JOURNAL OF PLANKTON RESEARCH | VOLUME 33 | NUMBER 8 | PAGES 1249–1262 | 2011

Effect of bacterial community dynamics on DOC seasonal changes in the north-western Mediterranean Sea

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Received August 17, 2010; accepted in principle February 16, 2011; accepted for publication February 21, 2011

Corresponding editor: Roger Harris

To better understand the seasonal variation in concentrations and accumulation of dissolved organic carbon (DOC) in north-western Mediterranean surface waters, we investigated changes in bacterial abundance, activity (production, BP; respiration, BR; growth efficiency, BGE) and DOC concentration at different depths during different periods: winter mixing, the phytoplankton bloom period and summer stratification. The highest DOC concentrations were observed during the stratified period, reaching 90 ± 10 μmol C L^{-1}. During this period, DOC concentrations were negatively correlated with the BP and BGE values at 5 m and positively correlated with BR at 5 and 20 m. DOC concentrations were also negatively correlated with viral abundances at 5 and 20 m depths. Furthermore, viral abundances were negatively correlated with bacterial abundance, production and growth efficiency, thus suggesting a viral influence on bacterial DOC assimilation. Inorganic nutrient depletion observed during the summer period had no significant relationships with bacterial activity. Characterization of the bacterioplankton assemblages by denaturing gradient gel electrophoresis of 16S rRNA profiles revealed the existence of seasonal trends in phylogenetic identity of the bacterioplankton. During the summer stratification period, the relative intensity of specific phylotypes was correlated with DOC concentrations. *Alphaproteobacteria* and *Synechococcus* sp. phylotypes were highly correlated with chlorophyll *a*, BP and BGE.

KEYWORDS: DOC seasonality; bacterial activity; community structure

INTRODUCTION

The western Mediterranean Sea is characterized by seasonal variability in hydrological structure and trophic regimes, ranging from oligotrophy with summer stratification to winter mixing followed by a period of mesotrophy with a bloom period occurring in spring. The seasonal changes correspond with shifts in concentrations of dissolved organic carbon (DOC). Several studies have demonstrated spring and summer accumulations of DOC in surface waters (Copin-Montégut and Avril, 1993; Carlson *et al.*, 1994; Zweifel *et al.*, 1995; Børsheim, 2000) and also have documented the accumulation of DOC in Mediterranean surface waters (Copin-Montégut and Avril, 1993; Avril, 2002). However, information on the factors limiting DOC degradation in the Mediterranean Sea is scarce. Heterotrophic bacteria are known to be the major consumers of DOC with removal of DOC

doi:10.1093/plankt/fbr024, available online at www.plankt.oxfordjournals.org. Advance Access publication April 21, 2011

via bacterioplankton uptake, a primary biological consumption process (Azam *et al.*, 1983). However, bacteria also require substrates other than carbon, e.g. those containing nitrogen and phosphorus (Azam, 1998). Hence, DOC accumulation may occur because of limitation of the bacterial community by the availability of substrates other than carbon and/or could also be caused by the refractory nature of the DOC (Carlson and Ducklow, 1995; Carlson *et al.*, 1998).

To determine the factors limiting carbon consumption by heterotrophic bacteria, many studies have investigated bacterial metabolic activity. Kirchman (Kirchman, 2000) reviewed a large body of information regarding heterotrophic microbial activity and the biological processing of organic carbon. Other studies have demonstrated an association between changes in bacterial growth and activity rates and the composition of bacterial assemblages (Pinhassi *et al.*, 1999; Riemann *et al.*, 2000; Kirchman *et al.*, 2004). However, direct links between *in situ* DOC changes, bacterial activity, phylogenetic identity and environmental factors are poorly understood. As noted above, the availability of nutrients might be among the factors limiting the processing of DOC by heterotrophic bacterioplankton (Carlson *et al.*, 1994; Williams, 1995). As the Mediterranean waters are known to be phosphorus (P)-limited (e.g. Dolan *et al.*, 1995; Thingstad and Rassoulzadegan, 1995, 1999), Thingstad *et al.* (Thingstad *et al.*, 1997) hypothesized that such a P-limitation may lead to DOC accumulation in the surface layers. During winter, the DOC accumulated in summer is redistributed in the water column due to convective mixing and constitutes the major sources of substrate supporting bacterial production (BP) (Lefèvre *et al.*, 1996).

In this study, we investigated the possible relationships among the seasonal changes in both the activity of heterotrophic bacteria (production, BP; respiration, BR; growth efficiency, BGE) and in community structure (denaturing gradient gel electrophoresis, DGGE of 16S rRNA profiles), in relation to environmental factors (temperature, Chl *a*, phosphate, nitrite and nitrate concentrations) in an attempt to improve understanding of the seasonal changes in DOC concentration in the north-western (NW) Mediterranean surface waters.

METHOD

Water sampling and environmental parameters

Sea water samples were collected using acid-cleaned Niskin bottles at the entrance of Villefranche Bay, Point B: a standard oceanographic station (43°41′10″N, 07°19′00″E). This study site can be influenced by temporal coastal anthropogenic and atmospheric inputs due to its proximity to the coast. Measurements were conducted during three different seasons with a total of 8 weekly series of experiments. The first measurements (December 2005 and January 2006) were conducted during winter mixing conditions. The second period of study (February and March 2006) was during a phytoplankton bloom period. The third period of the study corresponded with summer stratification, lasting from June to July 2006. The three depths (5, 20 and 50 m) of sampling were chosen to compare bacterial populations in relation to DOC at different depths.

Temperature was recorded using a Seabird SBE25 CTD and Chl *a* concentrations were analysed spectrophotometrically following the method of Vidussi *et al.* (Vidussi *et al.*, 1996). Nutrient data were obtained from the SOMLIT observation site from samples obtained on the same day of sampling.

DOC measurement

Total organic carbon (TOC) refers to the sum of particulate (POC) and DOC (μmol C L^{-1}) which was measured in subsamples collected directly from the Niskin bottles into pre-combusted (450°C for 6 h) glass ampoules. DOC samples were generated by removing POC, via syringe filtration through 25 mm pre-combusted GF/F filters (Norrman, 1993). Concentrations were measured with a Shimadzu TOC-5000 TOC analyser as described by Sempéré *et al.* (Sempéré *et al.*, 2003). The filtrates were collected directly in pre-combusted (450°C for 6 h) glass ampoules, acidified with 1.2 M HCl, sealed immediately after collection and stored (triplicates) in the dark at 4°C until processing. DOC concentrations were measured in triplicate; the reproducibility of the DOC measurements was 2 μmol C L^{-1}. The instrument blank was assessed using external standards and run multiple times during each run to establish stability of the analytical system (Certified Reference Materials, Hansell laboratory, Bermuda biological station). The instrument blank was 12 μmol C L^{-1} and was subtracted from the measurements.

Bacterial and viral abundances

For counts of bacteria and viruses, fixed subsamples (glutaraldehyde 2% final concentration) were stained with the nucleic acid stain SYBER Green (1% final concentration, Molecular Probes). Calibrations for bacterial abundance were carried out by addition of fluorescence beads (0.7−1 μm, Polyscience). The counts were

made using a FACS Calibur flow cytometer (Becton Dickinson) according to Marie *et al.* (Marie *et al.*, 1999).

Bacterial activity

BP was measured using the ^{3}H-Leucine filtration method (Kirchman, 2001). Tritiated leucine was added to fresh samples to a final concentration of 40 nM. Triplicates of 100 mL samples and two blank controls fixed with trichloroacetic acid (TCA, 5% final concentration) were incubated at *in situ* temperature in the dark for 2 h. The incubation was stopped by filtering the solution on 0.2 μm pore-size nitrocellulose filters (Millipore HA, 25 mm) and washing with three times 2 ml ice-cold 5 % TCA and 90% ethanol. Leucine incorporation rates were converted to BP by a factor of 1.55 kg C mol^{-1} with an assumption of isotope dilution 1 (Kirchman, 1993).

Before oxygen concentration was measured, water samples were filtered through 0.8 μm filters to eliminate grazers. The filtrate was distributed into 60 mL glass bottles in triplicate. Oxygen concentrations were determined by Winkler titration using an automated titrator (Anderson *et al.*, 1992). O_2 respiration rates were obtained by linear regression of oxygen concentration versus incubation time and transformed to carbon units assuming a respiratory quotient of 1. After 24 and 48 h dark incubation in isothermal incubators at the *in situ* temperature ($\pm 2^{\circ}$C), the oxygen consumption was computed as the slope of the linear regression of nine measurements, each set of three aliquots was measured at three different time points (t_{0h}, t_{24h}, t_{48h}). The regression slopes of the respiration rates versus time, corresponding to bacterial respiration (BR), showed a significant decrease in oxygen values ($P < 0.05$, $n = 9$) and indicated the linearity in the consumption of oxygen with incubation time in all our experiments.

BGE represents the efficiency of bacterioplankton in converting DOC to bacterial biomass. It was measured by dividing BP by the sum of BP and BR. Bacterial carbon demand (BCD) represents the gross flux of carbon channelled through bacterioplankton and was calculated as the sum of BP and BR.

Sampling and extraction of nucleic acid

To concentrate bacterial biomass, 0.5 L of seawater was filtered through 0.2 μm pore-size polycarbonate filters (0.47 mm diameter, Millipore) and stored at -80°C until DNA extraction. Bacterial nucleic acids were extracted from filters as described by Somerville *et al.* (Somerville *et al.*, 1989). DNA was purified with the QIAEX II gel extraction kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Products were checked by electrophoresis on 1% (w/v) agarose gels.

Polymerase chain reaction–denaturing gradient gel electrophoresis

DGGE analysis of the bacterial community composition was done as described in Schäfer and Muyzer (Schäfer and Muyzer, 2001). Polymerase chain reaction (PCR) mixtures (all SIGMA kit, CA, USA) with a final volume of 50 μL contained 5 μL of PCR buffer (10×), 1 μL dNTP (10 mM), 0.5 μL each primer (100 μM), 0.25 μL (5 U/μL) Taq DNA polymerase, 1 μL of DNA and 42.5 μL of sterile water. A "touchdown" PCR was performed to amplify the 16S rRNA using the primer pairs 341F-GC/907R (Muyzer *et al.*, 1993, 1998). PCR products were inspected on 1% (W/V) agarose gels using a molecular mass DNA ladder (Easy Ladder I, 100 lanes, Bioline). Fragments were resolved on 6% (W/V) polyacrylamide gels in 1% TAE buffer with denaturing gradients of 35–70% (urea/formamide), electrophoresis was performed at 60°C and 100 V for 18 h (Schäfer and Muyzer, 2001). DGGE gels were stained for 30 min with 10× SYBER Gold solutions. Imaging was performed with a gel documentation system (GelDoc EQ, BIORad, CA, USA).

Sequencing

Prominent DGGE bands were excised (Sambrook *et al.*, 1989), repeatedly cleaned with additional DGGE gels and re-amplified using the same primers used for the PCR. DNA was purified with the Qiaquick PCR purification kit (Qiagen), following the manufacturer's protocol. Products were checked by electrophoresis in 1% (w/v) agarose gels. Sequencing was performed by MWG Biotech, Ebersberg, Germany.

Statistical analysis

To test the significance of seasonal differences in DOC, BP, BR and BGE values, statistical analyses included analysis of variance (one-way ANOVA) in conjunction with the Tukey *post hoc* test for pair-wise comparison (differences were considered significant at *P*-values of <0.05).

Analysis of DGGE band patterns was carried out with the Quantity One (BioRad) software package. The program detects the presence and absence of bands in individual lines and their relative intensities (in %). The ordination by non-metric multidimensional scaling (nMDS) was performed as described by Kruskal and

Table I: Means and standard deviations (SD, in parentheses) during three different periods of study

$n = 3$	Date of sampling	T (°C)	Chl *a* ($\mu g\ L^{-1}$)	NO_3 ($\mu M\ L^{-1}$)	NO_2 ($\mu M\ L^{-1}$)	PO_4 ($\mu M\ L^{-1}$)	DOC ($\mu M\ C\ L^{-1}$)	POC/DOC (%)
Mixing period	6 December 2005	19.57 (3.36)	0.33 (0.05)	2.79 (1.95)	0.01 (0.01)	0.17 (0.04)	71 (1)	8.03 (0.52)
	13 December 2005	20.40 (0.48)	0.27 (0.01)	0.62 (0.41)	0.03 (0.01)	0.18 (0.04)	64 (5)	7.53 (1.17)
	20 December 2005	20.22 (0.08)	0.27 (0.08)	0.29 (0.24)	0.01 (0.01)	0.16 (0.01)	65 (3)	8.35 (0.58)
	27 December 2005	19.64 (0.41)	0.35 (0.05)	0.39 (0.09)	0.09 (0.01)	0.16 (0.05)	60 (2)	7.81 (0.67)
	3 January 2006	19.74 (0.09)	0.25 (0.04)	0.02 (0.03)	0.08 (0.01)	0.05 (0.02)	59 (2)	7.76 (0.34)
	10 January 2006	19.59 (0.03)	0.25 (0.01)	0.06 (0.06)	0.08 (0.01)	0.15 (0.05)	59 (4)	8.11 (0.45)
	24 January 2006	18.11 (0.02)	0.36 (0.01)	1.50 (0.13)	0.13 (0.03)	0.04 (0.05)	55 (3)	10.06 (041)
	31 January 2006	16.95 (0.01)	0.29 (0.04)	2.38 (0.01)	0.14 (0.03)	0.05 (0)	57 (2)	8.03 (0.52)
Phytoplankton bloom period	7 February 2006	12.76 (0.02)	0.63 (0.04)	2.12 (0.34)	0.14 (0.04)	0.12 (0.03)	60 (1)	11.34 (0.64)
	14 February 2006	12.77 (0.01)	0.32 (0.01)	1.95 (0.11)	0.11 (0.04)	0.18 (0.03)	57 (5)	10.22 (0.8)
	23 February 2006	12.74 (0.01)	0.48 (0.05)	3.13 (0.07)	0.12 (0.05)	0.13 (0.05)	57 (2)	8.91 (0.15)
	28 February 2006	12.69 (0.04)	0.33 (0.06)	2.95 (0.28)	0.13 (0.05)	0.09 (0.01)	57 (2)	8.46 (0.96)
	7 March 2006	12.67 (0.10)	0.34 (0.04)	3.11 (0.26)	0.15 (0.04)	0.08 (0.02)	57 (2)	9.29 (0.33)
	14 March 2006	12.70 (0.01)	2.45 (0.04)	3.51 (0.12)	0.14 (0.03)	0.08 (0.01)	61 (1)	11.00 (0.09)
	21 March 2006	12.48 (0.03)	1.95 (0.37)	1.43 (0.34)	0.24 (0.03)	0.08 (0.01)	77 (3)	12.33 (1.19)
	30 March 2006	13.16 (0.06)	1.95 (0.5)	1.43 (0.28)	0.23 (0.01)	0.07 (0.01)	73 (2)	13.59 (0.43)
Stratified period	1 June 2006	17.53 (3.41)	0.25 (0.07)	0.20 (0.07)	0.17 (0.09)	0.02 (0.01)	82 (2)	6.55 (0.15)
	6 June 2006	16.83 (2.47)	0.30 (0.08)	0.25 (0.59)	0.17 (0.08)	0.02 (0.02)	85 (4)	6.66 (0.40)
	13 June 2006	17.11 (3.24)	0.36 (0.15)	0.12 (0.03)	0.13 (0.04)	0.01 (0.01)	85 (2)	6.64 (0.17)
	20 June 2006	18.56 (3.22)	0.23 (0.05)	1.00 (0.52)	0.09 (0.08)	0.05 (0.03)	85 (7)	6.46 (0.30)
	27 June 2006	19.68 (4.19)	0.21 (0.18)	0.64 (0.13)	0.07 (0.06)	0.02 (0.01)	89 (5)	6.60 (0.08)
	4 July 2006	19.40 (4.95)	0.20 (0.03)	0.28 (0.08)	0.05 (0.05)	0.01 (0.01)	90 (10)	5.74 (1.28)
	11 July 2006	19.75 (5.12)	0.46 (0.24)	0.30 (0.12)	0.05 (0.04)	0.02 (0.01)	81 (7)	6.40 (0.31)
	18 July 2006	20.75 (5.40)	0.30 (0.25)	0.32 (0.16)	0.05 (0.03)	0.02 (0.01)	80 (9)	6.41 (0.32)

T, temperature; Chl *a*, chlorophyll *a*; NO_2, nitrites; NO_3, nitrates; PO_4, phosphates; DOC, dissolved organic carbon; POC, particulate organic carbon.

Wish (Kruskal and Wish, 1978) using the program Systat 9.0.

The associations among bacterial assemblage structure and abiotic factors (Chl *a*, T, phosphates, nitrates, nitrites) as well as the measures of carbon stocks and metabolism (DOC, BP, BR, BGE, BCD) were examined using the Mantel test (Mantel, 1967) using Primer software (Version 5; Clarke and Warwick, 1994). In the Mantel test, correlations of similarity matrices were made in a procedure using the Spearman correlation coefficients instead of the standard Pearson coefficient correlations as used by Clarke and Ainsworth (Clarke and Ainsworth, 1993). The species–environment correlations were measured between three similarity matrices. Data were not transformed. To construct the matrices, the DGGE banding patterns used the Bray–Curtis similarities. However, for the carbon stock and metabolism measures (DOC, BP, BR, BGE, BCD) and the abiotic data (temperature, Chl *a*, phosphate, nitrite and nitrate concentrations), the Euclidean distances were used to construct the similarity matrices.

To examine relationships between bacterial assemblage structure (DGGE band pattern) with both environmental factors and bacterial activity factors, canonical correspondence analysis (CCA) was performed as described by Legendre and Legendre (Legendre and Legendre, 1998) using PAST software (Version 1.67b). The analysis used DGGE band pattern (% of relative intensities) and variables including DOC, temperature (T), chlorophyll *a* (Chl *a*), concentrations of phosphates (PO_4^-), nitrates (NO_3^-), nitrites (NO_2^-) as well as BP, BR, BCD and BGE. The co-linearity of the different variables was assumed and the variance inflation factor of the different variables studied was not checked. The CCA was performed without transformation of data. The intraset correlation coefficients of the different variables as well as the eigenvalues of axes were selected according to their significance level ($P < 0.05$), after applying a permutation test (999 permutations).

RESULTS

The study site, Point B, exhibited typical seasonal changes with a mixed water column in winter followed by a phytoplankton bloom period in spring and a stratified water column from June to September with the thermocline at 30 m depth. The seasonal distribution of DOC and POC concentrations at the three depths is shown in Table I. Winter mixing conditions were characterized by low concentrations of Chl *a*, POC and DOC. During the phytoplankton bloom period, the first 5 weeks corresponded to pre-bloom conditions and

the last 3 weeks corresponded to an active phytoplankton bloom period where Chl *a* concentration reached highest levels of 2.45 $\mu g\,L^{-1}$. POC concentration was within 8.5–13.6% of the surface water DOC concentrations (Table I). The high DOC concentrations measured during the phytoplankton bloom period were significantly correlated with high stocks of phytoplankton, as measured by Chl *a* ($r = 0.725$, $P < 0.05$).

The summer stratification was characterized by a depletion of nutrients and low Chl *a* as well as the highest values of temperature recorded in July 2006 of 20.75°C; in comparison, the bloom period temperature was 13.16°C. The DOC concentration measured at the surface was also significantly higher than during the other periods ($P < 0.05$). The mean DOC values reached a maximum of $90 \pm 10\ \mu mol\ C\ L^{-1}$ in July.

Concerning the period of mixing, values ranged from 55 ± 3 to $71 \pm 1\ \mu mol\ C\ L^{-1}$, whereas those observed during the phytoplankton bloom period were below $57 \pm 2\ \mu mol\ C\ L^{-1}$, except for a peak of $77 \pm 3\ \mu mol\ C\ L^{-1}$ occurring in March during the last 2 weeks of the bloom. The POC/DOC ratio was the highest during the bloom period ranging between 8.5 ± 1 and 13.5 ± 0.5%. It varied between 7.5 ± 1.2 and 10 ± 0.5% during the well-mixed period and reached the lowest values during stratification (5.8 ± 1.3 to 6.7 ± 0.4%).

Seasonal variation of bacterial, viral abundances and bacterial activity

The highest bacterial abundances were observed during the stratified period ($P < 0.05$) and ranged between 8.5 ± 0.9 and $10.8 \pm 0.8 \times 10^8\ cells\ L^{-1}$, whereas the lowest abundances were measured during the well-mixed period attaining $6.24 \pm 0.33 \times 10^8\ cells\ L^{-1}$. In contrast, the lowest viral abundances were measured during the summer stratified period $7.67 \pm 0.6 \times 10^9\ cells\ L^{-1}$, while during the well-mixed period, viral abundances increased to $3.82 \pm 0.2 \times 10^{10}\ cells\ L^{-1}$. The greatest variations of bacterial and viral abundances with depth were observed during summer stratification ($P < 0.05$). The Pearson correlation coefficient analysis (r) between viruses and bacterial abundance and activity at three different depths in the surface water showed several significant relationships. Indeed, the viral abundances were negatively correlated with bacterial abundance at 5 and 20 m ($r = -0.782$ and -0.751, $P < 0.05$, $n = 8$). Also, viral abundances were negatively correlated at 5 and 20 m, with BP ($r = -0.684$, -0.671, $P < 0.05$, $n = 8$) and with BGE ($r = -0.598$, -0.573, $P < 0.05$, $n = 8$).

Figure 1 shows the changes in average BP. The observed values were: 0.09–0.32 and 0.03–0.15 $\mu mol\ C\ L^{-1}\ day^{-1}$ for the mixed and stratified periods, respectively. During the bloom period, BP increased significantly as did Chl *a* ($P < 0.05$). However, the highest BP values were observed during the bloom period. The highest BR rates were measured during the stratified period (Fig. 1); mean values ranged from 2.22 to 2.67 $\mu mol\ C\ L^{-1}\ day^{-1}$, followed by values of 0.77–1.59 $\mu mol\ C\ L^{-1}\ day^{-1}$ for the well-mixed period. The lowest BR values were measured during the bloom period, while during the same period, the BGEs were significantly higher ($P < 0.05$) than values observed during the stratification period (Fig. 1).

Relationship between bacterial activity and DOC values

During the well-mixed period, BR was positively correlated with DOC only at 5 m ($r = 0.776$, $P < 0.05$) (Table II). Also, within the phytoplankton bloom period, BR was correlated with DOC values at 5 m depth ($r = 0.706$, $P < 0.05$). However, both BP and BR at 20 m were correlated with DOC values ($r = 0.731$, $P < 0.05$ and $r = 0.751$, $P < 0.05$), respectively, as shown in Table II. The stratified period was characterized by the highest BR values and these were related to highest DOC values at 5 and 20 m depths ($r = 0.799$ and 0.951, $P < 0.05$), respectively. During the same period, BP as well as BGE were negatively correlated with DOC values observed at 5m ($r = -0.949$ and -0.955, $P < 0.05$), whereas no significant correlations were found at 20 and 50 m.

Relationship between bacterial community structure and carbon metabolism and environmental factors

The DGGE profile of summer samples had at least 14 distinguishable bands at 5 and 20 m (Fig. 2) and 17 bands at 50 m and two specific positions related to the summer stratification period (bands B23 and B25). The sequence data of the 28 excised bands representing five different phylogenetic groups is shown in Table III; most sequences were related to members of *Alphaproteobacteria*. Our samples originating from the three periods revealed different bacterial community structures (Fig. 2a). The ordination by nMDS, based on the DGGE bands, suggested similar bacterial community composition at the three different depths during the three study periods (Fig. 2b). The nMDS analysis indicated that the bacterial community composition during the summer stratification at 5 (D) and 20 m (E) was similar. However, the bacterial

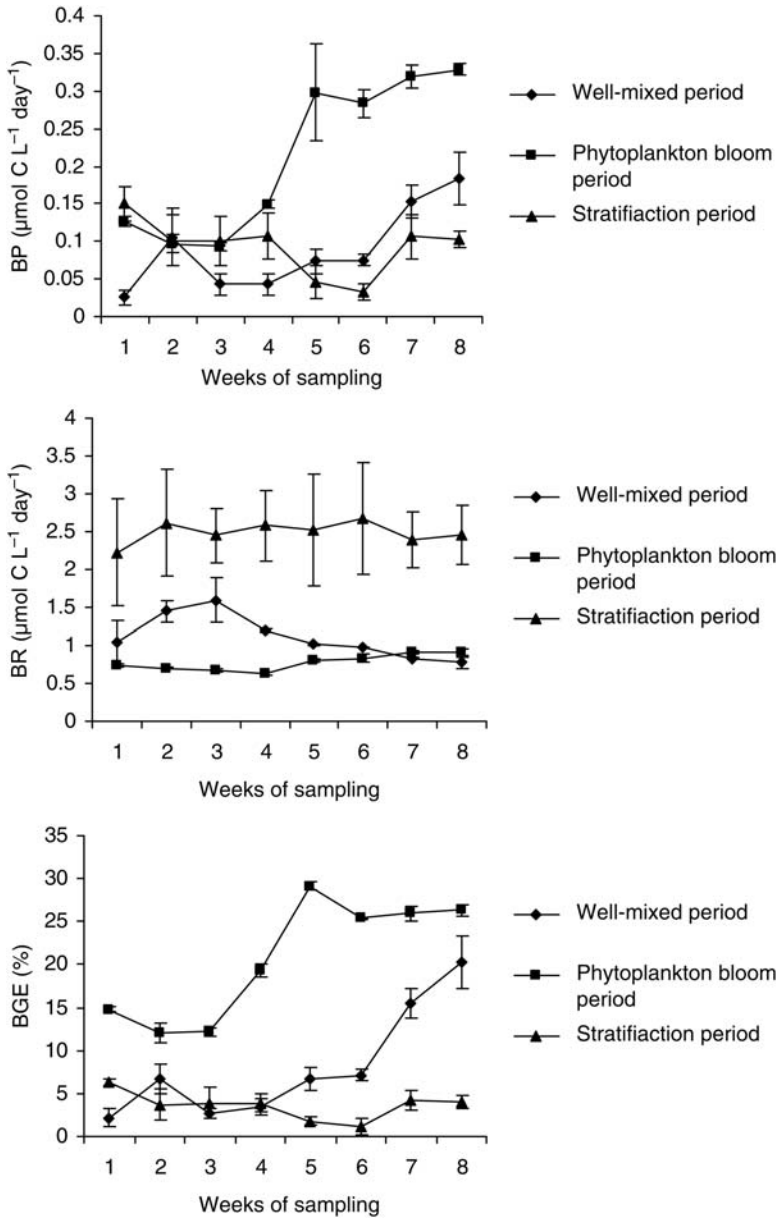

Fig. 1. Seasonal averages (±SD) of depth integrated bacterial activity: BP, BR, BGE, during three different study periods. Error bars are standard deviations for the three depths. The numbers from 1 to 8 refer to each week of sampling, $n = 8$.

community found at 50 m (F) during the summer period was more similar to the bacterial community of the spring bloom at (5, 20, 50 m) and corresponding to A, B, C.

According to the Mantel test analysis, the Spearman correlation coefficient (ρ), measured between the three similarity matrices of bacterial assemblage structure (DGGE banding patterns) and measures of carbon stock and metabolism carbon (DOC, BP, BR, BGE, BCD) and the abiotic data (*T*, Chl *a*, phosphates, nitrates, nitrites), showed stronger relationships between abiotic parameters and carbon variables ($\rho = 0.595$, $P < 0.05$) than between abiotic parameters and bacterial assemblage structure ($\rho = 0.351$, $P < 0.05$). There was also a significant relationship between both abiotic parameters (*T*, Chl *a*, phosphates, nitrates, nitrites) and carbon variables (DOC, BP, BR, BGE, BCD) with the bacterial assemblage ($\rho = 0.448$, $P < 0.05$).

In order to elucidate specific bacterial species–DOC interactions with the different environmental and bacterial activity factors, we performed CCA of bacterial phylotypes and the parameters measures or estimated (*T*, Chl *a*,

Table II: Correlation coefficients measured between DOC and bacterial activity at 5, 20 and 50 m during December 2005 and January 2006 (well-mixed period)

Correlation coefficient (r)	r (DOC-BP) at 5 m	r (DOC-BP) at 20 m	r (DOC-BP) at 50 m
Well mixing period D/J	n.s.	n.s.	n.s.
Phytoplankton bloom period F/M	n.s.	0.731	n.s.
Stratified period J/J	−0.949	n.s.	n.s.
	r (DOC-BR) at 5 m	r (DOC-BR) at 20 m	r (DOC-BR) at 50 m
Well mixing period D/J	0.776	n.s.	n.s.
Phytoplankton bloom period F/M	0.706	0.751	n.s.
Stratified period J/J	0.799	0.951	n.s.
	r (DOC-BGE) at 5 m	r (DOC-BGE) at 20 m	r (DOC-BGE) at 50 m
Well mixing period D/J	n.s.	n.s.	n.s.
Phytoplankton bloom period F/M	n.s.	n.s.	n.s.
Stratified period J/J	−0.955	n.s.	n.s.

During February and March 2006 (phytoplankton bloom period) and during June and July 2006 (stratified period). Significant r values were calculated for ($P < 0.05$), $n = 8$.

PO_4, NO_3, NO_2, DOC, BR, BP, BCD, BGE). Total variation that could be explained by the different environmental and bacterial activity factors accounted for 0.95, as indicated by the sum of all the canonical eigenvalues. The eigenvalues of CCA are shown in Table III and accounted for 0.33 for axis 1 and 0.24 for axis 2. The variance of the bacterial community and the factors studied were mainly explained by the first two axes (35.6% for axis 1 and 25.2% for axis 2). Intraset correlations were also measured between species and parameters (*T*, Chl *a*, PO_4, NO_3, NO_2, DOC, BR, BP, BCD, BGE). The variables, PO_4, BR and BCD, contributed particularly to the gradient, as indicated by the intraset correlation coefficient, respectively (0.941, 0.834, 0.818) with axis 1 (Table IV). However, the DOC variable was important, only with axis 2 (0.539). The CCA biplot of axes 1 and 2 (Fig. 3a) revealed the presence of two main groups of phylotypes, the first was influenced mainly by the variables (BCD, BR, PO_4) and composed by the bands (B4, B5, B8, B9, B11, B15, B16, B21, B22) and the second group mainly influenced by the variables (BGE, Chl *a*, NO2, NO3) and composed by the remaining phylotypes.

The relative intensity of different phylotypes was correlated with DOC concentrations and shown in Table V. Significant Spearman's correlations were observed between the phylotypes B2, B13, B27 and DOC concentrations, respectively ($\rho = 0.770$, -0.752, 0.861, $P < 0.05$). The presence of bands B23 and B25 that were specific to the summer stratification period were not correlated with DOC concentrations, but the same bands B23 and B25 were highly correlated with Chl *a* concentrations ($\rho = 0.849$, $r = 0.805$, $P < 0.05$), and the BGE rates, respectively ($\rho = 0.752$, 0.729, $P < 0.05$), showing an indirect effect on DOC *in situ* concentration.

DISCUSSION

Several factors can control the size of the DOC pool, including DOC production rates, composition of DOM released, the nutrient field supporting phytoplankton and heterotrophic bacteria (e.g. Ducklow *et al.*, 1999). The "malfunctioning of the microbial loop hypothesis" (Thingstad *et al.*, 1997) stated that various factors (such as P-limitation), other than labile DOC substrate concentration, can control bacterioplankton production and thus their capacity to consume utilizable DOC. In fact, low bacterial growth and biomass maintained at low levels by top-down controls and competition for nutrients could slow down DOC consumption and allow accumulation. To determine the factors contributing to DOC variability and accumulation in the NW Mediterranean Sea, we wanted to investigate the seasonal relationships between bacterial activity and community structure with the DOC *in situ* concentration changes.

Regulation of DOC by bacterial activity

During the well-mixed period, DOC was significantly correlated with BR at 5 m ($r = 0.776$, $P < 0.05$), while there was no significant correlation between DOC and BP. This absence of a relationship may be partly due to the small percentage of the DOC pool that supports the majority of bacterial carbon utilization. Legendre and Rivkin (Legendre and Rivkin, 2002) explained the absence of correlation between BP and DOC by the fact that *in situ* DOC reflects the balance between DOC production by the whole pelagic food web and its uptake by only the microbial heterotrophic plankton. DOC corresponded then to the fraction that had not been used by microbial heterotrophic at the time of sampling.

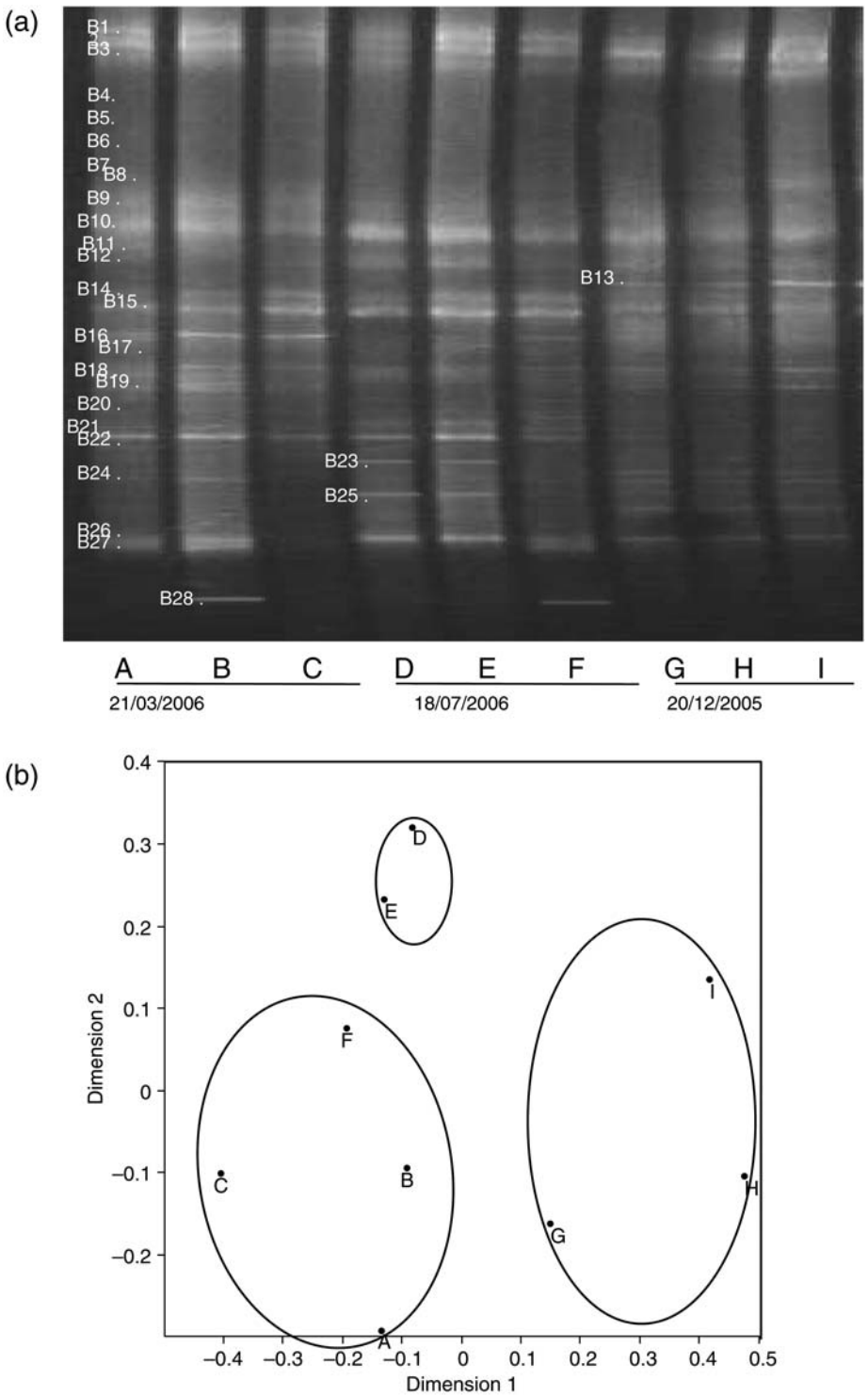

Fig. 2. (**a**). DGGE pattern of samples from different depths (5, 20 and 50 m) representing three periods: A, B, C, phytoplankton bloom; D, E, F, stratification period; and G, H, I, mixing period. Numbers 1–28 represent the different bands. (**b**) nMDS of bacterial composition data (DGGE banding pattern) of samples from different depths (5, 20 and 50 m) representing three periods: A, B, C, phytoplankton bloom; D, E, F, stratification period; and G, H, I: mixing period.

Table III: Phylogentic affiliation of sequences contained in DGGE bands

DGGE band	Closest relatives in GenBank, accession no. of closest relatives (accession no. of the sequence)	Sequence similarity (%) (number of bases)	Phylogenetic group
B1	Uncultured bacterium clone S-DCM-56, GU061963 (HQ 132263)	100 (108)	*Alpha*
B2	Uncultured SAR11 clone ESP450_K6II_21, DQ810671 (HQ 132264)	100 (350)	*Alpha*
B3	Uncultured alphaproteobacterium clone ARG_35, AM238568 (HQ 132264)	100 (235)	*Alpha*
B4	Uncultured marine bacterium clone ZA3821, EU019987 (HQ 132264)	100 (260)	*Alpha*
B5	Uncultured marine bacterium clone C48, EU010198 (HQ 132264)	100 (195)	*Alpha*
B6	Uncultured gammaproteobacterium clone bac 6 HM485304 (HQ 132264)	100 (525)	*Gamma*
B7	Uncultured cyanobacterium, isolate DGGE band 60, AM747456 (HQ 132264)	86 (130)	*Cyano*
B8	Uncultured actinobacterium clone SARG_14, AM238555 (HQ 132264)	100 (479)	*Actino*
B9	Uncultured bacteroidetes bacterium clone T41, DQ436746 (HQ 132264)	95 (554)	*CFB*
B10	Uncultured bacterium clone CEP-DCM-46, GU061792 (HQ 132264)	100 (220)	*Alpha*
B11	Uncultured actinobacterium T41-182, DQ436808 (HQ 132264)	100 (240)	*Actino*
B12	Uncultured actinobacterieum clone T41_69, DQ436799 (HQ 132264)	99 (478)	*Actino*
B13	Uncultured bacteroidetes bacterium clone B17, DQ436746 (HQ 132264)	100 (470)	*CFB*
B14	Uncultured alphaprotebacterium clone A014, DQ874999 (HQ 132264)	100 (234)	*Alpha*
B15	Uncultured actinobacterium clone SIMO_2719, DQ189694 (HQ 132264)	100 (245)	*Actino*
B16	Uncultured gammaproteobacterium clone T42_127, DQ436714 (HQ 132264)	99 (443)	*Gamma*
B17	Uncultured roseobacter sp isolate DGGE Band BL03_band 37, DQ778279 (HQ 132264)	100 (144)	*Alpha*
B18	Uncultured alphaproteobacterium clone G1-49, EU005292 (HQ 132264)	98 (528)	*Alpha*
B19	Uncultured bacterium isolate gel Band B19, EF221657 (HQ 132264)	98 (227)	*Alpha*
B20	Uncultured pelagibacter sp. DGGE gel band FD 13, DQ385018 (HQ 132264)	100 (129)	*Alpha*
B21	Uncultured marine bacterium clone WC0-165, FN435359 (HQ 132264)	99 (470)	*Alpha*
B22	Uncultured marine bacterium, clone WC5-139, FN435440 (HQ 132264)	99 (229)	*Alpha*
B23	Uncultured alpha proteobacterium clone BL03-AUTO3, DQ778230 (HQ 132264)	100 (300)	*Alpha*
B24	Uncultured bacterium clone HF130_C5_P1, DQ300602 (HQ 132264)	100 (272)	*Alpha*
B25	Uncultured Synechoccus sp. Isolate DGGE gel band D2Bn22, EF506910 (HQ 132264)	100 (290)	*Cyano*
B26	Prochlorococcus marinus str.MIT9211, AF115270 (HQ 132264)	89 (230)	*Cyano*
B27	Uncultured roseobacter sp. Isolate DGGE band BL03_band 43, DQ778280 (HQ 132264)	100 (324)	*Alpha*
B28	Prochlorococcus marinus subsp. pastoris str. NATL1 , AF311218 (HQ 132264)	100 (419)	*Cyano*

The sequences obtained in this study are registered in GenBank under accession numbers HQ 132263–HQ 132290. *Actino*, Actinobacteria; *Alpha*, Alphaproteobacteria; *CFB*, Cytophaga-Flavobacteria-Bacteroides; *Cyano*, Cyanobacteria; *Gamma*, Gammabacteria.

Table IV: Intraset correlation coefficients of the environmental and bacterial activity variables with the four axes produced by CCA and the eigenvalues measured for the four axes reported with their P*-values after permutation test (999 permutations)*

	Axis 1	Axis 2	Axis 3	Axis 4
Intraset coefficient correlations				
T	0.603	−0.118	−0.555	−0.377
Chl *a*	−0.589	0.598	0.432	−0.103
PO_4	0.941	0.145	−0.013	−0.090
NO_3	−0.554	0.244	0.654	−0.201
NO_2	−0.634	0.290	0.621	−0.08
DOC	0.285	0.539	−0.063	−0.592
BR	0.834	−0.01	−0.33	−0.171
BP	0.017	0.689	0.322	−0.443
BCD	0.818	0.111	−0.270	−0.241
BGE	−0.405	0.595	0.462	−0.331
Eigen values	0.33	0.24	0.12	0.09
P (permutation test)	0.56	0.53	0.36	0.26

CCA, canonical correspondence analysis. Environmental and bacterial activity variables (PO_4, phosphates; NO_2, nitrites; NO_3, nitrates; *T*, temperature; Chl *a*, chlorophyll *a*; DOC, dissolved organic carbon; BR, bacterial respiration; BP, bacterial production; BCD, bacterial carbon demand; BGE, bacterial growth efficiency).

However, during the phytoplankton bloom, DOC was positively correlated with BP at 20 m ($r = 0.731$, $P < 0.05$) and BR at 5 and 20 m ($r = 0.706$ and 0.751, $P < 0.05$), respectively. Indeed, the elevation of BP could be fuelled by the high primary production observed during the bloom period (e.g. Turley *et al.*, 2000), where Chl *a* concentration reached a maximum of 2.45 $\mu g\ L^{-1}$. The increasing BP and organic matter elevation due to the high primary production resulted in an increase in BGE estimates during the phytoplankton bloom period, as described previously by Carlson *et al.* (Carlson *et al.*, 1999) in the Ross Sea.

During summer stratification, we found a seasonal variability of BGE, with lowest rates measured (2.19%). Lemée *et al.* (Lemée *et al.*, 2002) found that in the open NW Mediterranean, BGE ranged from 0.1 to 43% throughout the study period and that BGE values were the highest during the spring bloom and during the fall. Our data also showed negative relationships between viral abundance and bacterial activity (BP and BGE) during summer stratification likely contributing to DOC accumulation. In fact, viral lysis can equal ~10–30% of daily bacterioplankton production lost (Fuhrman, 1992).

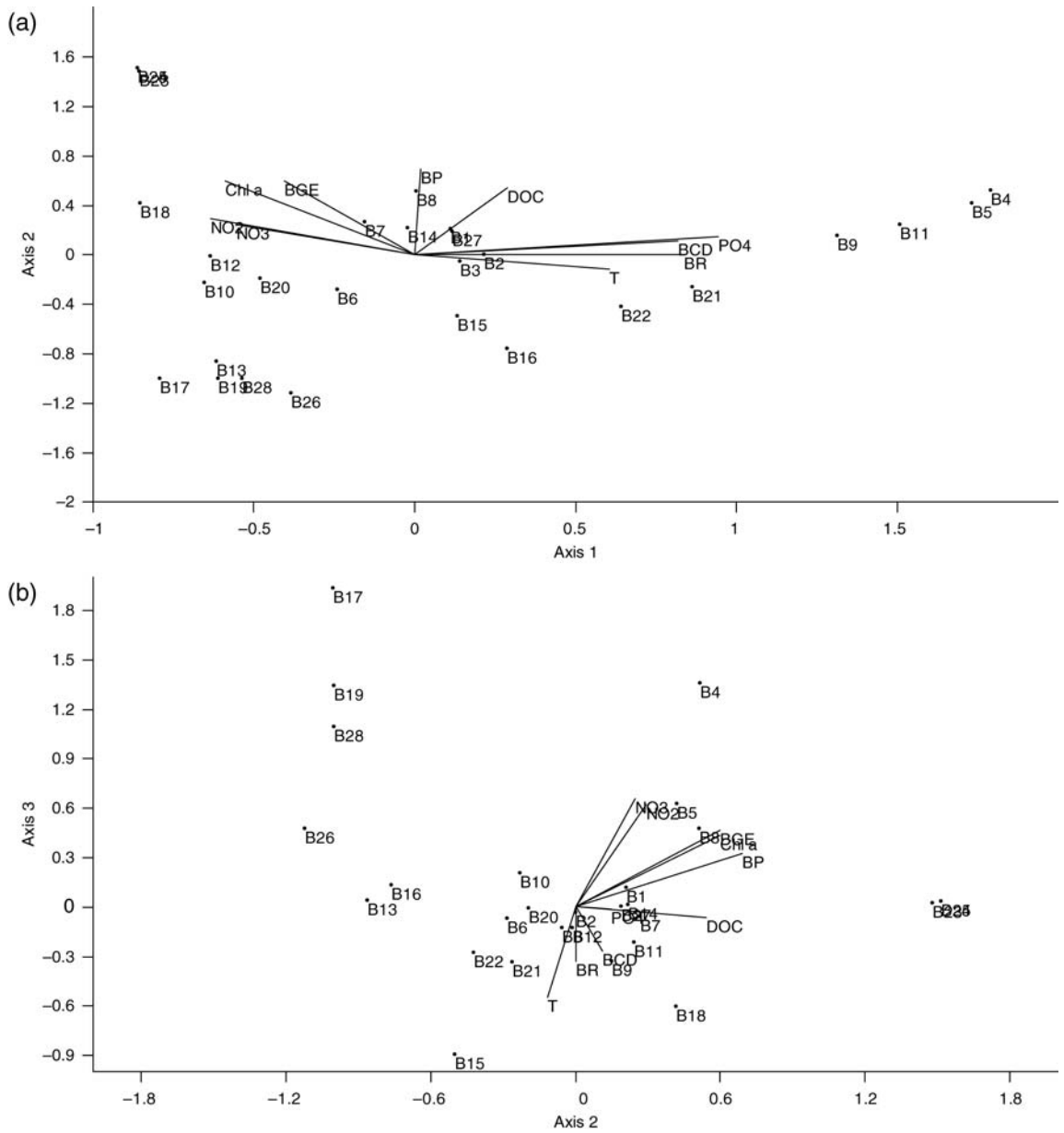

Fig. 3. CCA biplot of intersample and interspecies distances of DGGE fingerprints of the bacterial community from nine samples using 10 variables: PO_4, phosphates; NO_2, nitrites; NO_3, nitrates; *T*, temperature; Chl *a*, chlorophyll *a*; DOC, dissolved organic carbon; BR, bacterial respiration; BP, bacterial production; BCD, bacterial carbon demand; BGE, bacterial growth efficiency. Numbers of 1–28 represent the different phylotypes. Dates of sampling were not represented on the graphs. Lines indicate the direction of increasing values of respective variables, and the length of the lines indicates the degree of correlation of the variable with community data. (**a**) Axes 1 and 2 of CCA biplot. (**b**) Axes 2 and 3 of CCA biplot.

Within the stratified period, the high DOC values we recorded (Table I), in comparison to the other periods, coincided with low BGE estimates (Fig. 1). del Giogio and Cole (del Giogio and Cole, 2000) and del Giorgio and Duarte (del Giorgio and Duarte, 2002) suggested that low growth efficiencies could be related to the degree of energy limitation of the bacterial population. In fact, bacterial growth may be limited by the availability of inorganic nutrients such as N and P (Zweifel *et al.*, 1993; Cotner *et al.*, 1994). Consequently, such a nutrient limitation may impact bacterial DOC uptake, which in turn could affect the magnitude of carbon accumulation. The Mediterranean Sea is known to exhibit a predominance of P- rather than N-limitation (Jaques *et al.*, 1973; Fiala *et al.*, 1976). Zweifel *et al.* (Zweifel *et al.*, 1993) demonstrated how orthophosphate additions can stimulate both

Table V: Non-parametric correlation Spearman (ρ) coefficient between proportions of phylotypes and measured environmental parameters

	Spearman rank correlation coefficient (ρ)									
Band	*T*	Chl *a*	PO_4	NO_3	NO_2	DOC	BR	BP	BCD	BGE
B1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
B2	n.s.	n.s.	0.803	n.s.	n.s.	0.770	0.850	n.s.	n.s.	n.s.
B3	0.711*	−0.750*	n.s.	−0.867	−0.850	n.s.	n.s.	n.s.	0.683*	n.s.
B4	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
B5	n.s.	n.s.	0.80	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
B6	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	−0.667*
B7	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	0.881	n.s.	n.s.
B8	n.s.	0.678*	n.s.	0.695*	n.s.	n.s.	n.s.	n.s.	n.s.	0.881
B9	0.845	n.s.	0.835	n.s.	−0.695*	n.s.	0.842	n.s.	0.842	n.s.
B10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	−0.797	n.s.	−0.763	n.s.
B11	n.s.	n.s.	0.900	n.s.	n.s.	n.s.	0.782	n.s.	0.782	n.s.
B12	n.s.	n.s.	−0.891	n.s.	n.s.	n.s.	−0.661*	n.s.	n.s.	n.s.
B13	n.s.	n.s.	n.s.	n.s.	n.s.	−0.752*	n.s.	n.s.	−0.676*	n.s.
B14	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
B15	0.690*	−0.836	n.s.	−0.801	−0.740	n.s.	n.s.	n.s.	n.s.	−0.670
B16	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
B17	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
B18	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
B19	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	−0.730*	n.s.
B20	n.s.	n.s.	−0.669*	n.s.	n.s.	n.s.	n.s.	n.s.	−0.845	n.s.
B21	0.804	−0.836	n.s.	−0.679*	−0.731*	n.s.	0.923	n.s.	0.792	n.s.
B22	n.s.	−0.749*	n.s.	n.s.	n.s.	n.s.	0.731*	n.s.	n.s.	n.s.
B23	n.s.	0.849	n.s.	n.s.	n.s.	n.s.	n.s.	0.820	n.s.	0.803
B24	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
B25	n.s.	0.805	n.s.	n.s.	n.s.	n.s.	n.s.	0.788*	n.s.	0.772*
B26	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
B27	n.s.	0.861	n.s.	n.s.	n.s.	0.861	n.s.	n.s.	n.s.	n.s.
B28	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	−0.707	n.s.

Statistical significance of ρ: n.s. = $P > 0.05$; *$0.05 < P > 0.01$; otherwise $P < 0.01$. PO_4, phosphates; NO_2, nitrites; NO_3, nitrates; *T*, temperature; Chl *a*, chlorophyll *a*; DOC, dissolved organic carbon; BR, bacterial respiration; BP, bacterial production; BCD, bacterial carbon demand; BGE, bacterial growth efficiency.

bacterial growth and the degradation of DOC in the Mediterranean. However, despite the low concentrations of nutrients, including phosphate, measured during the stratified period (Table I), no significant relationships were observed between nutrient concentrations and BGE values. It should be noted that Goldman and Denett (Goldman and Dennett, 2000) argued that the availability of inorganic nutrients is not a universal control on BGE. The quantity and quality of available organic material with availability of inorganic nutrients can also play an important role in regulating BGE (del Giogio and Cole, 2000). We found that bacterial activity was consistently correlated with nutrient and DOC concentrations. However, this apparent lack of relationships might be explained by the different filtration steps and the conversion factors we employed that during the different periods and in the different depths were involved in obtaining BP, BR, BGE and DOC concentration values. Indeed, the low values of BGE at the beginning of the well-mixed period were similar to the BGE values reported during stratification, while the well-mixed period corresponded to higher phosphate concentration than those reported during the stratified period. The only difference was the elevation of the BR driving the elevation of the BCD and thus allowing more DOC bacterial uptake during the mixed period. Other factors can influence DOC seasonality and have to be considered. Indeed, biosynthesis of compounds resistant to rapid microbial degradation (Carlson *et al.*, 1998) and abiotic processes such as exposure to UV irradiation (Carlson and Ducklow, 1995) can also affect the accumulation of DOC.

Regulation of DOC by bacterial assemblage structure and environmental factors

In this study, several relationships between DOC concentration variability, bacterial assemblage, bacterial activity and environmental factors were found. The nMDS analysis demonstrated that the bacterial community composition sampled during the summer stratification period at 5 (D) and 20 m (E) were very similar and differed from 50 m (E). Indeed, during the summer stratification period, the thermocline was well established at 30 m depth separating the bacterial community of the upper 5

and 20 m from the deeper 50 m bacterial community sample. The Mantel test showed higher significant relationships between abiotic parameters (*T*, Chl *a*, phosphates, nitrates, nitrites) and carbon processing variables (DOC, BP, BR, BGE, BCD) ($\rho = 0.595$, $P < 0.05$) than between abiotic parameters and bacterial community composition ($\rho = 0.351$, $P < 0.05$). However, the intraset correlations measured between bacterial community composition and a suite of factors (*T*, Chl *a*, PO_4, NO_3, NO_2, DOC, BR, BP, BCD, BGE) revealed that the bacterial community composition was mainly correlated with the variables phosphates (PO_4), BR and BCD, as demonstrated by the CCA where the importance of the gradient influenced the community structure. The CCA biplot (Fig. 3a) showed the presence of two main groups of phylotypes, the first influenced mainly by the variables (BCD, BR, PO_4), composed of the bands (B4, B5, B8, B9, B11, B15, B16, B21, B22) and the second group mainly influenced by the variables (BGE, Chl *a*, NO_2, NO_3) and consisting of the remaining phylotypes. Working in the NE Atlantic, Alonso-Sáez *et al.* (Alonso-Sáez *et al.*, 2007) showed changes in phylogenetic composition associated with a wide range of variables: DOC, BP, BR and BB. Our data suggest that in the bacterial assemblages, the phylotypes *SAR11*, *Bacteroidetes*, *Roseobacter*, represented by the bands, B2, B13 and B27, were correlated with the DOC concentrations (Table V), especially the members of the *Roseobacter* clade. These results agree with Pinhassi *et al.* (Pinhassi *et al.*, 1999) who showed the possible role played by the *Roseobacter* clade in dissolved organic matter degradation. Furthermore, members of *Roseobacter* clade have been suggested to be active colonizers of particles under algal bloom conditions (González *et al.*, 2000). Also the *SAR11* group of *Alphaproteobacteria* was significantly correlated with the DOC concentration. Mou *et al.* (Mou *et al.*, 2007, 2008) showed that among several DOC-transforming assemblages, members of the *Roseobacter* and *SAR11* clusters, which are an integral component of the Mediterranean Sea microbial community (Zaballos *et al.*, 2006), can be potentially linked to the DOC heterotrophic assimilation. The *Bacteroidetes* cluster has also been shown to play an important role in the degradation of organic matter (Cottrell and Kirchman, 2000; Kirchman, 2002).

CONCLUSION

During summer stratification, Villefranche Bay is characterized by P-limitation of bacteria (Thingstad *et al.*, 1997). While the early spring bloom period was characterized by high nutrient levels and a high phytoplankton growth, the stratification period was characterized by low growth efficiency, and low concentrations of free mineral nutrients which implies a longer DOC bacterial consumption time. The accumulation of DOC during the summer stratification period could be linked to low BGE such as that observed at 5 and 20 m. During this period, BR was driving BCD and the quantity of carbon passing through the bacterial community was mainly used for respiration rather than used by the higher trophic levels (Legendre and Rasoulzadegan, 1995). However, even with similar values of BGE and different phosphate limitation conditions observed during the well-mixed period as well as during the stratified period, the DOC concentrations were very different. It is tempting to link the relationships between viruses and the decreases in bacterial activity during the summer stratified period to DOC accumulation as suggested previously by a double effect of "top down" viral control and nutrient limitation as in the microbial malfunctioning hypothesis. During the phytoplankton bloom, nutrient availability and the quantity of DOC increased, phytoplankton sustained bacterial metabolism, BP was higher than BR, the energetic cost of bacterial growth appeared to decrease, and a decrease in the BGE was also apparent.

The use of DGGE revealed that the bacterial community during summer stratification was very similar between the depths 5 and 20 m, isolated from the deeper community by the thermocline. The CCA showed the presence of two main groups of phylotypes, the first mainly influenced by the variables (BCD, BR, PO_4), and the second group mainly influenced by the variables (BGE, Chl *a*, NO_2, NO_3) and composed of the remaining phylotypes. However, direct correlations showed that among the microbial community, the three phylotypes *SAR11*, *Roseobacter* members of the *Alphaproteobacteria* and the *Bacteroidetes* (CFB) were potentially linked to the DOC *in situ* concentration variability.

ACKNOWLEDGEMENTS

We wish to thank M.D. Pizay for the help with DOC analysis, J.R. Dolan for his precious comments and corrections of the manuscript and we appreciate the constructive criticism of two anonymous reviewers that improved the manuscript.

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