 (0.01)	0.05 (0.00)	36.6	4.6
150	13.4	54.5 (0.8)	2.2 (0.1)	0.02 (0.01)	0.00 (0.00)	11.7 (0.1)	1.0 (0.1)	7.8	64.6	53.3	0.07 (0.00)	0.04 (0.01)	37.5	7.2

BA, bacterial abundance; BP, bacterial production; free, free-living bacteria; att., particle-attached bacteria; %att. BA, percent of particle-attached bacteria in the total bacterial abundance; %att. HNA, percent of HNA cells in the attached bacterial count; %free HNA, percent of HNA cells in the free-living bacterial count; %att. BP, percent of particle-attached production in the total bacterial production; BP cell^{-1} att./free, the ratio between attached and free-living bacterial production per cells. SD is indicated in parentheses.

between 48% and 52% of the free-living bacterial count (mean = 51.2, SD = 3.6, $n = 6$) (Table 1).

Bacterial production measured by [^{3}H] leucine incorporation was maximal in the upper layer and decreased with depth for the free-living bacteria (from 0.69 to 0.07 μg C L^{-1} day^{-1}), whereas bacterial activity of attached bacteria was rather stable in the first 150 m (mean = 0.07 ± 0.02 μg C L^{-1} day^{-1}, $n = 6$). The contribution of the attached fraction to the total activity increased with depth from 10% at 10 m to a maximum of 38% at 150 m (Table 1). Cell-specific activity of attached and free-living bacteria were in the same range in the upper layer (0–30 m), but attached cells were 1.7–7.2 times more active than free-living cells below (50–150 m).

Distribution of DNA- and RNA-derived CE-SSCP peaks

The influence of sampling size was tested by analysing samples from 100 mL to 5 L of seawater with no visible effect on either attached or free-living CE-SSCP peak pattern (data not shown). In general, free-living bacteria presented a more complex community structure than the particle-attached fraction (Fig. 1). Maximum number of DNA-derived CE-SSCP peaks was observed at the DCM, with 30 and 23 peaks for the free-living and attached fractions, respectively (Fig. 2). Detailed analysis of the peak pattern revealed that a large majority of DNA-related OTUs for both attached and free-living fractions were found at several depths (46% and 62%, respectively) rather than at all depth (36% and 30%, respectively), with more rare examples at only one depth (18% and 9%, respectively) (Fig. 2b). RNA-related results also show a closer proportion of OTUs found at several depths (> 44%) and at all depth (> 31%) in both attached and free-living fractions (Fig. 2b). The repartition of these three subgroups was similar and even more pronounced when compiling both DNA- and RNA-related OTUs (> 76% found at several depths and < 11% at all depths or only one depth for the attached and free-living fractions). Figure 3a shows that around 61% (range 52–69%, $n = 5$) of the attached DNA-derived peaks were identical to the peaks of the free-living fraction in the first 80 m, and a minimal identity of 24% was observed at 150 m. This percentage was much lower at the RNA level (31%; range 17–48%, $n = 6$), with the lowest identity observed at the DCM (Fig. 3b). In contrast with the DNA level, the number of RNA-derived peaks was smallest at the DCM for both fractions compared with other sampling depths (Fig. 2). In both attached and free-living fraction, DNA : RNA ratio was always > 1 (mean = 1.29 and 1.28, SD = 0.34 and 0.32, $n = 6$ for attached and free-living bacteria, respectively), or close to 1 at 150 m for attached bacteria (0.92) and at 30 m for free-living bacteria (0.95) (Fig. 2).

The percentage of identical peaks on the DNA and RNA levels was much higher for the free-living bacteria, compared

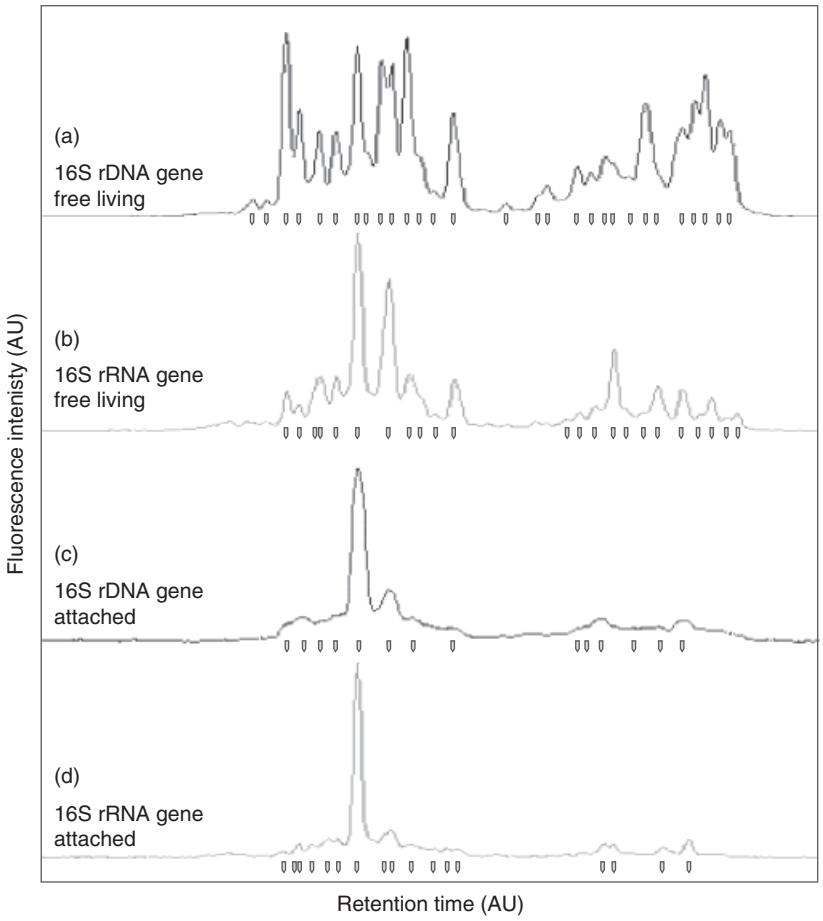

Fig. 1. Example of DNA- and RNA-derived CE-SSCP profiles from free-living and particle-attached bacteria at 50-m depth. Arrows indicate peak positions. Retention times are correlated to those for the internal controls.

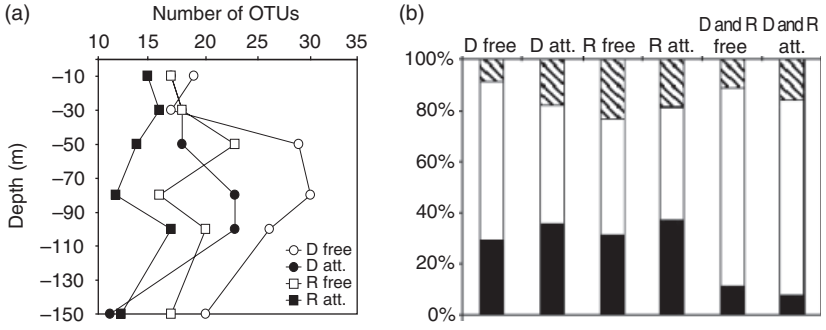

Fig. 2. (a) Total number of OTUs of attached and free-living bacteria using DNA- or RNA-derived CE-SSCP fingerprints and (b) percentage of attached and free-living DNA- and/or RNA-based OTUs found at only one depth (▨), at several depths (□) or at all depths (■). D, 16S rRNA gene; R, 16S rRNA; att., particle-attached fraction; free, free-living fraction.

with the attached fraction (Fig. 4). Indeed, when the peaks detected on the free-living fraction were compared on both DNA and RNA levels, 55% (range 38–71%, $n = 6$) of the peaks were found to be identical and 31% (range 23–47%, $n = 6$) of peaks were detected on the DNA level but not on the RNA level (Fig. 4a). For the attached bacteria, however, only 31% (range 21–39%, $n = 6$) were found to be identical and 42% (range 37–56%, $n = 6$) of peaks were detected on the DNA level but not on the RNA level (Fig. 4b). The percentage of peaks detected on the RNA, but not on the DNA level, was 14% for the free-living fraction (range 3–23%, $n = 6$) and 27% for the attached fraction (range 15–42%, $n = 6$).

Cluster analysis of DNA- and RNA-derived CE-SSCP profiles

PCA analysis clearly showed that the bacterial community structure of free-living bacteria was very different from the attached bacteria on both DNA and RNA level (Fig. 5a). Exceptions were found for 150-m samples that do not

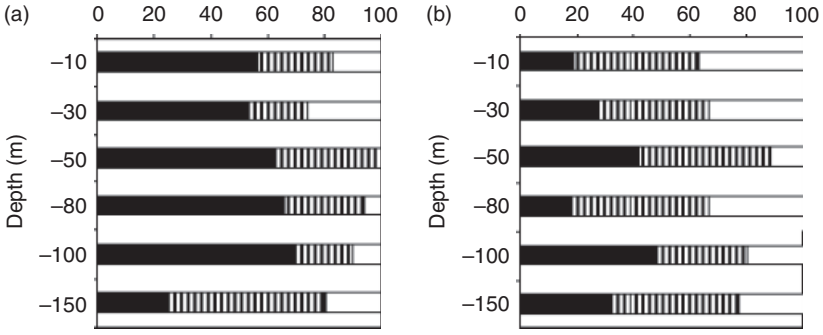

Fig. 3. Percentage of common OTUs found in both free-living and attached fractions (■), only in the free-living fraction (▮) or only in the attached fraction (□) from DNA- (a) and RNA-derived (b) CE-SSCP fingerprinting.

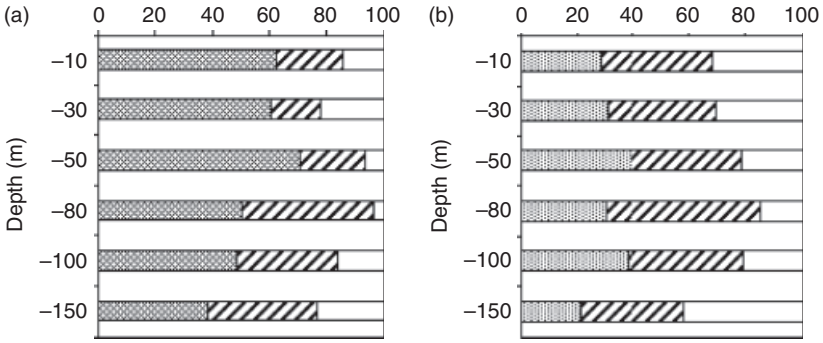

Fig. 4. Percentage of common OTUs found for both 16S rRNA genes and 16S rRNA (▦), only for 16S rRNA genes (▮) and only for 16S rRNA (□) CE-SSCP fingerprintings in the (a) free-living and (b) particle-attached fractions.

cluster with any of the free-living and attached group. The subroutine ANOSIM from PRIMER confirmed this result by showing a significant difference ($R = 0.371$, $P = 0.001$) between attached and free-living groups, when 150-m-originated samples were excluded from the analysis. In these groups, samples for a given depth showed only slight differences between DNA- and RNA-derived profiles. Maximum differences between DNA- and RNA-derived community structures were found in the attached fraction for samples taken at the DCM and at 100 m depth.

UPGMA cluster analysis also showed clear differences between the free-living and attached fractions (Fig. 5b). Within these groups, bacterial assemblages clustered according to three different layers. At a DNA level, clear differences were observed between the free-living bacterial community structure originating from the upper layer (10–30 m), the layers influenced by the DCM (from 50 to 100 m) and the deeper layer (150 m). The organization in three distinct layers was also found for the attached fraction, but the influence of the DCM appeared to be restricted to 50 and 80 m, but different from 100 m depth bacterial community structure. The organization in three different layers was also found for the free-living and attached fractions at the RNA level (Fig. 5b).

Discussion

Oligotrophic waters such as the NW Mediterranean Sea in summer are characterized by a high proportion of regenerated production, the dominance of the microbial loop (Hagström *et al.*, 1988), and a tight coupling between primary and bacterial production (Conan *et al.*, 1999). Here, we focused our study on the attached and free-living bacterial diversity and activity in the first 150 m in NW Mediterranean Sea. Our sampling was performed at the MOLA under classical summer oligotrophic conditions characterized by low concentrations of nutrients and DOC and by a thermal stratification of the water column. DOC and POC concentrations were in the range of summer concentrations found for oligotrophic area in the NW Mediterranean Sea (Cauwet *et al.*, 1997; Avril, 2002; Santinellia *et al.*, 2002). PP was low and limited to a DCM at 80 m depth ($PP = 2.3\ mg\ m^{-3}\ day^{-1}$, chlorophyll *a* maximum = 0.54 $\mu g\ L^{-1}$). The POC concentration and the ratio of the attached to the total bacterial abundance were maximum at the DCM (5.9 μM and 30%, respectively). As it was observed elsewhere, bacterial abundance and bacterial production of attached bacteria were always lower than that for free-living bacteria (reviewed by Simon *et al.*, 2002). As expected, the monitoring of 16S rRNA genes- and 16S rRNA-derived CE-SSCP dynamics of attached and free-living bacteria yielded more information about this common scenario.

It is generally admitted that metabolically active bacteria contain more rRNA than resting starved cells. Kemp *et al.* (1993) showed a highly significant relationship between RNA and growth rate for data pooled across different marine isolates, and they found that the correlation between growth rate and the RNA : DNA ratio accounted for 94% of variance when isolates were considered individually. Even if data based on culture media may be not really representative

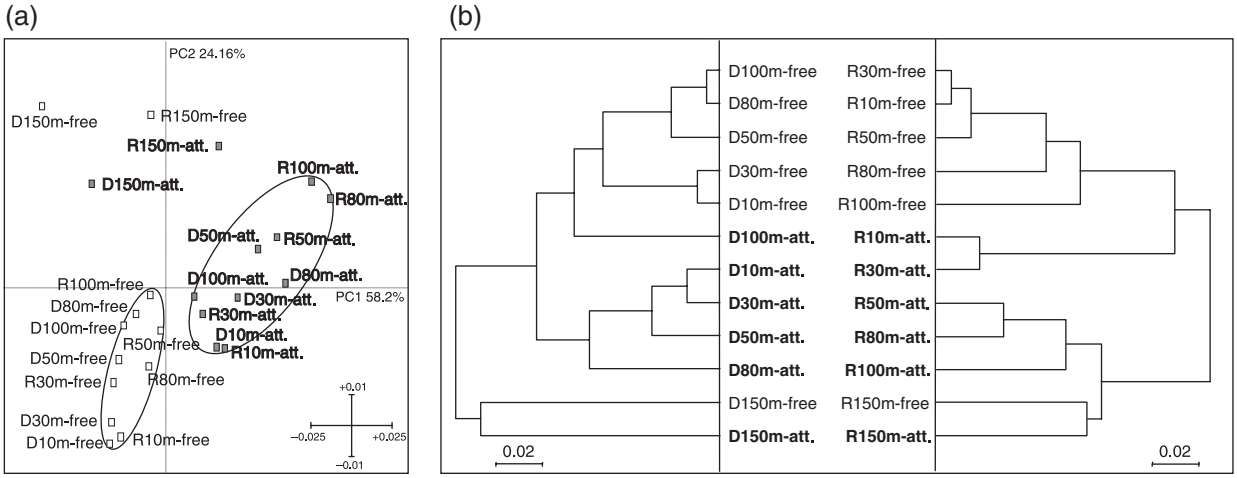

Fig. 5. Changes in DNA- and RNA-derived CE-SSCP fingerprints of both particle-attached and free-living bacteria with depth by (a) PCA (PC1 × PC2) explaining 82.3% of variability and (b) UPGMA clustering based on Euclidean distances. D, 16S rRNA genes; R, 16S rRNA; att., particle-attached fraction; free, free-living fraction. Scale bar indicates the Euclidean distance.

for oligotrophic microorganisms, several studies in molecular microbial ecology have used RNA as a marker of cellular metabolic activity (Troussellier *et al.*, 2002; Moeseneder *et al.*, 2005). On this basis, CE-SSCP results obtained from 16S rRNA genes are considered to reflect the presence of total bacterial community structure, while 16S rRNA-derived CE-SSCP results give an indication of the presence of bacterial population that contribute to the RNA pool, i.e. the metabolically active community structure. In this study, we could differentiate three groups based on the presence or absence of OTUs at the DNA and RNA level. We found a significant number of OTUs that were not detected at the DNA level but detected at the RNA level (between 3% and 42% of OTUs for a given sample). This suggests that their contribution to the community in terms of cell numbers was too low for detection, but due to their high metabolic activity and the accompanying 16S rRNA synthesis, these OTUs were readily detectable at the RNA level. Such result was also found in other studies (Teske *et al.*, 1996; Wawer *et al.*, 1997a, b, Moeseneder *et al.*, 2001, 2005; Winter *et al.*, 2001; Troussellier *et al.*, 2002). As mentioned before, we checked every sample for DNA contamination by PCR amplifying the DNAse digests before transcribing the remaining RNA into first-strand cDNA. In previous papers, we have verified the high reproducibility of the CE-SSCP technique (Ghiglione *et al.*, 2005) and we have shown that CE-SSCP do not miss any major known group detected by a clone library of the same sample (Rodriguez-Blanco *et al.*, 2009). Hong *et al.* (2007) showed that CE-SSCP resolution was higher in terms of number of peaks resolved, reduced comigration of distinct DNA sequences, and the length and legibility of the DNA-sequencing data of clones used to identify peaks compared with the classical denaturing gradient gel electrophoresis technique. Therefore, we are confident that the situation with a higher peak number on the RNA level than on the DNA level was not an experimental artefact. The observed mismatch between the DNA- and RNA-derived OTUs suggests that many bacteria may be low in abundance but metabolically highly active (Moeseneder *et al.*, 2001).

As suggested by other authors, three categories of particle-attached and free-living bacteria could be distinguished in our study: (1) bacteria specialized in colonizing particles (particle specialists), (2) free-living bacteria, and (3) bacteria that can grow in suspension as well as on particles (generalists) (Riemann *et al.*, 2000; Kirchman, 2002; Grossart *et al.*, 2005, 2006). In our study, more than half of the attached bacterial OTUs were also found in the free-living fraction in the productive layer (between 52% and 69% of identical DNA-derived OTUs in both fractions in the first 100 m depth), whereas diversity of bacterial OTUs found only at an attached state represent a minority (between 0% and 26% in the first 100 m depth) (Fig. 3a). The predominance of 'generalists' suggests that a large proportion of bacteria is able to colonize particles or detach from them in the water column. We are aware that the procedure used to separate the free-living from the attached bacteria may moderate this result. During the filtration process, some attached bacteria may have dislodged from particles and may have passed through the filter, resulting in an underestimation of the calculated attached bacterial abundance, activity or community structure. However, to minimize this problem, all filtrations were performed at a very low vacuum pressure. In contrast, free-living bacteria could be retained as a consequence of clogging. Because no difference was found in the CE-SSCP profiles of attached and free-living

communities for volumes ranging from 0.1 to 5 L of filtered seawater (data not shown), we can assume that in this study, clogging had no or little consequence on the genetic fingerprint of both fractions. Because we did not perform any prefiltration, templates other than bacterial DNA, such as plastids from phytoplankton, may have been amplified by PCR (Riemann *et al.*, 2000; Fandino *et al.*, 2001). However, a clone library made on the same 80-m sample but prefiltered on 3-μm filter (Rodriguez-Blanco *et al.*, 2009) showed that sequences from plastid origin were negligible (< 1.2% of the clone library), suggesting that plastid contamination should be minor in our free-living CE-SSCP profiles. Contamination of PCR products by plastid sequences in the attached fraction may result from phytoplankton higher than 3 μm, but such cells are generally 5000 times lower than the attached bacterial counts under summer oligotrophic conditions in NW Mediterranean Sea (Charles *et al.*, 2005). Together with the fact that the number of ribotypes specific to the attached state compared with the free-living state was rather small in all the profiles analysed (see results and Fig. 1), we can assume that contamination of our CE-SSCP profiles should also be minor for the particle-attached fraction. Exchanges between free-living and attached bacteria are consistent with previous results (Hollibaugh *et al.*, 2000; Moeseneder *et al.*, 2001; Ghiglione *et al.*, 2007) suggesting that colonization of particles is largely mediated by ubiquitous bacteria. However, others authors proposed that bacteria attached to particles may be phylogenetically different from free-living bacteria (Delong *et al.*, 1993; Crump *et al.*, 1999). Such discrepancies with our results may be due to different fractionation and molecular approaches, or to different types of particles and trophic conditions. As suggested by Riemann & Winding (2001) and because of the large number of similar OTUs found between attached and free-living bacteria, we propose that free-living and attached fractions are not separate entities but interacting assemblages.

Under our summer stratification sampling period, we found that attached bacteria played a non-negligible biogeochemical role in the NW Mediterranean carbon flux despite composing < 14% of total bacterial abundance. An indication of higher activity of attached bacterial cells was found by the higher percentage of HNA content cells on the attached compared with the free-living bacterial counts (Table 1). HNA cells measured by flow cytometry are considered to be not only larger but also more active, with higher specific metabolic and growth rates than the LNA cells (Troussellier *et al.*, 1999; Lebaron *et al.*, 2001). However, we cannot state that this result is sufficient to demonstrate the higher activity of attached bacterial cells, because the dichotomous view of HNA and LNA as active and inactive cells was not supported by others (Bouvier *et al.*, 2007; Morán *et al.*, 2007; Zubkov *et al.*, 2001). Moreover, in opposition to bacterial production and cell-specific activity measurements, we have not found clear relation between HNA cell counts and physicochemical changes with depth. Indeed, the contribution of the attached fraction to the total bacterial production showed a relationship with depth, increasing from 10% in the surface layer to 38% in deeper layer (150 m). Cell-specific activity of the attached fraction was greater than that of free-living fraction at the DCM and above, suggesting that the rapid colonization of freshly produced organic matter particles was an efficient strategy for bacteria to enhance their activity. This is consistent with the results reported by Turley & Stutt (2000) and Ghiglione *et al.* (2007) in the same period in the Ligurian Sea (NW Mediterranean Sea) or under other conditions (Alldredge & Gotschalk, 1990; Simon *et al.*, 1990; Smith *et al.*, 1995; Grossart & Simon, 1998). Here, molecular CE-SSCP fingerprinting shows that the DCM level was colonized by a large number of bacterial species, because the diversity of attached and free-living OTUs was maximal at the DCM (Fig. 2). High percentage of OTUs found in both attached and free-living fractions was also found at this depth (> 65%), suggesting a rapid exchange or co-occurrence between them (Fig. 3).

A large fraction of marine bacteria are motile (Grossart *et al.*, 2001), many of which have chemotactic behaviour (Blackburn *et al.*, 1998). Such mechanisms could be proposed to explain the large diversity of free-living bacteria that were found to colonize the POC newly produced at the DCM. However, our data based on RNA showed that only some of them were adapted to exploit such rich habitats. Indeed, less than half of the attached bacterial populations were found to be active at this depth, as regard to the high percentage (55.6%) of OTUs found at the DNA level but not at the RNA level for both fractions (Fig. 4). Interestingly, and in contrast to the results found at the DNA level, the identity between free-living and attached OTUs at the DCM was minimal at the RNA level, i.e. 16.7% (Fig. 3). It suggests that the activity of bacteria present at the DCM was dependent on their living mode, because only some of them were active in an attached state whereas others were active in a free-living state.

PCA and ANOSIM test showed that the main factor driving the bacterial community structure was the attached vs. the free-living life mode. This result is not in contradiction with the hypothesis of an exchange between free-living and attached bacteria, but more certainly a consequence of the lower number of attached OTUs per sample compared with free-living (Fig. 2). Such distinction between the free-living and attached bacterial community structure together with a low diversity of the attached fraction is a general feature in pelagic environments (Acinas *et al.*, 1997; Covert & Moran, 2001; Moeseneder *et al.*, 2001). In this study, the same trend was found in the first 100 m depth for the community

structure of active attached bacteria, coupled with a lower diversity of active attached OTUs compared with active free-living OTUs. Within the attached and free-living clusters, the community structure of total and metabolically active bacteria were generally organized according to their depth counterpart, suggesting that both total and active OTU diversity of attached and free-living bacteria were equally influenced by depth stratification. The influence of depth was more perceptible by the complementary UPGMA analysis (Fig. 3b). At both DNA and RNA levels, free-living and attached bacteria clustered according to the three layers above (10–30 m), around (50–100 m) and below (150 m) the DCM level. Such in-depth organization of the bacterial community suggests a selection of bacteria according to the presence of primary producers as well as the assemblages of protists and zooplankton together with changes in particle quality and quantity that are generally different from the surface to the DCM and deeper levels (Estrada & Salat, 1989; Dolan & Marrasé, 1995). These results reinforce the idea that attached and free-living total community structure and metabolically active bacteria are greatly influenced by the DCM in the first 150 m depth. Many bacteria exhibit chemotactic behaviour that allows them to cluster in the nutritionally rich environment, such as the steep gradients of extracellular polymers and nutrients surrounding the phytoplankton at the DCM (Grossart *et al.*, 2007). Chemotaxis is also a prerequisites for active colonization on suspended aggregates or phytoplankton cells, leading to a multitude of possible interactions (see a review by Cole, 1982) that results in changes in community structure.

The structure of particle-attached bacterial communities was not more stable throughout the water column than that of free-living bacterial assemblages, as might be expected if colonizers of newly formed particles were sinking through the water column. This reinforces the idea that both colonization and detachment occurred at all depths in the euphotic zone. Even if the structure of the community structure of total and active bacteria differed with depth, a large proportion of DNA- or RNA-based OTUs was found at several depths, and to a lower extent at all depth, but OTUs found at only one depth were much less abundant with no distinction between attached and free-living fractions (Fig. 2b). This suggests a large plasticity of these organisms to adapt to the changing conditions in the first 150 m. The predominance of OTUs found at several depths was even greater when compiling DNA- and RNA-based data (Fig. 2b), suggesting that particular presence or activity of given species at only one depth or at all depths was rather exceptional in the first 150 m.

The change in bacterial community structure below the productive layer (i.e. 150 m depth) corresponds to a decline in the number of OTUs, especially for the attached bacteria (Fig. 2). The same tendencies was found by Moeseneder *et al.* (2001) suggesting that the metabolic activity of attached bacteria might lead to a depletion of labile molecules on particles that sink through the water column, which, in turn, might cause a decline in the complexity of the attached bacterial community structure.

In conclusion, our study shows that during summer stratification in NW Mediterranean Sea, bacterial diversity of attached and free-living OTUs varied with depth. At the DCM (80 m) and down to 150 m, cell-specific activity of the attached fraction was greater than that of free-living fraction, with a contribution of the attached fraction to the total bacterial production between 33% and 38%. A large number of CE-SSCP OTUs was found at the DCM layer, mostly present on both free-living and particle state (generalists), supporting the view that free-living and attached OTUs are not separate entities but interacting assemblages. Interestingly, only some of these OTUs were found to take part of the dominant active bacterial pool, suggesting that freshly produced particles may act simply as refugia for some populations or as direct (on particles) or indirect (on patches of DOC) sources of nutriments for others.

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