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Bottom up effects on bacterioplankton growth and composition during summer-autumn transition in the open NW Mediterranean Sea

F. Van Wambeke[1], J.-F. Ghiglione[2,3], J. Nedoma[4], G. Mével[5,6], and P. Raimbault[1]

[1]Laboratoire de Microbiologie, Géochimie et Ecologie Marines, CNRS, Université de la Méditerranée, Centre d'Océanologie de Marseille, UMR6117, Campus de Luminy, case 901, 13 288 Marseille Cedex 09, France
[2]CNRS, UMR7621, Laboratoire d'Océanographie Biologique de Banyuls, Avenue Fontaulé, BP 44, 66 650 Banyuls-sur-Mer, France
[3]UPMC Univ Paris 06, UMR7621, Laboratoire ARAGO, Avenue Fontaulé, BP 44, 66 650 Banyuls-sur-Mer, France
[4]Biological Centre of the Academy of Sciences of the Czech Republic, Hydrobiological Institute, Na sádkách 7, 37005 České Budějovice, Czech Republic
[5]CNRS, UMR7144, Equipe de Chimie Marine, Station Biologique de Roscoff, 29 682 Roscoff, France
[6]UPMC Univ Paris 06, Equipe de Chimie Marine, Station Biologique de Roscoff, 29 682 Roscoff, France

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Abstract. We examined the vertical and temporal dynamics of nutrients, ectoenzymatic activities under late summer-fall transition period (September–October 2004) in NW Mediterranean Sea in relation to temporal change in factors limiting bacterial production. The depth of the mixed layer (12.8±5.3 m) was extremely stable until the onset of the destratification period after 11 October, creating a zone where diffusion of nutrient from the much deeper phosphacline (69±12 m) and nitracline (50±8 m) was probably strongly limited. However after 1st October, a shallowing of nutriclines occured, particularly marked for nitracline. Hence, the nitrate to phosphate ratio within the mixed layer, although submitted to a high short term variability, shifted the last week of the cruise from 1.1±1.2 to 4.6±3.8, and nitrate increased by a factor 2 (0.092±0.049 μM). A corresponding switch from more than one limitation (PN) to P-only limitation of bacterial production was observed during the month as detected by enrichment bioassays. Differences in the identity of the limiting nutrient in surface (5 m: N and P at the beginning, strictly P at the end of the study) versus 80 m (labile carbon) influence greatly bacterial community structure shift between these two layers. The two communities (5 and 80 m) reacted rapidly (24 h) to changes in nutrient concentrations by drastic modification of total and active population assemblages resulting in changes in activity. For bacterial production values less than 10 ng C l^{-1} h^{-1} (associated to deeper layers), aminopeptidase and lipase exhibited higher activity relative to production whereas phosphatase varied in the same proportions than BP on the range of activities tested. Our results illustrate the effect of bottom-up control on bacterial community structure and activities in the epipelagic NW Mediterranean Sea.

Correspondence to: F. Van Wambeke
(france.van-wambeke@univmed.fr)

1 Introduction

Bacterioplankton plays a central role in the nutrient and energy flux in marine ecosystems as the main significant consumers of dissolved organic matter in the oceans. Bacterial growth may be limited by several factors such as temperature, DOM, labile organic carbon, inorganic nutrient or micronutrient such as iron. In the Mediterranean Sea, the availability of P can limit photosynthesis, nitrogen uptake and heterotrophic bacterial production (Fiala et al., 1976; Thingstad et al., 1998; Moutin and Raimbault, 2002). Notably, P Limitation of heterotrophic bacterial production has been demonstrated on different places (Thingstad et al., 1998; Zohary and Robarts, 1998; Van Wambeke et al., 2002). Transition between P and labile carbon limitation has been shown along vertical profiles (Sala et al., 2002; Van Wambeke et al., 2002), or seasonally in a given place (Pinhassi et al.,

2006). The methodology generally used to track factors limiting bacterial production (BP) are enrichment bioassays which consists of enclosing seawater in a flask where different amendments are made and then bacterial growth or abundance is followed over time. However, confinement effects play a role not only on bacterial production rates, but also on diversity of bacterial populations whatever the initial volume of seawater incubated (from bottles to mesocosms: Schäfer et al., 2000; Massana et al., 2001). Only a small fraction of dissolved organic matter can be directly taken up by bacteria, therefore, expression (genetic potential) and physiological regulation of ectoenzymatic activity play a major role in heterotrophic bacterial growth. Maximum hydrolysis rates of ectoenzymes, determined by fluorogenic substrates (Hoppe et al., 1998), has been related to a variety of biogeochemical parameters. The presence of ectoenzymes is widespread among marine bacteria (Martinez et al., 1996). Alkaline phosphatase, also expressed by some autotrophic organisms, has been widely studied in the Mediterranean Sea and lakes because its expression is a good indicator of P-deficiency. More recently, phosphatase to aminopeptidase ratio has been used for indication of P versus N deficiency (Sala et al., 2001). We could also expect that the expression of phosphatase (as P provider) aminopeptidase (as N provider) and lipase (as C provider) would be determined by the composition of available organic matter (Zoppini et al., 2005) and thus, that patterns of enzyme expression will change along depth quality/stoechiometry of organic matter as well as factors limiting bacterial production.

The PECHE program was dedicated to investigate summer-autumn seasonal transition at the DYFAMED site (NW Ligurian Sea). In the frame of this program, we focused on the study of heterotrophic activities and factors controlling bacterial production at the month time scale.

The main objectives of the study were (i) to compare the effect of enrichment experiments on bacterial production in accordance with short term (month) temporal changes among in situ biological metrics related to heterotrophic activity: bacterial production, ectoenzymatic activities (phosphatase, aminopeptidase, lipase), nutrient inventories and stoechiometry (phosphate, nitrate) and (ii) to determine whether the diversity of total vs. metabolically active bacteria were substantially different within the epipelagic layer (surface to 150 m depth) and how and which community reacted to enrichments. For this purpose, we used an integrated approach combining DNA- and RNA-based capillary electrophoresis single strand conformation polymorphism (CE-SSCP). We hypothesized that differences in the composition of the bacterial community structure with and without nutrient enrichment would depend on the type and extent of in situ nutrient limitation.

2 Materials and methods

2.1 Study area and sampling for in situ measurements and bioassays

Water samples were taken near the DYFAMED site during one cruise aboard the r/v Thalassa between 14 September and 17 October 2004, in the frame of the PECHE program which was dedicated to investigating summer-autumn seasonal transition at the DYFAMED site (NW Ligurian Sea, see Andersen et al., 2009). The sampling strategy of the cruise was settled to follow drifting sediment trap and to perform high frequency CTD casts during the cruise particularly during 4 cycles C1 (17 September 04:00 LT- local time to 22 September 17:00 LT), C2 (24 September 16:00 LT to 29 September 20:00 LT), C3 (3 October 18:00 LT to 8 October 20:00 LT) and C4 (C4: 10 October 16:00 LT to 15 October 21:00 LT). During these cycles, the minimum interval between two CTD casts was 6 h, with some shorter periods of 3 h (see Andersen et al., 2009).

For experimental studies of nutrient limitation of bacterial growth, water was sampled at different depths from the Niskin bottles from selected CTD rosette at 12:00 LT on 18, 21 and 26 September and on 4 and 7 October. Depth levels of 5 m and 80 m were investigated for bioassays, whereas a more detailed profile (0–150 m) was sampled for leucine incorporation rates, ectoenzymatic activities, DOC and nutrients. For bioassays, aliquots of sea water were transferred to 250 ml acid-washed polycarbonate bottles and inorganic nitrogen (N: 1 μM as $NaNO_3$+1 μM as NH_4Cl), inorganic phosphorus (P: 0.25 μM as NaH_2PO_4) or glucose (G: 10 μM C-glucose) were added. A control was left unamended, and a flask received all four components (GNP). The flasks from 5 m depth were placed in a water bath with neutral density screens simulating 50% incident light. The flasks from 80 m depth were placed in a dark, cooled incubator at 14°C for 24 h. After incubation of 24 h, flasks were subsampled for bacterial production. Samples were also taken for CE-SSCP analysis were also sampled on two occasions (21 September and 7 October) along vertical profiles and after 24 h enrichments at 5 and 80 m depths. Finally, on 27–28 September, and on 12–13 October, we investigated whether bacterial production response to enrichment at 5 m depth differed according the sampling time of the day. Seawater was sampled at 18:00, 00:00, 06:00 LT on 27–28 September and 19:00, 00:00, 06:00 and 12:00 LT on 12–13 October. In all cases, flasks were left in sea-surface circulating water bath with natural incident light and where incubated during 24 h exactly before bacterial production measurements. There were not replicated bottles for the different enrichments, as we preferred to carry out more frequently experiments, however previous experience with duplicates and triplicates showed a good reproducibility and suggest that factors of increase more than $\times$1.5 are highly significant (Pinhassi et al., 2006; Van Wambeke et al., 2008).

Table 1. Synthesis of the criteria used to determine depths of mixed layer (Z_m) phosphacline (Z_P) and nitracline (Z_N). SRP: soluble reactive phosphorus NO_3: nitrate, σ: density excess, z: depth of Z_m, Z_p, Z_N, surf: first surface layer sampled (between 1 and 3 m according ctds), Δz: difference between 2 successive layers sampled for NO_3 and SRP. For σ Δz=1 m.

Criterion	mixed layer Z_m	phosphacline Z_P	nitracline Z_N
Depth z for which			
$[SRP]_z$-$[SRP]_{surf}$, $[NO_3]_z$-$[NO_3]_{surf}$	0.05 kg m^{-3}	0.01 μM	0.5 μM
$[\sigma]_z$-$[\sigma]_{surf}$ is more or equal to	0.1 kg m^{-3}	0.05 μM	1 μM
Depth z for which			
$[SRP]_{(z-\Delta z)}$-$[SRP]_z$, $[NO_3]_{(z-\Delta z)}$-$[NO_3]_z$	0.02 kg m^{-3}	0.05 μM	0.5 μM
$[\sigma]_{(z-1)}$-$[\sigma]_z$ is more or equal to	0.01 kg m^{-3}	0.1 μM	1 μM

2.2 Physico-chemical parameters

Dissolved organic carbon (DOC) by HTCO was analyzed as described by Ghiglione et al. (2008). Samples for nutrient analysis obtained from CTD casts were immediately analyzed on board by automated colorimetric procedures using a Technicon Autonanalyser® as described in Marty et al. (2008). Nitrate (abbreviated as NO_3 in the text) concentrations were determined applying a sensitive method (Raimbault et al., 1990). Soluble reactive phosphate (SRP) concentrations were measured according to Tréguer and Le Corre (1975). Detection limits (and analytical precision) are 0.003 ($\pm$0.003) μM, 0.02 ($\pm$0.01) μM for NO_3 and SRP, respectively. Depth of nitracline (Z_N), phosphacline (Z_P) and mixed layer (Z_m) were calculated from a mean depth determined from 4 different criterions (Table 1). These criterions were based on thresholds established either from absolute difference of density (or concentration) between the depth of the mixed layer Z_m (or nutricline) and the surface, or from gradient of density (or concentration) between two successive layers.

2.3 Ectoenzymatic activities

Ectoenzymatic activities were measured fluorometrically, using fluorogenic model substrates that were L-leucine-7 amido 4 methyl coumarin (Leu-MCA), 4 methylumbelliferyl – phosphate (MUF-P), 4 methylumbelliferyl – palmitate (MUF-palm) to track aminopeptidase (AMPase), alkaline phosphatase (APase), and lipase, respectively (Hoppe, 1983). Stocks solutions (10 and 5 mM) were prepared in methycellosolve and stored at -20°C. Release of the products of AMPase activity, MCA, and APase and lipase activities, MUF, were followed by measuring increase of fluorescence at 365 nm excitation and 450 nm emission. The spectrofluorometer (Kontron SFM 25) was calibrated with standards of MCA and MUF solutions diluted in $<$0.2 μm filtered seawater. For measurements, 20 ml samples were supplemented with the fluorogenic substrate (stock or sister dilutions made in sterilized sea water) and aliquots of 2 ml were subsampled with time for lecture of fluorescence. Incubations were run in the dark in themostated incubators reproducing in situ temperature ranges. Boiled water blank were run sometimes to check for abiotic activity.

The analogue substrate concentration 50 μM of Leu-MCA for AMPase, and 50 μM of MUF-palm for lipase were representative of saturation concentrations, this was verified on selected samples during the cruise where a large set of concentrations was tested for concentration kinetics (from 0.05 to 100 μM, data not shown). Concentration kinetics were established more systematically for APase at 5 m depth. Six concentrations of MUF-P were added (25, 50, 100, 250, 500, 1000 nM) and from varying velocities obtained, we determined the parameters V_{max} (maximum hydrolysis velocity) and Km (Michaelis constant which reflects enzyme affinity for MUF-P) by fitting the data using an non-linear regression on the following equation:

$$V = V_{max} \times S/(Km + S)$$

Where V is the MUF-P hydrolysis rate, and S the MUF-P concentration added. The prism 4 (Graph Pad software, San Diego, USA) was used to perform non linear regressions.

2.4 Bacterial abundance and production

Total abundance, attached fraction (retained by a 0.8 μm filter) and HNA fraction (fraction with high nucleic acid content) were determined by flow cytometry as described in Mével et al. (2008). Bacterial production was measured using the ^{3}H leucine incorporation technique coupled with the centrifugation method (Kirchman et al., 1993; Smith and Azam, 1992). For each sample, duplicate aliquots (1.5 ml) and a trichloracetic acid killed control were incubated for 2 h at in situ temperature in the dark. We used the same protocol for centrifuge treatments as in Van Wambeke et al. (2002): 20 nM final concentration of leucine, addition of Bovine Serum Albumin, 2 series of centrifugation at 16 000 g, no ethanol rinse. Saturation was checked for in situ data but not after NPG enrichments where very high signals could be obtained (up 2.3 nmol l^{-1} h^{-1}). It is thus possible that such

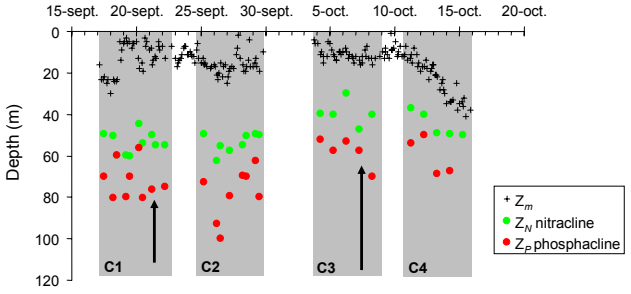

Fig. 1. Evolution of mixed layer depth (Z_m), phosphacline and nitracline from 17 September to 15 October, during DYNAPROC cruise, when the ship was at the fixed station DYFAMED. Shaded areas shows period of high frequency sampling of CTD casts, defined as cycle 1 (C1, 17–22 September), cycle 2 (24–29 September), cycle 3 (C3; 3–8 October) and cycle 4 (C4: 10–15 October). Arrows shows the two dates (21 September, 7 October) when enrichment experiment coupled to diversity study was made (see Figs. 6 and 7).

high rates were underestimates. Variability between duplicates was, on average, 6% and the blank represented a mean of 7% of the signal.

2.5 DNA and RNA extractions for capillary electrophoresis single strand conformational polymorphism (CE-SSCP) analysis

Two liters of each sample were pre-filtered through 3 μm-pore-size filters (47 mm, Nuclepore) to remove most eukaryotic organisms and to prevent clogging of the final filter. Bacterial cells were concentrated onto 0.22 μm-pore-size filters (47 mm, PC Nuclepore) and stored in 2 ml Eppendorf tubes at -20°C until extraction. Frozen filters were cut with sterilized scissors into small strips and 840 μl of alkaline lysis buffer (50 mM Tris hydrochloride pH 8.3, 40 mM EDTA, 0.75 M sucrose) were added. Cell lysis was accomplished by an initial incubation for 45 min at 37°C after adding 50 μl of freshly prepared lysosyme solution (20 mg ml^{-1}), and a second incubation at 55°C for 40 min after adding 100 μl of 10% sodium dodecyl sulfate and 10 μl of proteinase K (20 mg ml^{-1}).

Six hundred μl of lysate was treated with 10 μl of a 100 mg ml^{-1} RNase A solution (Qiagen) before DNA extraction with the DNeasy Tissue kit (Qiagen). DNA was used as a template for PCR amplification of the variable V3 region of the 16S rRNA gene with primer w49 and w34 (*Escherichia coli* positions 329–533), as previously described (Ghiglione et al., 2005). The primer w34 was fluorescently labelled at the 5'-end position with phosphoramidite (TET, Applied Biosystems).

Another 400 μl of lysate was treated with DNase I for RNA extraction using a SV Total RNA Isolation kit (Promega). The efficiency of the DNA removal from RNA was checked by amplifying each RNA extract by PCR (conditions as indicated above), and PCR reactions that did not give a product were used for cDNA synthesis. RNA was reverse transcribed (RT) into single-strand cDNA using MMLV reverse transcriptase (Promega), according to the manufacturer instructions. PCR amplification of cDNA was performed with the same conditions as for DNA. CE-SSCP and analysis of the electropherograms were performed as described in a companion paper (Ghiglione et al., 2008), except that cluster analyses along with their corresponding dendrograms were generated by the unweighted-pair group method using average linkages (UPGMA).

3 Results

The cruise period was chosen to encompass the transition period between summer and fall, expecting then to include the beginning of winter mixing. However, meteorological conditions were remarkably stable during the period of the cruise. We observed a strong stratification of the water column with a mixed layer depth (Z_m) stabilized at 12.8±5.3 m depth between 17 September and 11 October. The stratification partially disrupted after this date as seen on Z_m depth which started to deepen at 12 October (Fig. 1, down to 40 m) after two successive strong wind events (11 and 16 October, Andersen et al., 2009).

3.1 Evolution of nutrients

Vertical distributions of phosphate and nitrate exhibited the same trend during the cruise, with a strongly depleted layer within Z_m. Nutriclines appeared at layers much deeper than Z_m, and shifted during the cruise, as seen in Fig. 1 and in the vertical profiles obtained from the data set acquired during cycles C1, C2, C3 and C4 (Fig. 2). From analysis of vertical distributions of mean concentrations from C1 to C4, differences were particularly marked between 60 and 90 m. There was a shallowing of the phosphacline (Z_P) as well as sharper gradient of concentration along Z_P. For instance at 70 m mean concentrations of soluble reactive phosphorus (SRP) switched from 0.10±0.05 μM and 0.10±0.06 μM at C1 and C2 to 0.28±0.09 μM and 0.22±0.05 μM at C3 and C4, respectively (all comparisons of means between C1 and C2 on one hand, and C3 and C4 on the other hand, are significant, p is at least <0.01). However mean concentration of SRP did not change within Z_m, with concentrations at the lower range of sensitivity (0.030±0.017 μM: 5 and 10 m data together for the whole cruise, n=54). Indeed, there was no significant increase of SRP within the mixed layer during the cruise (no significant differences betweens means of concentrations from C1 to C4, $p>0.05$ for all comparisons). We noticed also a shallowing of the nitracline (Z_N) particularly marked between 30 m and 80 m. For instance at 60 m mean concentrations of NO_3 switched from 3.9±1.1 μM and

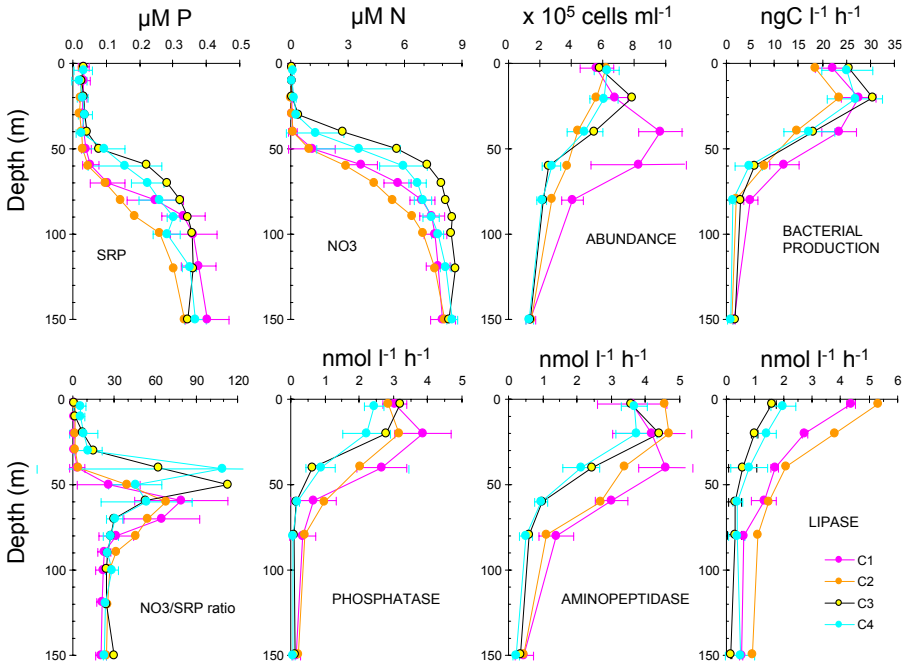

Fig. 2. Mean vertical distributions of nutrients : nitrate (NO_3), phosphate (SRP), nitrate to phosphate ratio NO_3/SRP ratio), total bacterial abundances (abundance), heterotrophic bacterial production, and ectoenzymatic activities (phosphatase, aminopeptidase, lipase) along 0–150 m water column during cycles C1, C2, C3 and C4. Errors bars (standard deviations of the means) are indicated only for C1 and C4 for clarity. For lipase, error bar delineates only absolute differences between 2 profiles, as only 2 vertical profiles per cycle were made.

2.9±1.1 μM at C1 and C2 to 7.2±1.1 μM and 5.9±1.2 μM at C3 and C4, respectively (all comparisons of means between C1 and C2 on one hand, and C3 C4 on the other hand, are significant, p is at least <0.001). Average stocks of NO_3 within Z_m (data from 0–10 m layers) were: 0.030±0.034, 0.038±0.033, 0.045±0.017 and 0.092±0.049 from C1 to C4, respectively. The increase during C4 period (mean statistically higher than for other cycles, p is at least <0.005) was particularly marked for the last CTD cast (15 October) with nitrate concentrations increasing more than 0.1 μM.

The NO_3 to SRP ratio (N/P ratio, Fig. 2) exhibited the classical shape of vertical distribution determined in the Mediterranean Sea during the stratified period, with a marked increase of N/P ratio located at the base of the photic zone between Z_N and Z_P. Particularly, we observed a shallowing and an increase of these maximum values from C1-C2 to C3-C4: the peaks of N/P ratio were not statistically different in terms of depth where it is observed and value N/P reached at C1 and C2 (60±6 m and N/P 83±30 during C1, 62±10 m and N/P 78±24 during C2, p>0.05 for both comparisons). At C3, the peak was at 46±6 m (N/P 116±79) and was at 47±9 m during C4 (N/P 145±103). Mean depth at C3 and C4 were not different (p>0.05) but significantly shallower than during C1 and C2 (p at least <0.005). However, although means were increasing at C3 and C4, they were not statistically different from those during C1 and C2, due to the high variability of the data related to a strong short time variability in the displacements of nutriclines. Within Z_m, means of N/P ratio were 0.9±1.0, 1.6±1.6, 0.9±0.5 and 4.6±3.8 for C1 to C4, respectively. Again, even with higher mean values, ratios obtained during C4 within Z_m were not statistically different from other cycles (ANOVA, p>0.05), due to the high sd values, in turn, related to the very low concentration (close to the detection limits) of NO_3 and SRP within the Z_m. However, the shallowing of the nitracline was clearly associated to an increase of N/P ratio within subsurface layers after 11 October.

3.2 Evolution of heterotrophic activities

Vertical distribution of bacterial production was stable throughout the cruise, with maximum values in subsurface layers (means of data from 5 and 20 m: 25±4, 19±4, 28±6 and 26±6 ng C l^{-1} h^{-1} for cycles C1, C2, C3 and C4, respectively; the mean was only statistically lower during C2, ANOVA, p<0.005). During all the cycles there was a similar decrease with depth (Fig. 2). However, this was not the case for ectoenzymatic activities which exhibited two distinct types of mean profiles: one group including C1 and C2 cycles, and another one C3 and C4 in which roughly, the layer where activities decreased was found shallower in the water column (from 40 m to 20 m depth for phosphatase, from 60 m to 40 m for aminopeptidase). However the range of phosphatase activities at 5 m depth stayed constant during

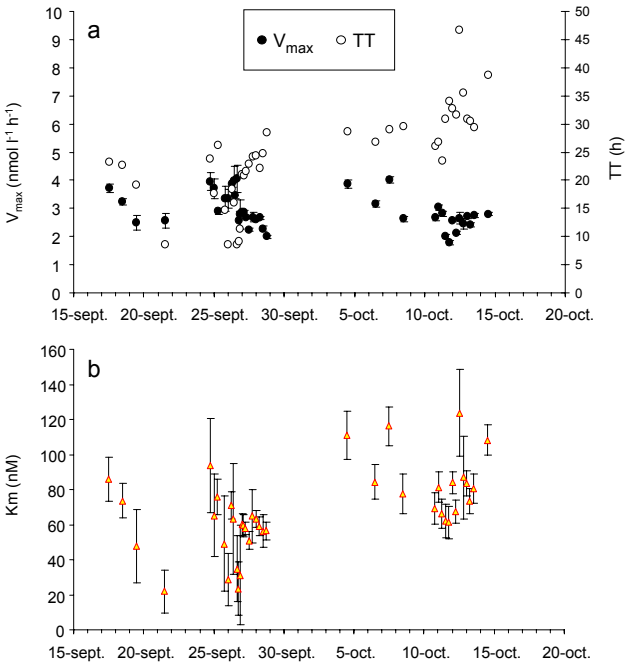

Fig. 3. Evolution of kinetic parameters determined from concentration kinetics realized on phosphatase activity at 5 m depth. **(a)** Maximum velocities (V_{max}, nmol MUF-P hydrolyzed $l^{-1}\,h^{-1}$), and turnover time (TT=Km/V_{max}, h); **(b)** half saturation constant (Km, nM). Bars on V_{max} and Km data indicate standard deviations computed from of the non linear regression analysis of velocity versus concentration.

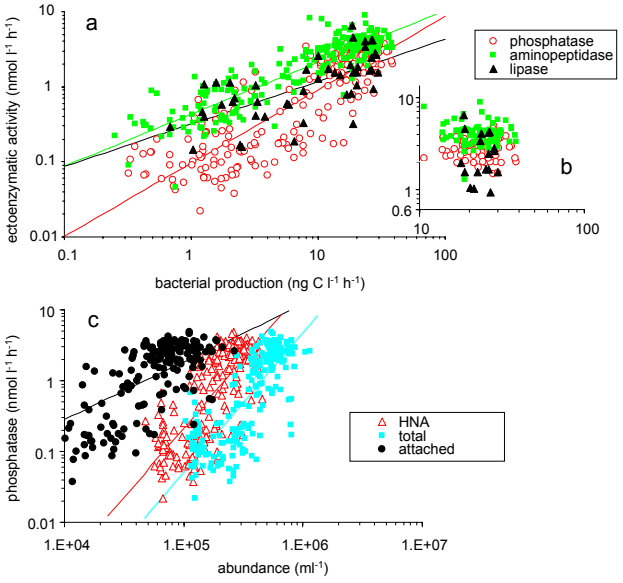

Fig. 4. Relation between **(a)** ectoenzymatic activities (phosphatase, aminopeptidase and lipase) versus bacterial production and **(c)** phosphatase alkaline activity versus total abundance, attached bacterial abundance and HNA-group. All data set 0–150 m is presented. The smaller graph in **(b)** focuses only on data corresponding to 5–20 m depths. Log-Log regression fit is indicated, details of equations corresponding are given in Table 3.

the 3 first cycles (means of data from 5 m: 3.0±0.3, 2.9±0.5, and 3.2±0.4 nmol MUF-P hydrolysed $l^{-1}\,h^{-1}$ for cycles C1, C2, C3 and C4, respectively) and decreased significantly only during C4 (2.4±0.3 nmol MUF-P hydrolysed $l^{-1}\,h^{-1}$, ANOVA, $p<0.05$). A similar slight trend of decrease at the end of the cruise was noticed for aminopeptidase activities at 5 m and 20 m depth (means of data from these 2 depths: 3.9±1.1, 4.6±1.5, 4.0±0.8 and 3.7±0.5 nmol MCA-leu hydrolysed $l^{-1}\,h^{-1}$ for cycles C1, C2, C3 and C4, respectively, difference significant only between C2 and C4, ANOVA, $p<0.005$). For lipase, activities decreased at all depths between C1-C2 and C3-C4 cycles. At 5 m depth, mean activities decreased from 4.4±0.2 and 5.3±1.2 nmol MUF-palmitate hydrolysed $l^{-1}\,h^{-1}$ during C1 and C2, respectively to 1.6±0.1 and 2.0±0.4 nmol MUF-palmitate hydrolysed $l^{-1}\,h^{-1}$ during C3 and C4 (all comparisons significantly different between C1, C2 on one hand and C3, C4 on the other hand, at least for all comparisons $p<0.02$).

At 5 m depth, kinetic parameters of alkaline phoshatase activity showed high variability at all time scales (Fig. 3). V_{max} ranged 1.8 to 4.0 nmol $l^{-1}\,h^{-1}$, Km 22 to 124 nM and turnover time TT (Km/V_{max} ratio) 9 to 47 h. Nevertheless, the trend with time is significant with all 3 parameters. Time explained only 12.4% of the V_{max} variance (negative trend with time, $p<0.05$), 22.8% of the Km variance (positive trend with time, $p<0.005$) but the regression was much better with TT (positive trend with time, r^2=0.49, $p<0.0001$).

3.3 Relationships between heterotrophic activities and biomasses

Including the whole 0–150 m data set, the three types of ectoenzymatic activities correlated very well with bacterial production, with correlations coefficients (log-transformed data) of 0.85, 0.89 and 0.68 for phosphatase, aminopeptidase and lipase, respectively (Table 3, Fig. 4a). However such correlations were absent when restricted only to the surface layers (between 0 and 20 m depth, see Fig. 4b). The slope of the log-log regression is less than 1 for aminopeptidase (0.69±0.02) and lipase (0.56±0.08), showing that these ectoenzymatic activities decrease less rapidly than production. This implies that for low bacterial production values, aminopeptidase and lipase exhibited higher activity relative to production whereas phosphatase (slope 0.97±0.04, not statistically different than 1) varied in the same proportions than BP on the range of activities tested. Aminopeptidase and lipase per unit BP (nmol ng C^{-1}) were indeed higher for a range of BP less than 10 ng C $l^{-1}\,h^{-1}$ (0.41±0.29 nmol ng C^{-1} for 0.19±0.09 nmol ng C^{-1}, ANOVA, $p<0.001$ or aminopeptidase, 0.25±0.21 nmol ng C^{-1} for 0.11±0.08 nmol ng C^{-1},

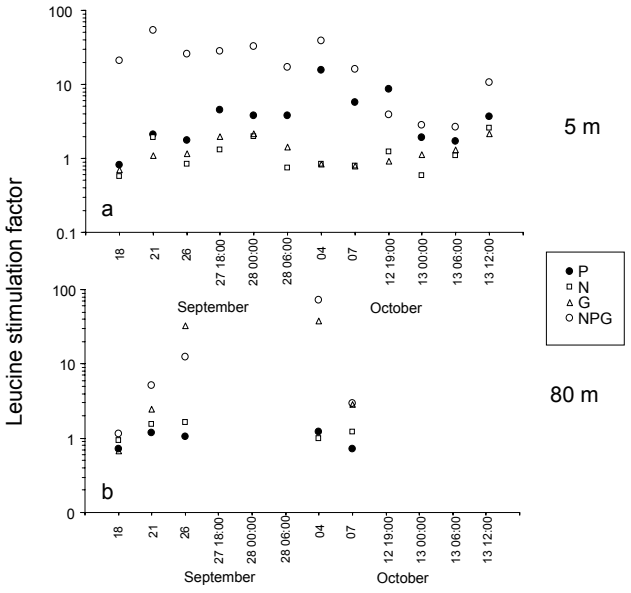

Fig. 5. Factor of stimulation of leucine incorporation rates after 24 h addition of phosphate (P), nitrate + ammonium (N), glucose (G) and 4 elements (NPG), compared to the control unamended (C), i.e. for C the value is set to 1. **(a)** enrichment experiments made at 5 m depth, **(b)** at 80 m.

ANOVA, $p<0.005$ for lipase). In contrast, phosphatase activity per unit BP remained constant throughout the range of BP (mean±sd: 0.12±0.09 nmol ng C^{-1}).

Correlation coefficients between ectoenzymatic activities and bacterial abundances were approximately in the same range as those obtained with bacterial production for the same ectoenzyme and were also quite invariant considering the category considered (i.e. total abundance, attached bacteria or HNA-group: 0.76–0.80 for phosphatase, 0.85–0.90 for aminopeptidase and 0.54–0.60 for lipase, Table 3). Considering total counts, the slope of log-log regressions where significantly higher than 1 for phosphatase (1.97±0.10) and aminopeptidase (1.57±0.05). Thus, phosphatase and aminopeptidase activities per cell were significantly higher for a range of abundance higher than 4×10^5 cells ml^{-1} (mean±sd: 3.9±1.9 amol $cell^{-1}$ h^{-1} for 1.4±1.6 amol $cell^{-1}$ h^{-1}, ANOVA, $p<0.0001$ for phosphatase, 6.4±2.2 amol $cell^{-1}$ h^{-1} for 3.9±2.8 amol $cell^{-1}$ h^{-1}, ANOVA, $p<0.0001$ for aminopeptidase). Lipase activity per cell was stable within the range of abundances encountered (mean±sd 3.3±2.7 amol $cell^{-1}$ h^{-1}). Finally, for a given enzyme, the slopes of log-log regressions decreased significantly with regard to attached bacteria, whereas for HNA group they remained equal to those obtained with total counts (Table 3, Fig. 4c).

Table 2. Initial conditions prevailing at the two depth profiles used for bioassays where bacterial diversity was investigated. Z_m : mixed layer, P stocks: soluble reactive phosphorus, N stocks: sum of nitrate + nitrite, Leu inc rates: leucine incorporation rates into proteins, AMPase: leucine aminopeptidase activities, APase: alkaline phosphatase activities.

		Units	21 Sep	7 Oct
Z_m	Depth	m	13	15
	mean temperature	°C	22.2	20.9
P stocks	Phosphacline depth	m	76	57
	Integrated 0–80 m	mmol m^{-2}	4.6	6
N stocks	Nitracline depth	m	49	47
	Integrated 0–80 m	mmol m^{-2}	137	211
Leu inc rates	Integrated 0–80 m	μmol m^{-2} h^{-1}	2.2	1.1
APase	Integrated 0–80 m	μmol m^{-2} h^{-1}	225	108
AMPase	Integrated 0–80 m	μmol m^{-2} h^{-1}	254	189

3.4 Enrichment experiments

The average factor of increase of leucine incorporation rate after 24 h of incubation in the unamended control was low (mean±sd: 1.8±0.9), excluding the experiences made at 5 m depth at the beginning of the cruise (×6 on 18 September) and at 80 m depth at the end of the cruise (×9 and ×46 on 4 and 7 October). Enrichment experiments resulted in variable stimulations of leucine incorporation rates according depth and date (Fig. 5). At 5 m depth, the main trend was a negligible stimulation of leucine incorporation rate when a single element was added alone between 18 and 26 September (up to ×1.9, ×1.2 and ×1.7 compared to the control for N, G and P, respectively, Fig. 5a) whereas addition of NPG simultaneously could stimulate leucine incorporation rates up to ×53 in 24 h (21 September, Figs. 5a and 6). On 27–28 September, stimulation by P alone started to be important, with the stimulation factor (compared to the control) varying between ×3.7 and ×4.4. Between 4 and 12 October, the P alone stimulation factor were the highest obtained during the cruise (×5.6 to ×15, Fig. 6). When the mixed layer started to deepen, after 12 October, the stimulation factors after NPG amendments decreased (×2.6 to ×10 compared to ×15 to ×50 before), and stimulation of leucine incorporation rate after addition of P alone was still higher than the control (×1.7 to ×8.6). At 5 m depth, addition of nitrate + ammonium alone and addition of glucose alone, even if sometimes stimulating up to a factor 2, never stimulated leucine incorporation rates more than in the flask enriched with P alone.

We paid special attention to the biogeochemical conditions prevailing during experiments made on 21 September and 7 October, because these were devoted also to the study of bacterial diversity. The main differences between the 2 situations were a shallowing of the phosphacline from 76 m (21 September) to 57 m (7 October), whereas the depth of

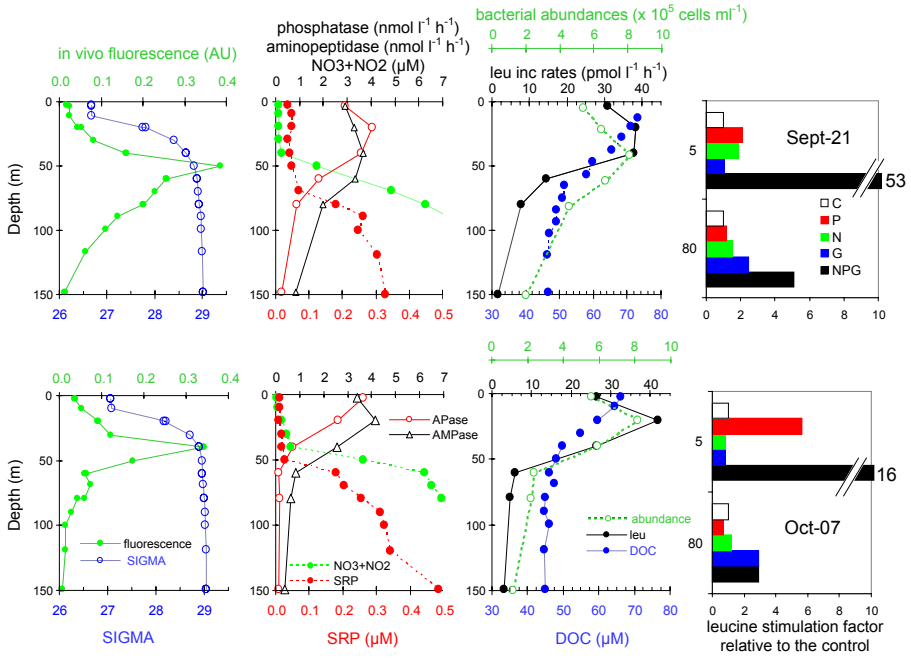

Fig. 6. Detailed information on vertical distributions of density, in vivo fluorescence, phosphate and nitrate concentrations, dissolved organic carbon concentration, bacterial abundance, leucine incorporation rates, ectoenzymatic activities measured on the same CTD cast that was used for sampling sea water used for making enrichment experiments at 5 m and 80 m depth on 21 September and 7 October. The response to enrichment (histograms) is presented in terms of relative response of leucine incorporation rate compared to the control unenriched after 24 h enrichment (i.e. for C the value is set to 1).

Table 3. *Y* intercepts and slopes of the Log-Log (base 10) regressions of ectoenzymatic activities versus bacterial production and abundances of total, attached and HNA-group bacteria. *R*: correlation coefficient, *N*: number of data. All these regressions were highly significant ($p<0.0001$ for all regressions). Before transformation in Log units were: nmol l^{-1} h^{-1} for ectoenzymatic activities, ng C l^{-1} h^{-1} for bacterial production, ml^{-1} for abundances. * Plot corresponding Fig. 4a, ** Plot corresponding Fig. 4c.

	Y intercept	slope	*R*	*N*
	BP			
Phosphatase*	−1.015±0.040	0.974±0.040	0.856	220
Aminopeptidase*	−0.365±0.024	0.691±0.023	0.890	222
Lipase*	−0.496±0.092	0.564±0.089	0.680	48
	Total abundance			
Phosphatase**	−11.175±0.558	1.976±0.100	0.800	220
Aminopeptidase	−8.534±0.274	1.575±0.049	0.906	222
Lipase	−5.124±0.991	0.921±0.178	0.608	48
	Attached bacteria			
Phosphatase**	−3.800±0.180	0.813±0.040	0.808	220
Aminopeptidase	−2.623±0.086	0.640±0.019	0.912	222
Lipase	−1.608±0.345	0.361±0.076	0.573	48
	HNA group			
Phosphatase**	−10.44±0.58	1.955±0.111	0.768	219
Aminopeptidase	−7.496±0.324	1.527±0.061	0.857	221
Lipase	−4.383±1.001	0.836±0.190	0.548	47

the nitracline was more or less constant (49 m and 47 m, respectively, Table 2). This resulted in higher stocks of SRP integrated from the surface to 80 m on 7 October (6 for 4.6 mmol P m^{-2}). Not only the depth of nutricline changed but also the slopes of nutrients gradient versus depth were higher on 7 October (Fig. 2).

N/P ratios of nutrients within subsurface layers (i.e. NO_3/SRP ratio) ranged 2.3–3 (2 m–30 m) on 21 September and 3.9–4.9 (2–10 m) on 7 October and increased with depth. The differences were mainly due to concentrations of SRP, which were low but detectable in the depleted layers on 21 September (range 0.037 to 0.047 μM) whereas they were close to detection limit on 7 October (range 0.013 to 0.017 μM).

On 21 September, the unamended control stayed stable at both depths tested. Indeed, the leucine incorporation rates after 24 confinement in a 250 ml flask were very close to in situ values (enhancement factor ×1.1 at 5 m depth, ×0.9 at 80 m depth). The situation was different on 7 October when incubation without additions resulted in a ×3 and a ×46 increase of leucine incorporation rates at 5 m and 80 m depth, respectively, compared to in situ conditions. Stimulation by addition of P alone was observed in both cases at 5 m, but to a larger extent on 7 October (increase by a factor 5.6 compared to the unamended control). Only addition of all three elements together resulted in huge stimulation of

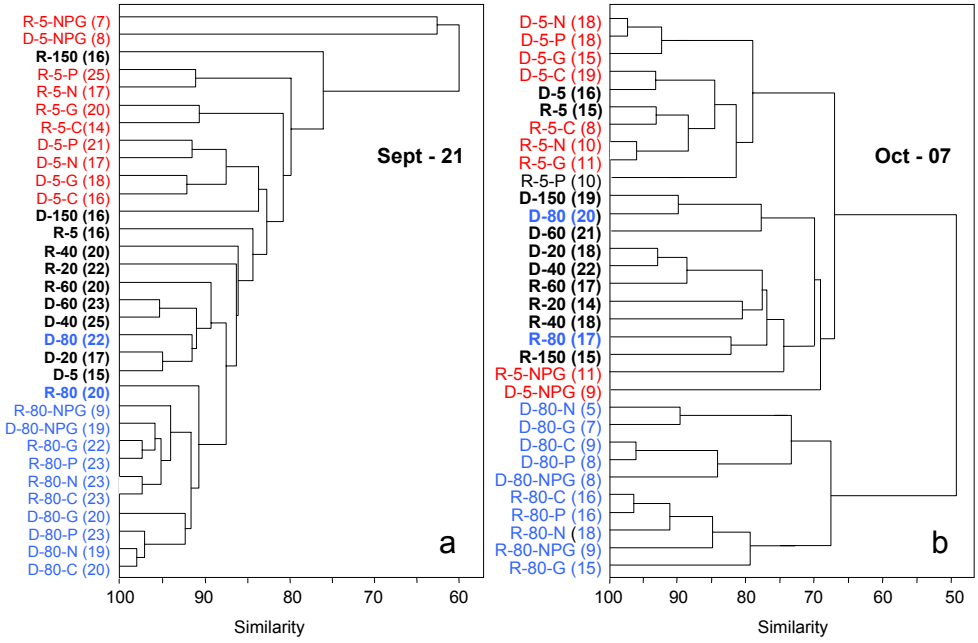

Fig. 7. Cluster analysis dendrogram based on16S rDNA (D) and 16 rRNA (R) CE-SSCP profiles taken from 21 September **(a)** and 7 October **(b)**. Samples originate from vertical profile (referred with the depth: 5, 20, 40 60 80 150 m in bold characters) or correspond to enrichment experiment (C: unamended control, N: +nitrogen, P: +phosphate, G: +glucose, NPG: +the three elements) from water taken at 5 m (red characters) or 80 m (blue characters). The numbers of ribotypes identified in each bacterial community are indicated in brackets.

leucine incorporation rates ($\times$53 and $\times$16 for 21 September and 7 October, respectively). In contrast, at 80 m depth only addition of glucose alone stimulated leucine incorporation rate, but with a low extent mostly equal in both dates (factor 2.4 and 2.8 compared to the control for 21 September and 7 October, respectively). Finally, at 80 m depth, stimulation of leucine incorporation rates after addition of the 3 elements were close to those obtained after addition of glucose alone (factor 5 and 2.9).

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

Changes in the composition of bacterioplankton assemblages were followed by cluster analysis of DNA- and RNA-based CE-SSCP profiles of samples from in situ water column (5 to 150 m depth) and from enrichment bioassays (5 m and 80 m depth) in 21 September (Fig. 7a) and 7 October (Fig. 7b). Overall, three main clusters could be distinguished for both dates: samples from the in situ vertical profile, samples from enrichment experiments at 5 m and at 80 m depth. In each cluster, CE-SSCP profiles were generally organised in two separated DNA- and RNA-based groups. In the water column, samples from 5 m and 80 m showed distinct community structures based on both DNA and RNA-based profiles for both dates. The effect of incubation on bacterial community structure was observed for all the enrichment experiments, except on 7 October at 5 m where in situ samples clustered together with non-amended controls on both DNA and RNA levels.

In the enrichment experiments, most important changes in bacterial community structure were generally observed for factors that stimulate greatly the bacterial production. For 21 September enrichment experiment, drastic changes in bacterial community structure were noticed at 5 m after addition of the 3 elements (NPG) in both DNA and RNA levels. Interestingly, these samples present the lowest number of CE-SSCP peaks (8 and 7 compared to 16 and 18 in the corresponding controls on DNA and RNA levels, respectively), clustered separately from the other samples (Fig. 7a) and presented the highest bacterial production (1850 pmol l^{-1} h^{-1}). Addition of one element (N, P, or G) resulted in smaller changes, especially for glucose addition in both DNA and RNA levels. Addition of NPG in 80 m seawater resulted also in clear changes in both DNA and RNA levels but in a lesser extent compared to 5 m, and decrease in the number of CE-SSCP peaks on NPG treatment was observed only at the RNA level. Samples with only one compound added (G, N or P) clustered together on both DNA and RNA levels. For 7 October bioassays, addition of the 3 elements (NPG) on 5 m depth sample resulted also in drastic changes in both DNA- and RNA-based community assemblages associated to a slight decrease in the number of CE-SSCP peaks compared to the unamended control. The effect of individual N, P or G

addition on bacterial community structure was much lower on both DNA and RNA levels. One exception was found for P addition that resulted in clear changes of CE-SSCP profiles compared to others individual amendments at the RNA level but not on the DNA level. Interestingly, this sample corresponds to significant increase of leucine incorporation rate compared to the control ($\times$5.6, 469 pmol l^{-1} h^{-1}).

4 Discussion

4.1 Nutrient status during summer to autumn transition

The hydrological situation observed during the cruise was remarkably stable and characterized by a strong stratification. The expected destratification in the late summer-fall transition period started to occur only at the end of the cruise, after 12 October. Nevertheless, during the whole month study, distribution along vertical profiles exhibited temporal changes mainly reflected by vertical displacements of nutriclines. The high variabilities obtained inside each cycle were related to short term fluctuations in both depths of phosphacline and nitracline, associated with a clear trend of shallowing during the cruise and a relative increase in the maximum N/P ratio of nutrients.

As previously observed by Moutin and Raimbault (2002), nitrate becomes detectable at shallower depth than SRP. That is, subsurface NO_3/SRP was significantly greater than the usual value 15–16 generally found in most of the world ocean (Mc Gill, 1965). Ratios of about 20–22 that characterize Mediterranean deep waters, may reach 100 at 50 m depth. High values of NO_3/SRP ratio have already been observed in subsurface layers of the Western Mediterranean Sea (Diaz et al., 2001) and especially at the DYFAMED site (Marty et al., 2002). Several hypotheses have been proposed to explain the high nitrate versus phosphate ratio as biological nitrogen fixation (Béthoux and Copin-Montégut, 1986) or phosphate adsorption on iron-rich dust particles (Krom et al., 1991) that both lead to increase in P depletion. Recently, Diaz et al. (2001) suggested that high subsurface ratios may be primarily attributed to the depletion in P relative to N in deep Mediterranean waters. Upward diffusion of deep nutrients taken biologically in constant Redfield ratio of 16:1 may theoretically lead to NO_3/SRP ratios as high as those observed. This biogeochemical evidence, as well as many observations from enrichment bioassays studying autotrophs and heterotrophs (from Fiala et al., 1976 to Pinhassi et al., 2006), confirms the P-deficiency in Mediterranean waters and the major role that P can play in the control of biological processes.

4.2 Bacterial community response to nutrient status through ectoenzymatic activities

The range of ectoaminopeptidase activities obtained during this survey was similar that previously described for the NW Mediterranean Sea using the same set of concentration added (50 μM for aminopeptidase, Genoa Gulf: Misic and Fabiano, 2006; Thyrehnnian Sea, Misic et al., 2008). Interannual variation related to the seasonal changes in hydrological and weather conditions may also account for some of the observed variability in activity measurements (Marty et al., 2002). For instance at the same DYFAMED site, same season (30 September 2000; Van Wambeke et al., 2002), we obtained higher phosphatase activities (up to 12 nmol l^{-1} h^{-1}; TT 14 h) than during this survey. Higher V_{max} of phosphatase were obtained in coastal areas in NW Mediterranean Sea in spring (Thingstad et al., 1998, using a range of MUF-P concentration of 5–200 nM: V_{max} 28 nmol l^{-1} h^{-1}) but on the same range as ours in eastern ultra-oligotrophic Mediterranean Sea in May (Thingstad and Mantoura, 2005: 2–5 nmol l^{-1} h^{-1}). For higher concentrations of analog substrate used by some other authors (Sala et al., 2001: 200 μM; Zoppini et al., 2005: 125 μM), comparison is impossible due to multiple kinetics appearing according the range of concentration tested (Bogea et al., 2006).

Bacterial lipase activities have received little attention and rate measurements in marine ecosystems are scarce (Bourguet et al., 2008) and, to our knowledge, inexistent in Mediterranean Sea. Furthermore, different lipases exhibit specific preferences for their triacylglycerol substrates, depending on chain length, optical and positional isomers, and the characteristics of the lipid/water interface. Thus, depending on the choice of the fatty acid chain probably different information in terms of V_{max} and Km is expected due to the different specificity of ectoenzymatic "lipase" (Ruiz et al., 2002). Bourguet et al. (2003) reported lipase rates (with 12.5 nM MUF-oleate as substrate) in the range 0.9–5.8 nmol l^{-1} h^{-1}, in a New-Caledonia lagoon. Goutx et al. (unpublished results) measured lipase activities in the range 5–7 nmol l^{-1} h^{-1} using 30 μM saturating MUF-oleate concentrations in the 0–60 m layer in Western Mediterranean including the DYFAMED site. Using also MUF-oleate analog substrate (20 μM), Martinez et al. (1996) reported lipase activities per cell ranging 3 orders of magnitude (0.2–584 amol cell^{-1} h^{-1}) on pure cultures and 20–70 amol cell^{-1} h^{-1} in coastal Californian waters, i.e. higher values than we obtained at DYFAMED site (mean sd 3.3$\pm$2.7 amol cell^{-1} h^{-1}). However, this author noticed also large day-to day fluctuations of ectoenzymatic activity and a prominent presence of lipase activity among isolates. Finally, nothing is known about the difference that can be obtained by using MUF-oleate (unsaturated fatty acid analog 18:1) and MUF-palmitate (saturated fatty acid analog 16:0 that we used) as fluorogenic substrates (Taylor et al., 2003).

Because variations of ectoenzymatic activities are expressed (mainly) by bacteria, rates can change not only because of the varying status of the quality of organic matter, but simply can follow abundance or bacterial production. It is then worthwhile considering in some detail the relationships between ectoenzyme activities and bacterial abundance and production. For instance in mesocosms experiments, the strong dependence between ectoenzymatic activity and BP as been attributed to substrate-limited conditions (Chrost and Rai, 1993). During our survey, the correlation coefficients between ectoenzymatic activities and production, or between ectoenzymatic activities and total abundance were very close (0.85 versus 0.81, 0.89 versus 0.91, 0.68 versus 0.60 for phosphatase, aminopeptidase and lipase, respectively, Table 3). Rosso and Azam (1987), investigating a deeper water column with L-leucyl-β-naphthylamide as substrate, found a better correlation between aminopeptidase and bacterial production than with abundance (0.94 versus 0.8, respectively). In our study, interestingly, the main difference in relationships between ectoenzymatic activities and BP and ectoenzymatic activities and abundances was not reflected by correlation coefficients, but by the slopes of the log-log regressions, which were lower for BP. For instance when aminopeptidase activity decreases, it decreases less rapidly than bacterial production (slope smaller than 1) but more rapidly than abundance (slope greater than 1). As lower values of abundances and BP were found above the deep chlorophyll maximum depth (80–150 m) in these layers aminopeptidase activity per unit production was higher, but aminopeptidase activity per cell was lower, than in surface layers. Such a trend was described in the subarctic Pacific, where Fukuda et al. (2000) noticed a probable smaller proportion of aminopeptidase-active cells in deep layers. Lipase is less dependant on abundances (the activity per cell was constant) but increased also in relative proportion to BP in deep layers. This is consistent with a labile carbon limitation in deep layers, protease and lipase being more expressed to sustain BP. Phosphatase activity per cell was significantly higher at layers with high abundances but did not show a particular trend with bacterial production. Phosphatase activity is well known to be controlled by availability of SRP which was a highly dynamic pool during this study. This is well evidenced by the high variability of kinetic parameters obtained at 5 m depth.

4.3 Role of attached and/or HNA bacteria

Although ectoenzymatic activities were estimated only on the total fraction, there was not any particularly better correlation coefficient between ectoenzymatic activity and abundances of attached bacteria compared to that one obtained with total abundance. This suggests that ectoenzymatic activities were not especially under the control of a distinct bacterial category (total versus attached), implying a versatility of both attached versus free bacteria to exhibit ectoenzymatic activity. According Unanue et al. (1992), examining assimilation of thymidine and glucose, there is a dynamic equilibrium between the two communities, regulated by the concentrations of particulate matter and nutrients and by other environmental factors. Hoppe et al. (1998) investigated a large eutrophication gradient in different seasons and showed that chitinases were more specifically related to attached bacteria ($>3\,\mu$m fraction) whereas aminopeptidase was more associated to free living bacterial fraction (0.2–3 μm). In our study, attached bacteria were nevertheless probably more efficient to work in low abundance condition (deeper layers) as confirmed by the difference between the two log-log regression slopes of enzyme activity versus attached or total bacteria (0.81 versus 1.97 for phosphatase, 0.64 versus 1.57 for aminopeptidase, 0.36 versus 0.92 for lipase, Table 3).

HNA group was not especially better related to bacterial production compared to what was obtained with total abundance (Mével et al., 2008). In addition, the HNA group was not representative of any "high ectoenzymatic activity group" either. Thus both HNA and LNA group, recognized as important components of the active community member (see Bouvier et al., 2007 and references therehein), are also equally active in terms of actors of phosphatase, lipase and ectoaminopeptidase activities. The low range of variability found during the one month survey probably also prevented correlations generally found with larger scales and larger ranges of activities; for instance the percentage of attached bacteria and HNA varied on a rather low range (14–25% and 44–59%, respectively, Mevel et al., 2008). Some inverse relationships between BP and aminopeptidase have been obtained (trophic gradient in Carribean Sea, Rath et al., 1993) as well as absence of correlation (subtropical North Pacific ALOHA station, Donachie et al., 2001). In addition to phosphatase, aminopeptidase activity might be also not solely of heterotrophic bacterial origin, as *Synechococcus* might synthesize some (Martinez and Azam, 1993) as well as some eukaryotic algae (Dyhrman, 2005). Tools combining cell cytometry and flow sorting with adequate functional fluorescing sensors of ectoenzymatic activity, like ELF-97 for instance, might provide valuable data (Duhamel et al., 2008). Finally, presence of significant correlations is not evidence of causal and unique relationship, due to the multiplicity of variables varying with depth. Bacterial diversity structure showed a temporarily stable vertical stratification in three zones in the first 0–150 m layers: above, in or just below the chlorophyll maximum and deeper (Ghiglione et al., 2008). The inoculum, but mainly the source of organic matter influences the activity of most ectoenzymes as seen in switch experiments (Kirchman et al., 2004). Interestingly, both inoculum and organic matter source had particularly different characteristics at 5 m depth and at 80 m depth. For instance sources of DOM (reflected by pigment composition), stoechiometry of nutrients, chloroplast lipids (Goutx et al., 2009) were some of the markers explaining the best vertical stratification of bacterial communities (Ghiglione et al., 2008).

4.4 Bioassay: evidence for nutrient status and bacterial response

We observed a frequent enhancement of growth by P addition within surface layers suggesting that P was the major nutrient limiting factor in the mixed layer whereas labile carbon limited bacterial production above nutriclines (80 m experiments). Stimulation of leucine incoporation rates after 24 h P amendment by more than $\times 2$ compared to the control is not rare in stratified conditions in Mediterranean Sea samples (Pinhassi et al., 2006; Van Wambeke et al., 2002). On a large transect over Mediterranean Sea in autumn, it has been shown that the P stimulation factor was increased when the tunovertime of phoshatase activity decreased, which is also a general indicator of P deficiency (Van Wambeke et al., 2002). Such relationship was not found on this data set, probably because of the low range of activities measured here (range of V_{max} 0.6–2.9 nmol l^{-1} h^{-1}, range of TT 9–47 h), compared to 0.6–12.6 nmol l^{-1} h^{-1} and 14–786 h for the same parameters, respectively, in Van Wambeke et al. (2002). P limitation conditions also occurred at different areas in Mediterranean Sea and at different periods of the year; in summer, winter, spring (Zohary and Robarts, 1998; Sala et al., 2002; Van Wambeke et al., 2002; Pinhassi et al., 2006).

The frequent enhancement of growth by P addition alone suggests that the nutrient pool otherwise satisfied growth requirements at least during the 24 h incubation period. Indeed, regeneration process and DOM fueling by high-sized organisms was still present, because samples were unfiltered. Thus, stimulation of leucine incorporation rate, if any, is a consequence of both direct and indirect effects. We did not follow response of phytoplankton in our enrichment experiments and thus it is possible, like described in Zohary et al. (2005) that phytoplankton and heterotrophic bacteria could not be limited by the same elements (or combination of elements). P limitation of heterotrophic bacterial production within surface layers is not well evidenced in our study on the 2 first experiments (18 and 21 September) probably because a dual limitation NP. Note however, that only the combination NPG has been tested here, not NP. Thus, labile carbon could be also the second limiting factor during the first part of the survey but the slight stimulation obtained with N alone ($\times 1.9$) on 21 September as well as low nitrate concentrations suggests that N was effectively the second limiting factor. Although the absence of N$\times$P combination precludes to respond firmly to what were the second limiting factors in surface at the beginning of the cruise (N, G or both N and G), these results showed that the apparent severity of limitation of P is thus related to the availability of the other limiting factors after P (Pinhassi et al., 2006). What we show here during this monthly scale is that, even in stable stratification conditions when P is frequently the primarily factor limiting bacterial production, small scale physical changes (i.e. winds) can rapidly alter the stoechiometry of different elements within the mixed layer. Indeed, due to the large difference (20 m) between the depths of nitracline and phosphacline, wind events visibly favoured a supply of N in regard to P at the end of the cruise and then, provoked a stricter P-limitation only of bacterial production at the end of the cruise. The particularity of significantly large layer between phosphacline and nitracline encountered most of the year in Mediterranean stratified conditions may enhance such rapid variations in stoechiometry of nutrients and factors limiting BP.

4.5 Role of confinement and nutrient enrichments on bacterial community structure

During the bioassay experiments, we used molecular fingerprint to assess the influence of confinement as well as enrichment on bacterial community structure. Although molecular fingerprinting is not as complete technique for describing microbial diversity as cloning and sequencing approach, it offers the best compromise between the need to process a significant number of samples and the information generated (Muyzer et al., 1998). Two findings concerning the changes in composition of the bacterioplankton assemblage in our enrichments were conspicuous.

First, we found an effect of the enclosure experiment on the bacterial community structure, a phenomenon recognized for a long time now – the so-called "bottle-effect", generally resulting in an increase of bacterial abundance (Zobell and Anderson, 1936). More recent studies have showed a significant influence of confinement on culturable bacterial assemblages (Haldeman et al., 1994; Eilers et al., 2000) as well as total bacterial communities (Suzuki, 1999; Schäfer et al., 2000; Massana et al., 2001, Pinhassi et al., 2006; Agis et al., 2007). Our study extends previous findings in several aspects. We present the response of bacterial community changes due to confinement in short time scale (24 h), whereas studies by Schäfer et al. (2000) and Massana et al. (2001) were conducted for longer periods (from 161 h to 10 days). In our experiments, changes in community structure occurred more rapidly than reported by Suzuki (1999) or Pinhassi et al. (2006), who did not detect taxonomic shifts in confined samples compared to control in situ seawater for a period of 24 h. In our study, samples originated from different depths (5 m and 80 m) and give a better view of confinement effect on communities from diverse water masses. Here, bottle effect was observed at both depths, with the exception of the 5 m samples on 7 October that shows similar bacterial community structure between in situ and confinement control samples. We found that the magnitude of the changes between in situ and confined samples was more important than between samples from different depths in the water column. Moreover, none of the previous studies were taking into account the active bacterial communities. Indeed, it is generally admitted that results obtained from 16S rRNA genes (DNA-based molecular fingerprinting) are considered to reflect the presence of total bacterial

community structure, while 16S rRNA-derived CE-SSCP results (RNA-based molecular fingerprinting) give an indication of the presence of bacterial population that contribute to the RNA pool, i.e. the metabolically active community structure. Because changes in RNA pool depend directly on the metabolic activity of the cells, a faster response may be expected based on the active bacterial community than from the total bacterial community, especially in our short experiment of 24 h duration. In our study, we found that the time and intensity of the response of total community to confinement was as rapid as for the active community and these changes were not a consequence of a drastic decrease in diversity, as it has been observed in the other above cited studies. Such discrepancy may be due to the shorter time scale of our study, distinct seawater community or to sensitivity of the molecular fingerprinting method, since we generally observed almost the same number of CE-SSCP peaks per sample before (in situ sample) and after 24 h contamination (enrichment control sample). These results suggest that in our conditions, changes in total and active bacterial community structure due to confinement may not be related to predation by protozoan or viral lysis (Suzuki, 1999), but more to (i) proliferation of specific microbial types encouraged by sample perturbation or incubation conditions and/or (ii) previously undetected cells resuscitation due to sample handling (Haldeman et al., 1994). In this regard, perturbing as less as possible biological components and reducing the so-called bottle effect could be resolved using large-scale in situ fertilization experiments. To our knowledge, this was realized only once in Mediterranean Sea (Thingstad et al., 2005), but this team did not investigate bacterial community. Previous experience exists, however, on iron enrichment experiments, mainly in Southern Hemisphere. In some of these studies, bacterial diversity was studied within and out of the fertilized patch (with terminal restriction fragment length polymorphism (T-RFLP) and some differences were seen in ectoenzymatic activity but not in bacterioplankton community (Arietta et al., 2004). The more activity of a group increase, the more a change of diversity of that group is expected. Thus changes in activity and diversity of heterotrophic bacteria are more expected in systems where activity and diversity of phytoplankton also varied a lot (Pinhassi et al., 2004), although this is not systematic (Hutchins et al., 2001). Small bottle volumes are not ideal restitution of nature, but it allows multiplication of measurements, and thus help to confirm reproducibility of results in different time or space scales (different depths, short time scale).

Second, we observed significant changes in bacterial community structure between enrichments conditions compared to non-amended controls, systematically co-occurring with changes in bacterial production. Drastic changes were observed with NPG treatments that generally induce a decrease of total and/or active bacterial diversity (i.e. number of CE-SSCP peaks), especially for communities originating from the surface layer (5 m depth). This resulted in clear changes in the structure of both total and active communities in combination with a drastic increase in bacterial production in the NPG enrichments. For the other treatments, total and metabolically active community structures generally grouped in separate clusters, but were influenced with the same magnitude (i.e. similarity index) by the different enrichments. Exception was found on 7 October for P enrichment at 5 m, where changes were observed for metabolically active assemblage but not for the total community structure, and resulted in an increase of bacterial production. Such co-occurrence of changes in bacterial community structure together with increase in bacterial production is in agreement with another enrichment experiment by Pinhassi et al. (2006). They concluded that changing nutrient concentrations are likely to have both direct and indirect effect on bacterioplankton growth and composition. Here we also show that the magnitude of bacterial community changes (i.e. similarity index) depend on the composition of the initial inoculum (5 m or 80 m). This result reinforces the role of nutrient regime as driving force behind bacterial community structure shifts in the water column, as depicted in a companion paper (Ghiglione et al., 2008). Finally, the use of RNA-based data show that metabolically active communities may sometimes react faster than total bacterial community and point out the interest of both DNA- and RNA-based fingerprints for monitoring rapid bacterial dynamics changes in short term experiments.

5 Conclusions

The main correlations obtained in this study were related to shifts of the variables with depth, implying an organization of communities along the water column. The 5 m depth used for bioassay experiment was always situated in the mixed layer depleted in both nitrate and phosphate. The 80 m layer sampled corresponded in both situations to a layer below both nitracline and phosphacline, situated also below the deep chlorophyll maximum. The specificity of the Mediterranean Sea in stratified conditions is the large (up to 20 m depth) difference between the layers of phosphacline and nitracline. This implies that rapid shifts in N, P stoechiometry can happen rapidly due to vertical motions, internal waves, wind events.

The epipelagic bacterial community can react rapidly (24 h) to such variations. Enrichment experiments showed that changes in nutrient concentrations may induce drastic modification of both total and metabolically active bacterial community structure and consequently changes in bacterial activity.

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