Biogeosciences

Role of environmental factors for the vertical distribution (0–1000 m) of marine bacterial communities in the NW Mediterranean Sea

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Abstract. Bacterioplankton plays a central role in energy and matter fluxes in the sea, yet the factors that constrain its variation in marine systems are still poorly understood. Here we use the explanatory power of direct multivariate gradient analysis to evaluate the driving forces exerted by environmental parameters on bacterial community distribution in the water column. We gathered and analysed data from a one month sampling period from the surface to 1000 m depth at the JGOFS-DYFAMED station (NW Mediterranean Sea). This station is characterized by very poor horizontal advection currents which makes it an ideal model to test hypotheses on the causes of vertical stratification of bacterial communities. Capillary electrophoresis single strand conformation polymorphism (CE-SSCP) fingerprinting profiles analyzed using multivariate statistical methods demonstrated a vertical zonation of bacterial assemblages in three layers, above, in or just below the chlorophyll maximum and deeper, that remained stable during the entire sampling period. Through the use of direct gradient multivariate ordination analyses we demonstrate that a complex array of biogeochemical parameters is the driving force behind bacterial community structure shifts in the water column. Physico-chemical parameters such as phosphate, nitrate, salinity and to a lesser extent temperature, oxygen, dissolved organic carbon and photosynthetically active radiation acted in synergy to explain bacterial assemblages changes with depth. Analysis of lipid biomarkers of organic matter sources and fates suggested that bacterial community structure in the surface layers was in part explained by lipids of chloroplast origin. Further detailed analysis of pigment-based phytoplankton diversity gave evidence of a compartmentalized influence of several phytoplankton groups on bacterial community structure in the first 150 m depth.

1 Introduction

Microorganisms represent the most abundant, biogeochemically important biological component in the oceans. At concentrations ranging between 10^4 and 10^6 cells ml^{-1}, an estimated 20 to 50% of marine primary productivity is channeled through bacterioplankton (Azam et al., 1983; Cho and Azam, 1988, 1990). Bacterial species composition is an important variable controlling the rates and patterns of organic matter remineralization (Martinez et al., 1996; Pinhassi et al., 1997). Thus, knowledge of the composition of bacterial communities, and how that composition varies over space and time, is likely to be of major importance for understanding

the role of bacteria in marine biogeochemistry (Riemann et al., 1999). The application of molecular techniques to microbial ecology has led to an increased appreciation of bacterial community changes with depth as demonstrated in a wide variety of oligotrophic seawaters, including the Pacific and Atlantic Oceans (Lee and Fuhrman, 1991), the Mediterranean Sea (Acinas et al., 1997; Moeseneder et al., 2001; Ghiglione et al., 2005, 2007), the Arabian Sea (Riemann et al., 1999) and Antarctic zones (Murray et al., 1998). The presence of the same pattern in such widely distant ocean basins suggests that vertical stratification is a very important factor determining the bacterial species inhabiting the ocean (Acinas et al., 1997).

Previous studies formulated different hypotheses to explain the vertical distribution of bacterial communities pointing to depth-related changes in environmental conditions or interactions with other planktonic components. More generally, theoretical models and empirical surveys indicate that microbial growth or population size are controlled by resource availability, including nutrient and energy sources ("bottom-up" control) and microbial biomass is controlled by mortality, including factors such as predation or viral lysis ("top-down" control) (Pernthaler, 2005). The relative importance of "top-down" and "bottom-up" controls on bacteria have been poorly investigated (Fuhrman and Hagström, 2008), and our knowledge on the interplay between the different factors involved within "top-down" and "bottom-up" controls is still limited in the field. Protistan predation may often be most influential in limiting the total abundance and biomass of the bacterioplankton, whereas viruses are considered to more profoundly affect prokaryotic community diversity (Pernthaler, 2005). On the other hand, clear bottom-up effects on bacterial community structure have been observed in controlled laboratory conditions (Pinassi et al., 1999; Carslon et al., 2002) but few studies demonstrated statistically robust, predictable patterns in the field (Korona et al., 1994; Furhman et al., 2006). Such complex ecological questions may be addressed by robust statistical analysis. Although indirect gradient multivariate statistical analyses have been used to link microbial community profiling to environmental parameters (Roeling et al., 2001; Edlund et al., 2006), the use of direct gradient analyses like canonical correspondence analysis (CCA) in combination with high-throughput molecular technologies is scarce in spite of the power of this method for this purpose (Ramette, 2007; Rooney-Varga et al., 2005).

The NW Mediterranean Sea is characterized by oligotrophic conditions and strong isothermic stratification of the water column during the summer. The great depth (2300 m), and weakness of horizontal advection currents. (Andersen and Prieur, 2000), makes the French JGOFS-DYFAMED sampling area situated at the central zone of the Ligurian Sea (NW Mediterranean) a great model zone to follow the mineralization fluxes from the prokaryotic compartment along the water column. In this work, we examined the verti-

cal distribution (0–1000 m) of bacterial community structure at this station during a one month cruise, by using capillary electrophoresis-single strand conformation polymorphism (CE-SSCP). This technique, originally developed for mutation detection, has been widely used for genetic profiling of microbial communities within a variety of ecosystems (Lee et al., 1996; Sunnucks et al., 2000; Godon et al., 2001; Ghiglione et al., 2005). CE-SSCP high reproducibility permits comparisons of microbial community fingerprinting profiles from a large set of samples (Ghiglione et al., 2005). In a recent paper (Rodriguez et al., 2008), we show that CE-SSCP does not miss any major known group detected by a clone library of the same sample and that this technique is a reasonably quantitative measure of the relative abundance of the only taxon we could independently measure (*Prochlorococcus*, by flow cytometry). As a first objective we sought to determine the vertical distribution of bacterial communities during the late summer-autumn transition period by using multivariate non-parametric statistical methods. Secondly, we took quantitatively unparalleled measurements of environmental parameters including physico-chemical variables, lipid class biotracers and phytoplankton pigments, to investigate their influence as potential driving forces for shifts in bacterial community structure along the water column using multivariate statistical direct gradient ordination methods. In a companion paper, the vertical and temporal dynamics of bacterial abundance and activity during this cruise are reported in Mével et al. (2008).

2   Materials and methods

2.1   Study site and general sampling methods

Sampling took part during a one month cruise conducted from mid-September to mid-October 2004 at the French-JGOFS time-series station DYFAMED ($43°25'2$ N, $07°51'8$ E; 2350 m max depth) in the NW Mediterranean Sea on the RV "Thalassa". We collected samples once a week in 19 September (Julian Day-JD 263; CTD46) (sample A), 26 September (JD 270; CTD 101) (sample B), 06 October (JD 280; CTD 167) (sample C) and 12 Oct (JD 286, CTD 215) (sample D) using a rosette system equipped with 24 Niskin bottles. For each sampling day, samples were collected at depths between the surface and 1000 m (i.e. 5, 20, 40, 60, 80, 150, 200, 400, 500, 700, 1000 m).

2.2   Physico-chemical parameters

We used standard sensors of the CTD – SeaBird SBE 911 plus installed in the rosette system to measure pressure, temperature, conductivity, oxygen, salinity and photosynthetically active radiation (PAR).

For nutrients analysis (nitrate, nitrite, phosphate, silicate) seawater was collected into polyethylene flasks and analyses were performed on board on a Technicon AutoAnalyzer

(AAII) according to Tréguer and LeCorre (1975). Ammonia was measured on board according to Holmes et al. (1999) on a Turner Design TD-700 fluorimeter. Detection limits were $3\,\mathrm{nmol\,l^{-1}}$ for nitrate and nitrite, $0.02\,\mu\mathrm{mol\,l^{-1}}$ for phosphate and $5\,\mathrm{nmol\,l^{-1}}$ for ammonium. To ensure reproducibility in nutrient measurements between analyses, a unique type of in-house standards was used, which was regularly compared to commercial products (OSIL). Precision was also tested, through the participation in the European inter-calibration exercise QUASIMEME (Aberdeen, UK).

For dissolved organic carbon (DOC), seawater was filtered through two pre-combusted (24 h, 450°C) glass fiber filters (Whatman GF/F, 25 mm) and collected into precombusted glass tubes closed with a screw cap and a Teflon liner. Samples were acidified with orthophosphoric acid (H_3PO_4) and DOC was analyzed immediately on board on a Shimadzu TOC-V analyser using the high temperature catalytic oxidation (HTCO) technique (Sugimura and Suzuki, 1988; Cauwet, 1994). Analytical accuracy of measurements was less than $2\,\mu\mathrm{M}$. Deep Sargasso Sea reference water ($47\,\mathrm{mol\,l^{-1}C}$, ± 0.5 SE, (http://www.rsmas.miami.edu/groups/biogeochem/CRM.html) was injected every 10 samples to insure stable operating conditions.

Colored dissolved organic matter (CDOM) substances were extracted from 500 ml sea water samples by adsorption on C18 microcolumns. CDOM was eluated from the microcolumn by methanol and quantified by light-absorbance at 412 nm.

2.3 Lipid biomarkers

Four liters of seawater were filtered onto pre-combusted glass fiber filters (Whatman GF/F, 47 mm). Dissolved lipids were extracted from 2 liters of the filtrate by liquid-liquid extraction, using dichloromethane (two extractions at sea water natural pH, followed by two extractions at pH 2). Each lipid extract was separated into classes of compounds according to polarity, using chromarods SIII (0.9 mm diameter, 150 mm length, 75 μm silica thick) and quantified using a thin layer chromatography-flame ionization detection (TLC-FID) Iatroscan TH10 apparatus model MK-IV (Iatron, Japan; hydrogen flow, $160\,\mathrm{ml\,min^{-1}}$; air flow, 2000 ml min-1). The elution scheme previously described (Striby et al., 1999) separated twelve classes of acyl-lipids and free lipids, including reserves (wax esters (WE) and triacylglycerols (TG)), lipids from chloroplasts (LC) (pigments and glycolipids), cellular membrane lipids (phospholipids (PL) which include phosphoglycerides, phosphatidylethanolamines and phosphatidylcholines), and their hydrolysis metabolites (Met) (alcohols, free fatty acids, monoglycerides and 1,2- and 1,3-diglycerides).

2.4 Phytoplankton pigments

Two liters and a half of seawater were filtered on glass fiber filters (Whatman GF/F, 25 mm), which were immediately frozen in liquid nitrogen until analysis. Pigments analysis employed a Agilent Technologies HPLC system with a RP-C8 (Agilent) column 150 mm long, 3 mm internal diameter, 3.5 μm granulometry (Zorbax Eclipse) and a Diode array detector (1100 model). All steps of extraction and analysis were performed under dim light conditions. The use of an internal standard (Vitamin E Acetate) allowed for the correction of possible losses during extraction as well as for the presence of water in the filter. The analytical method was based on a separation gradient between a methanol: ammonium acetate (70:30 v:v) mixture and 100% methanol as in Vidussi et al. (1996) with a few improvements in sensitivity.

Identification of pigments was performed using retention-time data and by comparison of on-line collected absorption spectra with those of a library established from standards and reference cultures. Response factors for eight external pigment standards (peridinin, 19'-butanoyloxyfucoxanthin, fucoxanthin, 19'-hexanoyloxyfucoxanthin, alloxanthin, zeaxanthin, chlorophyll b, chlorophyll a) were determined by spectrophotometry (Perkin Elmer) followed by HPLC analysis. These response factors were then derived to compute the specific extinction coefficients of these pigments for the HPLC system. The specific extinction coefficients from others pigments were derived from the literature (Jeffrey et al., 1997). The concentration of key chlorophyll and carotenoid pigments was used to determine phytoplankton community composition. The CHEMTAX matrix factorization program developed by Mackey et al. (1998) was applied to the phytoplankton data set to determine the contribution of principal taxonomic phytoplankton groups to the total chlorophyll a (Marty et al., 2008).

2.5 Nucleic acid extraction and PCR of 16S rDNA

Two liters of seawater were pre-filtered through 3 μm-pore-size filters (47 mm, Nucleopore) to remove most eukaryotic organisms (containing plastids that complicate the interpretation of CE-SSCP fingerprints) and to prevent clogging of the final filter. Such pre-filtration do not exclude the presence of cyanobacteria on the filtrate. Bacterial cells were concentrated onto 0.22 μm-pore-size filters (47 mm, PC Nucleopore) and stored into 2 ml Eppendorf tubes at −20°C until extraction. The procedure used for DNA extraction was modified from Furhman et al. (1988). This is a relatively gentle method that employs enzymatic and detergent-based lysis to avoid excessive shearing of DNA, producing DNA of suitable quality for PCR amplification, and reducing the risk of chimera formation during PCR. Previously frozen filters were cut with sterilized scissors into small strips and vortexed briefly in 840 μl of alkaline lysis buffer (50 mM Tris hydrochloride pH=8.3, 40 mM EDTA, 0.75 M

sucrose). Cells were lysed 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 50°C for 10 min after adding 100 μl of 10% sodium dodecyl sulfate and 10 μl of a proteinase K solution (20 mg ml^{-1}). The lysate was extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and an equal volume of chloroform: isoamyl alcohol (24:1). Nucleic acids were precipitated at −20°C overnight with 1 volume of isopropanol, and resuspended in TE (10 mM Tris, 1 mM EDTA). Molecular weight distribution and purity of the DNA were assessed by 1% agarose gel electrophoresis and quantified either by visual comparison with molecular weight markers in ethidium bromide stained agarose gels (rough estimate) or by optical density measurements using GeneQuant II (Pharmacia Biotech.).

PCR amplification was performed by using 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 with phosphoramidite (TET, Applied Biosystems). Both primers were synthesized commercially (Eurogentec). Each 50 μl reaction mixture contained 50 μM of each primer, 1X Pfu reaction buffer, 20 mM dNTPs, 1.0 U of Pfu DNA polymerase (Promega) and 0.1 μg of template DNA. PCR amplification consisted of 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, and extension at 72°C for 30 s, and a final elongation step at 72°C for 10 min using a Robocycler (Stratagene). Correct size (ca. 200 bp length) of PCR products were determined by 2% agarose gel electrophoresis with a DNA size standard (Low DNA Mass Ladder, GIBCO BRL).

2.6 Capillary Electrophoresis – Single Strand Conformation Polymorphism (CE-SSCP) procedure

Labelled PCR amplicons were purified with a QIAquick PCR purification kit (Qiagen) according to manufacturers instructions followed by quantification by visual inspection as above. Samples were diluted in sterile TE (10 mM Tris, 1 mM EDTA) in order to obtain 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. The procedure used for CE-SSCP analysis was a modification of that described by Delbès et al. (1998). Samples were electrokinetically injected (5 s, 12 kV) in a capillary tube (47 cm×50 μm) filled with a mixture composed of 5.6% GeneScan polymer (Applied Biosystems) and 10% autoclaved glycerol in sterile TBE buffer (90 mM Tris-borate, 2 mM EDTA [pH

8.0]). Electrophoresis was carried out at 15 kV and 30°C for 30 min per sample and phosphoramidite (TET)-labelled amplicons were detected by a laser with a virtual filter C (detection wavelengths 532, 537, and 584 nm). Data were collected with ABI Prism 310 collection software (Applied Biosystems). In order to normalize mobilities from different runs, all electropherograms were calibrated by fixing the positions of peaks produced by the size standard and by using a second-order least square curve (i.e. linear regression) to provide the best interlane comparison (Genescan analysis software, Applied Biosystems). Electropherograms were then transferred to the software SAFUM (Zemb et al., 2007), which renders a profile of fluorescence intensity as a function of retention time per sample taking therefore into account the presence and intensity of each individual signal. This software corrects for sample intensity differences rendering normalized profiles for all samples.

2.7 Ecological statistical analysis

Comparative analysis of CE-SSCP fingerprints was carried out with the PRIMER 5 software (PRIMER-E, Ltd., UK). Ordination of Bray-Curtis similarities among normalized sample profiles was performed by non-metric multidimensional scaling (MDS). We used this ordination technique to determine the relationships among sample profiles as representative of the bacterial community structure of each sample site. This method attempts to preserve the ranked order of the similarity of any two communities as an inverse function of the distance between plotted sample points (Kruskal, 1964). The degree to which the plot matches the similarity matrix can be judged by examining stress values (Kruskal's stress). MDS was performed using 30 random starting configurations of sample points to avoid local minima. Additionally, hierarchical agglomerative clustering of Bray-Curtis similarities was performed using the group average method of PRIMER software. To test the null hypothesis that there was no difference between bacterial communities of different depth layers we conducted an analysis of similarities with the subroutine ANOSIM of PRIMER. ANOSIM is a nonparametric technique designed to allow statistical comparisons for multivariate data sets in a manner similar to univariate techniques (ANOVA) (Clarke and Warwick, 2001). 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 (Monte Carlo test) to test whether within-group samples are more closely related to each other than would be expected by chance.

To investigate relationships between bacterioplankton community structure and physico-chemical parameters, lipids biomarkers and phytoplankton pigments, we used a direct gradient approach, canonical correspondence analysis (CCA) using the software package CANOCO, version 4.5

for Windows (ter Braak and Šmilauer, 2002). CCA generates an ordination plot that shows the main pattern of variation in community composition as accounted for by measured environmental variables. In other words, the resulting ordination axes are linear combinations of the environmental variables that best explain microbial diversity composition data (ter Braak, 1986). We first imported OTUs abundance data from spreadsheets using WCanoImp program within the CANOCO package. We then used CANOCO program to perform CCA with species scaling on intersample distances so that samples and environmental variables formed a biplot. To statistically evaluate the significance of the first canonical axis and of all canonical axes together we used Monte Carlo permutation full model test with 199 unrestricted permutations. Finally, to represent biplots we used the program CANODRAW within CANOCO package. Additionally, Spearman's rank pairwise correlations between the environmental variables mentioned above helped to determine their significance for further ecological analysis.

3 Results

3.1 Bacterial community structure analysis

Bacterial community structure was characterized through CE-SSCP fingerprinting profiling once per week within 0–1000 m depth during September-October 2004. An example of the community fingerprints obtained at different depths for julian day 263 is shown in Fig. 1. Of a mean of 49 peaks (SD=0.82, n=4) found at each date, 19.4% ($\pm$5.23) were found at all depth whereas 14.9% ($\pm$0.82) appeared only once or twice in the water column. For comparative analysis of community fingerprints we used multivariate MDS and hierarchical agglomerative clustering. Both analyses show a recurrent in-depth pattern along the sampling period with distinct bacterial communities at three different layers: 0–40 m, 60–150 m and 200–1000 m (Fig. 2). MDS ordination plot stress value was low (0.14) which corresponds to a good ordination with no real prospect of a misleading interpretation (Clarke and Warwick, 2001). We then used ANOSIM to determine the significant differences among bacterial communities from the three depth layers. This analysis showed the highest difference between surface and deeper community structures (R=0.754, p=0.001). Bacterial communities from the intermediate layer were more similar to the upper layer than to the deeper layer but still significantly different from both (R=0.32 p=0.001 and R=0.22 p=0.004, respectively).

3.2 Bacterial community structure and physico-chemical variables ecological analysis

The upper layer (0–40 m) was nutrient depleted with nitrate and phosphate concentrations close to the detection limit. Concentrations increased along a nitracline up to maxima
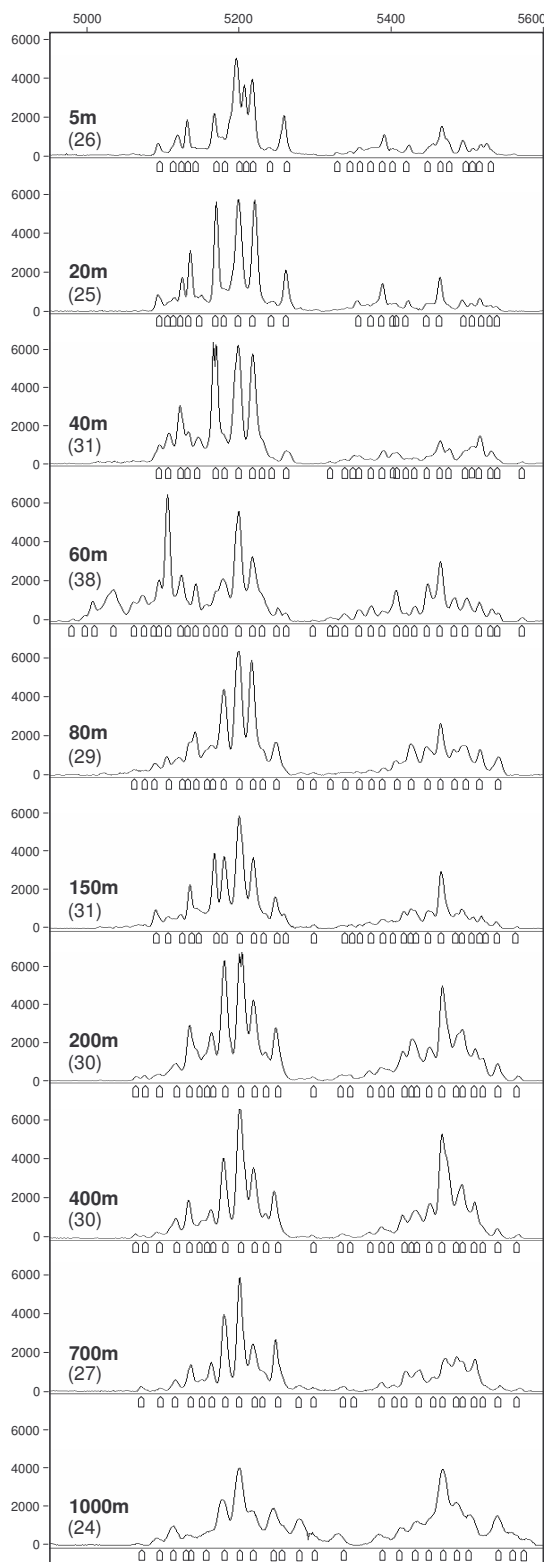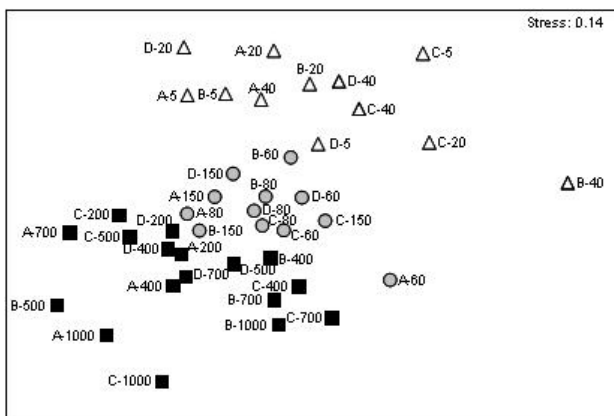

Fig. 1. Depth profiles of CE-SSCP fingerprints of bacterial communities of sample A (Julian Day 263).

Table 1. Summary of results from canonical correspondence analyses of the bacterioplankton community structure data when constrained by physico-chemical, lipid biomarkers or phytoplankton environmental variables, respectively.

Environmental variables	Physico-chemical (0–1000 m)		Lipid biomarkers (0–1000 m)		Phytoplankton (0–150 m)	
Total inertia	0.518		0.415		0.449	
Sum of all canonical eigenvalues	0.237		0.093		0.200	
	Axis 1	Axis 2	Axis 1	Axis 2	Axis 1	Axis 2
Eigenvalues	0.091	0.042	0.050	0.018	0.064	0.048
Species-environment correlations	0.926	0.876	0.761	0.714	0.959	0.850
Cumulative percentage variance of						
– species data	17.6	25.8	9.6	13.0	14.3	25.1
– species-environment relation	38.4	56.3	53.6	72.5	32.0	56.2

Fig. 2. MDS plot based on Bray-Curtis similarities of CE-SSCP fingerprints of bacterial communities samples at different time and depth. Depth repartition in three groups: 0–40 m ($\triangle$), 60–150 m (O) and 200–1000 m.

(7 and 0.2 μM, respectively) around 80 m depth. Silica varied from 1.0 μM at the surface to 13.5 μM) at 1000 m depth. DOC concentrations varied from 72.8 μM to 38.8 μM from surface to depth. We first performed CCA using all physico-chemical parameters as constrained variables of the bacterial community diversity from CE-SSCP fingerprints of 0–1000 m depth samples. CCA revealed values higher than 20 of the variance inflation factor for nitrate and silicate, which indicated that these variables were almost perfectly correlated with other variables in the dataset. A strong Spearman's rank pairwise correlation between nitrate and silicate (R=0.91, p<0.01) allowed us to use nitrate as a proxy of silicate to perform CCA together with the rest of physico-chemical parameters. A summary of the results of this CCA is shown in Table 1. The total variance of fitted 0–1000 m samples diversity data when using physico-chemical variables to explain their bacterial community structure was 0.237, as indicated by the sum of all canonical eigenvalues (ter Braak and Šmilauer, 2002). The cumulative percentage variance of species-environment relation indicates that the first and second canonical axes account for 38.4 and 17.9% of this variance respectively (Table 1). Consequent axes accounted for less than 10% of the variance each, and are not further considered here. The eigenvalues of the ordination analyses, which also measure their relative importance are small (<0.3) implying that CCA does not strongly separates species niches and therefore the biplot rule must be used to interpret the fitted relative abundances of samples with respect to physico-chemical variables in the CCA ordination diagram (Fig. 3). Monte Carlo test for first and all canonical axes were highly significant (p<0.01) indicating that the parameters selected are good explanatory variables of the community structure. The first canonical axis is highly negatively correlated with depth, salinity, nitrate and PO$_4$ (ca. -0.5) and positively correlated with oxygen and DOC (ca. 0.4) and to a lesser extend with temperature and PAR (ca. 0.2). This first synthetic gradient clearly separates samples in the three depth layers observed when using diversity data alone indicating that their community composition varies with different concentrations of these parameters. Because depth was not collinear with the other variables correlated with first axis, we include this variable in the analysis as a proxy of pressure effect on bacterial community structure. Surface water samples further spread out along the second canonical axis (Fig. 3). We infer that 60 m depth samples have similar concentrations of nitrite, ammonia and CDOM variables elements than 40 m samples and together form a group independent from that of the 5–20 m samples (with the exception of C-20 m sample). The significant contribution of these variables in defining surface bacterial community structures was confirmed by additional CCAs using only shallower samples (data not shown).

3.3 Bacterial community structure and lipid biomarkers ecological analysis

Total dissolved lipids varied from 0.4 to 4 μM. Detailed analysis of this fraction of the organic matter is given in Goutx et
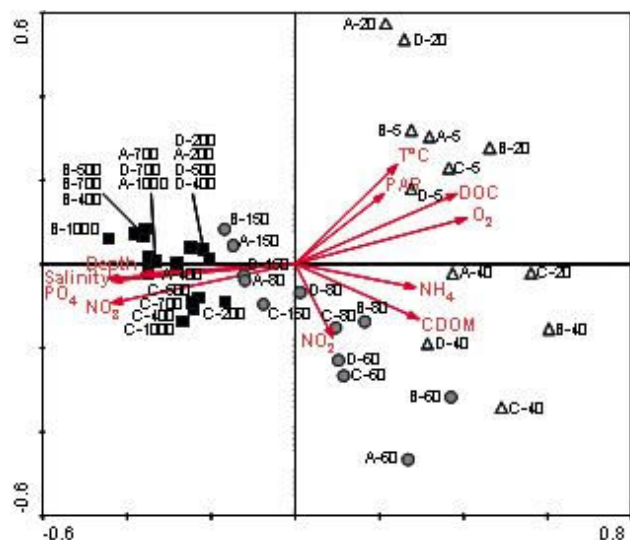
Fig. 3. Canonical correspondence analysis of bacterioplankton community structure from samples from 0 to 1000 m depth using physico-chemical parameters. Arrows point in the direction of increasing values of each variable. The length of the arrows indicates the degree of correlation with the represented axes. The position of samples relative to arrows is interpreted by projecting the points on the arrow and indicates the extent to which a sample bacterial community composition is influenced by the environmental parameter represented by that arrow. Samples from different depth groups (0–20 m, 60–150 m and 200–1000 m) are indicated by different symbols.

Fig. 4. Canonical correspondence analysis of bacterioplankton community structure from samples from 0 to 1000 m depth using lipid biomarker parameters. For interpretation see Fig. 3. LC: lipids from chloroplastic origin, TG: triglycerides, Met: metabolites, PL: phospholipids, WE: wax esters.

al. (2008). Chloroplast lipids (LC) dominated the lipid pool (38.7 ± 8.5% on average, n=166), which indicated a phytoplankton source for DOM. Triglycerides (TG) were minor lipids (<10% of total lipids). CCA total variance of the fitted CE-SSCP diversity from 0–1000 m depth samples when using dissolved lipid biomarkers to explain this diversity is 0.093 with the first two axes accounting for 72.5% of this variance (Table 1). Eigenvalues for these axes were 0.050 and 0.018 respectively. The Monte Carlo test for first and all canonical axes rendered a significant p-value of 0.02. The lipid biomarkers explain therefore the difference in bacterial community structures among samples according to the CCA biplot in Fig. 4. The first synthetic gradient was strongly positively correlated with lipids from chloroplast origin (LC, ca. 0.8) followed by triglycerides (TG, ca. 0.4). Samples from the upper 0–20 m layer, and especially samples from time B-5 m and 20 m, time C-5 m and 20 m and time D-20 m were separated from the rest according to this first axis. The rest of the samples formed a relatively similar group with no clear difference between layers at this first axis indicating the homogeneity of LC at these samples (Fig. 4). Some samples from this last deeper group further spread out along the second axis, which is slightly positively correlated with TG (ca. 0.3), and to a lesser extent with the remaining lipid biomark-
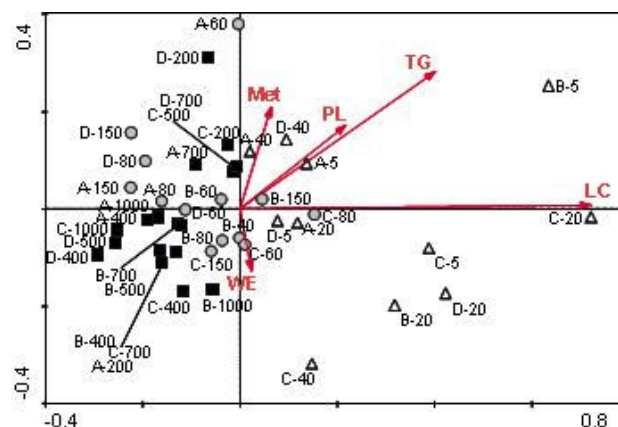
ers analyzed in this study. However, samples appear mixed with respect to their depth along this axis.

3.4 Distribution of phytoplankton pigments

Because phytoplankton pigments were almost undetectable below 150 m depth, all analyses with phytoplankton diversity data included 0–150 m depth samples only. Total chlorophyll a (TChl a) maxima increased during the one month cruise period of this study from 0.45–$0.44 \mu g \, l^{-1}$ at 50 m depth for times A and B, respectively, to $0.60 \mu g \, l^{-1}$ at 40 m depth for time C and $0.80 \mu g \, l^{-1}$ at 50 m depth for time D. The study period was characterized by the predominance of small species (nano- and pico-phytoplankton) over microphytoplankton. The principal classes observed were prochlorophytes, cyanobacteria (*Synechococcus*) together with prymnesiophytes, pelagophytes and prasinophytes (with concentrations of these classes varying from 15 to 19% of TChl-a). Diatoms and dinoflagellates were lesser constituents of the phytoplankton (from 3 to 5% of TChl-a). We observed very important variations in the vertical distribution of specific biomasses. As an example, euglenophytes were minor constituents of the TChl-a biomass (<2%) and present at very low concentrations during the whole sampling period except for their high concentration (almost 10% of TChl-a) in the surface layer 0–20 m at date A (Marty et al., 2008).

3.5 Bacterial community structure and phytoplankton pigments ecological analysis

CCA when using pigment analysis-based phytoplankton diversity to explain CE-SSCP bacterial community structure gave a total variance of fitted 0–150 m samples diversity data of 0.200 (Table 1). Axes 1 and 2 accounted for 56.2% of
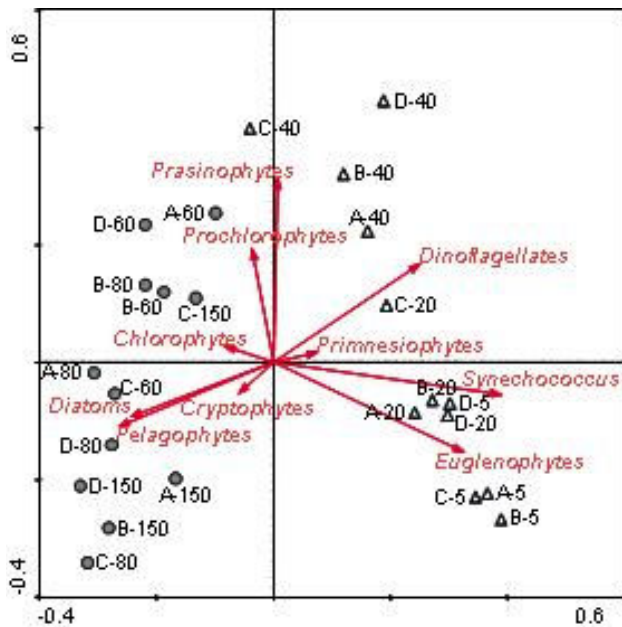
Fig. 5. Canonical correspondence analysis of bacterioplankton community structure from samples from 0 to 150 m depth using phytoplankton pigments variability. For interpretation see Fig. 3.

this variance being the other axes negligible. Eigenvalues for the two first axes were 0.064 and 0.048 respectively. Monte Carlo permutation tests of first and all canonical axes were highly significant (p-values≤0.01). Canonical axis 1 is highly positively correlated with *Synechococcus* and euglenophytes (ca. ∼0.5) and, to a lesser extend, positively correlated with dinoflagelates and negatively to pelagophytes and diatoms (ca. ±0.3) (Fig. 5). This first axis clearly distinguished between samples from the two shallower depth layers described above based on higher concentrations of *Synechococcus*, euglenophytes and dinoflagelates in the 0–20 m depth samples vs. the higher levels of pelagophytes and diatoms in the deeper 60–150 m layer. The second axis was correlated with higher abundances of prasinophytes and prochlorophytes further distinguishing the bacterial communities from samples at 40–60 m with the exception of B-60 m and C-60 m samples. Primnesiophytes, chlorophytes and cryptophytes variation had slight influence on the distribution of samples as indicated by their small arrow length (Fig. 5).

4   Discussion

4.1   CE-SSCP incomes to bacterial community profiling studies

Culture-independent, sequence-based community finger-printing techniques such as CE-SSCP (Lee et al., 1996), ter-

minal restriction fragment length polymorphism (T-RFLP) (Marsh, 1999), or automated ribosomal intergenic spacer analysis (A-RISA) (Borneman and Triplett, 1997) allow comparison of a theoretically infinite number of samples. Application of these techniques to environmental samples yields considerable amount of information on their microbial diversity composition that can be used to assess how environmental factors contribute to changes in microbial community structure. However, the introduction of experimental biases during analysis and the interpretation of the complex data generated still represent major challenges for the application of these methods in microbial ecology. As it was pointed out elsewhere (Riemann et al., 2000; Fandino et al., 2001), we cannot exclude the presence of peaks originating from eukaryotes in the CE-SSCP profiles since the PCR primers used in this study can amplify plastid 16S rRNA genes. However, this should not greatly affect our results, since picoeukaryotes represented less than 1% (mean=0.22, SD=0.03) of total cell counts in the fraction 0.2 to 3 μm (data not shown). Pre-filtration does not exclude the presence of cyanobacteria, a component of the bacterioplankton that may slightly influence our interpretation of the driving force exerted by phytoplankton species or given classes of lipids biomarkers on the total bacterial community distribution in the water column (especially at the deep chlorophyll maximum). Biases during nucleic acid extraction, PCR amplification (Wintzingerode et al., 1997) and 16S rDNA copy number variability (Farrelly et al., 1995) may lead to a distorted view of bacterial community structure.

In this study, several tests were performed to investigate the efficiency and reproducibility of the DNA extraction and PCR-CE-SSCP protocols. Cell lysis was monitored several times by microscopy, which assured that there was no cells recalcitrant to our lysis procedure. The quality of the DNA was carefully assessed before proceeding to 16S rDNA gene amplification. Only single PCR products were used for subsequent analyses. The use of an internal standard co-migrating with each sample PCR product allowed us to normalize peak mobilities for comparative sample analysis. This procedure results in a high reproducibility of both, peak pattern and peak intensities, as previously observed (Ghiglione et al., 2005). Finally, because all samples in this study are subject to similar biases, comparative analysis of microbial community structure and relative abundances are still possible (Hughes et al., 2001). Molecular fingerprinting techniques in general may be restricted in giving information about microbial diversity, since they result in 30 to 40 peaks (or bands) in marine waters. This is due to co-migration of PCR-products from different species within the same peak (or band), as depicted by several authors (Wintzingerode et al., 1997; Schmalenberger and Tebbe, 2003). Similarly, Schauer et al. (2000) mentioned the subjectivity in deciding whether a very weak DGGE band is a real band or a background artefact. In our study, this problem was, at least partly, overcome by taking into account the presence or

absence of individual CE-SSCP peaks and the relative contribution of each peak to the total surface area of the pattern. The advantage of analysis fingerprinting data this way has been recently enlightened by numerical simulation (Loisel et al., 2006). This study underlines that fingerprinting patterns contains extractable data about diversity although not on the basis of a number of bands or peaks, as is generally assumed to be the case, and can be considered as an "image" of the whole microbial ecosystem free of inventory (cloning) limitation.

On the other hand, the use of adequate numerical and statistical tools overcomes the difficulty of analyzing complex datasets. Until now most studies have used indirect gradient analysis such as principal component analysis or cluster analysis to infer the influence of environmental variables on microbial diversity data, leaving the use of direct gradient analysis approaches such as redundancy analysis or canonical correspondence analysis (CCA) neglected (Ramette, 2007). Here we compared both indirect and direct gradient multivariate approaches to explore relationships between bacterial community structure as obtained by CE-SSCP with respect to concomitant measurements of physico-chemical parameters, lipid class biotracers and pigment analysis-based phytoplankton diversity.

4.2 Indirect gradient multivariate analysis to study bacterial community composition

The DYFAMED offshore station is an ideal sampling site for 1-D studies because it is far enough from the Ligurian current to be protected from lateral transport (Andersen and Prieur, 2000). During the late summer-autumn oligotrophic conditions, biotic parameters showed a temporally stable organization along the 0–1000 m water column. Bacterial community structure appeared differentiated in three layers, shallow (5–40 m), intermediate (60–150 m) and mesopelagic (200–1000 m), when using multivariate (Fig. 2) and cluster analyses. The robustness of this strong stratification with depth pattern was confirmed by a greater similarity within than between samples from each of these layers. This study adds to previous findings of a strong stratification pattern with depth of bacterial communities in oligotrophic conditions (Lee and Fuhrman, 1991; Acinas et al., 1997; Murray et al., 1998; Riemann et al., 1999; Moeseneder et al., 2001; Ghiglione et al., 2007). This is also consistent with genotypic and phenotypic properties of stratified *Prochlorococcus* "ecotypes" for example, that are suggestive of depth-variable adaptation to light intensity and nutrient availability (Rocap et al., 2003). However, none of these studies tested for the statistical significance of vertical trends (autocorrelation), nor did they test for the predictability of vertical pattern from environmental parameters, so we do not know whether these studies show spatial patterning or predictability comparable to what we observed. Recent metagenomic approaches showed that vertical zonation of taxonomic groups was concomitant with

functional gene repertoires and metabolic potential (DeLong et al., 2006) and future efforts are needed to interpret the observed genomic pattern as a function of contextual environmental parameters (Ramette, 2007).

4.3 Linking bacterial community profiling to the environment by direct gradient multivariate analysis

To unravel the environmental parameters eventually responsible for the diversity pattern observed, we chose direct gradient canonical correspondence analysis (CCA). Long ago developed by community ecologists to elucidate the relationships between biological assemblages of species and their environment, it is only recently that CCA has been applied to microbial assemblages from marine (Cordova-Kreylos et al., 2006; Hannig et al., 2006; Klaus et al., 2007; Sapp et al., 2007), lake (Yannarell and Triplett, 2005) and soil (Salles et al., 2004) systems. Problems with this method may arise when explanatory variables are intercorrelated, making indistinguishable their separate effects. To avoid this problem, we performed separate analysis of measured environmental variables by organizing them in three ranks: physico-chemical variables, lipid biomarkers and phytoplankton pigments. We also observed Spearman's rank pairwise correlations between variables within each rank and avoided colinear variables when performing CCA.

4.4 CCA with physico-chemical parameters

Our CCA analyses, using physico-chemical data as explanatory variables, showed a temporally stable vertical stratification of the bacterial community. At the DYFAMED station, under the oligotrophic conditions observed, the diversity data alone is statistically significantly related to changes in concentrations of salinity, nitrate and phosphate variables and to a lesser extend oxygen, DOC, PAR and temperature (Fig. 3). Physical and chemical parameters are usually studied in combination with bacterial activity or bacterial growth to demonstrate their influence on the prokaryotic component of the marine food web (Caron, 1994; Downing et al., 1999), sometimes with specific changes along the water column (Riemann et al., 1999). At the DYFAMED station, bacterial activity was limited by phosphorus above the deep-chlorophyll maximum (DCM) level (0–40 m) and by carbon at intermediate depth (60–150 m) for the same seasonal sampling time as in our study (Van Wambeke et al., 2002). Our results are consistent with Fuhrman et al. (2006) that reported high predictability and significant influence of physico-chemical parameters (temperature, oxygen, salinity, dissolved nitrite and dissolved silicate) in annually reoccurring surface marine bacterial communities. The originality of the present study is to unravel such question in spatial (0–1000 m depth) gradients and we demonstrate that physico-chemical parameters are acting in synergy with nearly equal contribution to the bacterial vertical stratification. A complex interaction of

physico-chemical parameters is therefore necessary to predict bacterial community structure in marine environments.

4.5 CCA with lipid class biotracers

Since lipid classes are useful markers to determine sources and fate of organic matter in marine systems (Parrish, 1988) they have widely been used as biological tracers (Parrish et al., 1995; Derieux et al., 1998; Goutx et al., 2000), but only once in conjunction with bacterial community structure changes (Ghiglione et al., 2007). In the present study CCA analysis showed that dissolved lipids from chloroplast origin (LC), which indicate phytoplankton source of DOM (Goutx et al., 1990), were the main biotracer associated with bacterial community structure, due surely to their high concentration at particular samples of 5–20 m depths (Fig. 4). Inputs of LC from microalgae to the dissolved carbon pool might occur through cell lysis, grazing by herbivorous and/or viral lysis. However, we found a significant correlation between dissolved LC concentrations and both *Synechoccocus* cells number and *Synechoccocus* to total picophytoplankton ratio (R^2=0.55 and R^2=0.61, p<0.01, n=36, respectively). This result, together with pico-phytoplancton (*Synechococcus* and *Prochlorococcus*) dominance in the sampling site (see Results) let us infer that *Synechoccocus* cells are the major source of LC pools in our samples. The other four classes of dissolved lipid biomarkers contributed to a lesser extent to bacterial community structure differences among samples along the water column perhaps because their biological signature is more difficult to track. Phospholipids (PL) are membrane compounds in biological cells (Gérin and Goutx, 1993), wax esters (WE), are storage compounds in zooplankton and bacteria (Sargent et al., 1977; Ishige et al., 2003), trigycerides (TG) are reserve compounds in zooplankton, microalgae, bacteria and flagellates (Lee et al., 1971; Parrish and Wangersky, 1987; Alvarez and Steinbüchel, 2002; Méjanelle et al., 2005) and metabolites (Met) are products of acyl-lipid hydrolysis, free fatty acids, free alcohols, diglycerides and monoglycerides (Goutx et al., 2003). Their marginal effect upon the sample scores in the ordination diagram is probably because, taken together, these biomarkers indicated the presence of freshly biosynthesized organic matter not exclusively from phytodetritus.

4.6 CCA with pigment analysis-based phytoplankton diversity

It is well known that marine phytoplankton biomass and species distributions vary along vertical profiles, an obvious example being the deep chlorophyll maximum found in many areas of the world's oceans. This distribution may affect prokaryotic assemblages as well, since phytoplankton distribution appears to be one of the most important biotic variables influencing planktonic bacterial growth (Azam et al., 1983; Cole et al., 1988). Some studies have proposed a

possible relation between bacterial community structure and phytoplankton, but most of them were limited to the study of chlorophyll-*a* pigments. Phytoplankton pigment signatures obtained by high-pressure liquid chromatography are widely used as a proxy of phytoplankton diversity in marine systems (Bustillos-Guzman et al., 1995; Mackey et al., 1998; Marty et al., 2002). We used this signature to determine how phytoplankton taxonomic composition explained bacterial community CE-SSCP profiles. We found that the stratification of bacterial community structures in two shallow layers, 0–40 m and 60–150 m, coincided with the higher presence of *Synechococcus*, euglenophytes and dinoflagelates in the first 40 m samples, while diatoms and pelagophytes were preferentially present at samples bellow 60 m. Furthermore, separation of samples situated at the chlorophyll-*a* maximum corresponds with a higher abundance of prochlorophytes and prasinophytes at this depth. The general preferential abundance of some of these groups along the water column coincides with our findings, particularly for *Synechococcus* (Veldhuis et al., 2005), diatoms (Latasa et al., 1992), and pelagophytes (Claustre et al., 1994). It is also the case for prochlorophytes which are preferentially located at the base of the chlorophyll maximum (Li and Wood, 1988; Marty et al., 2002) and for prasinophytes which have been described as forming localized maxima at depth in the Mediterranean Sea (Marie et al., 2006). Euglenophytes and dinoflagelates presence at the surface layers is particular to this study. On the other hand, because of their uniform distribution in the first 150 m depth, it is not surprising that prymnesiophytes, cryptophytes and chlorophytes had low influence on bacterial community changes with depth. Our results are the first study to demonstrate a compartmentalized influence of each phytoplankton group on bacterial community structure stratification in the first 150 m of the water column.

5 Concluding remarks

Our study propose a complex biogeochemical dataset, which includes physico-chemical parameters, lipid class biotracers and pigment analysis-based phytoplankton diversity, analyzed together with microbial community profiles to unravel the environmental parameters responsible for bacterial community structuring with depth in the marine environment. Direct gradient multivariate canonical analyses using these parameters as explanatory environmental variables of the molecular fingerprinting profiles along the water column revealed that the observed vertical zonation of bacterial communities in three layers, above, in or just below the chlorophyll maximum and deeper, is due to synergetic driving forces pertaining to the three biogeochemical levels analyzed in this study. Similarly, the interaction of several of these parameters further distinguished samples situated above and in the chlorophyll maximum (up to 150 m). The role of the quality of the organic matter (DOC and CDOM)

is significant in the upper layers (Fig. 3) and we have been able to attribute it to a phytoplankton origin (Figs. 4 and 5). The influence of phytoplankton was not limited to exudates released at the surface levels since different phytoplankton phylogenetic groups explain the bacterial community structure zonation in the first 150 m depth (Fig. 5). This finding is consistent with a notion of specific association between bacteria and phytoplankton.

In our conditions, resources ("bottom-up" control) as well as other factors such as pressure, temperature, light, O_2 and PAR controlled simultaneously the vertical zonation of bacterial communities in the water column. Unfortunately, the parameters measured in our study do not permit to evaluate the effect of predation by viruses or protists ("top-down" control). There are indications in the literature that bacteria are tightly controlled by protistan predation in highly oligotrophic systems, whereas their growth is limited by competition for nutrients in more productive waters (Strom, 2000; Gasol et al., 2002). On the other hand, virus-induced mortality seems more substantial when bacterial productivity is enhanced or in anoxic conditions (Weinbauer et al., 2004). "Sideways controls" such as competition for resource, allelopathy or syntrophy may also influence bacterial community structure in marine environments and the new insights given by metagenomics approach should allow more thorough investigation of this subject in the near future (Fuhrman and Hagström, 2008; Long and Azam, 2001; Long et al., 2003). Numerous factors can regulate microorganism population dynamics, often simultaneously, and our study together with other evidences found in the literature (Fuhrman et al., 2006) underline the importance of relevant statistical analysis for further multidisciplinary investigations to investigate the relative importance of environmental factors in predicting the bacterial community dynamics in marine systems.

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