

*Biogeosciences Discussions* is the access reviewed discussion forum of *Biogeosciences*

## **Short scale variations in nutrients, ectoenzymatic activities and bottom-up effects on bacterial production and community structure during late summer-autumn transition in the open NW Mediterranean Sea**

**F. Van Wambeke<sup>1</sup>, J-F. Ghiglione<sup>2,3</sup>, J. Nedoma<sup>4</sup>, G. Mével<sup>5,6</sup>, and P. Raimbault<sup>1</sup>**

<sup>1</sup>Laboratoire de Microbiologie, Géochimie et Ecologie Marines, CNRS, Université de la Méditerranée, Centre d'Océanologie de Marseille, UMR6117, Campus de Luminy, case 901, 13288 Marseille Cedex 09, France

<sup>2</sup>CNRS, UMR7621, Laboratoire d'Océanographie Biologique de Banyuls, Avenue Fontaulé, BP 44, 66650 Banyuls-sur-Mer, France

<sup>3</sup>UPMC Univ Paris 06, UMR7621, Laboratoire ARAGO, Avenue Fontaulé, BP 44, 66650 Banyuls-sur-Mer, France

687

<sup>4</sup> Biological Centre of the Academy of Sciences of the Czech Republic, Hydrobiological Institute, Na sádkách 7, 37005 České Budějovice, Czech Republic

<sup>5</sup> CNRS, UMR7144, Equipe de Chimie Marine, Station Biologique de Roscoff, 29682 Roscoff, France

<sup>6</sup> UPMC Univ Paris 06, Equipe de Chimie Marine, Station Biologique de Roscoff, 29682 Roscoff, France

Received: 31 October 2008 – Accepted: 16 November 2008 – Published: 13 January 2009

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

Published by Copernicus Publications on behalf of the European Geosciences Union.

## 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 ( $12.8 \pm 5.3$  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 phosphocline ( $69 \pm 12$  m) and nitracline ( $50 \pm 8$  m) was probably strongly limited. However during the second half of the cruise, a shallowing of nutriclines occurred, 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  $1.1 \pm 1.2$  to  $4.6 \pm 3.8$ , and nitrate increased by a factor 2 ( $0.092 \pm 0.049 \mu\text{M}$ ). 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  $10 \text{ ng C l}^{-1} \text{ h}^{-1}$  (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.

689

## 1 Introduction

Bacterioplankton plays a central role in the nutrient and energy flux in marine ecosystems as the only 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). Phosphatase alkaline, 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

690

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/stoichiometry of organic matter as well as factors limiting bacterial production.

5 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 stoichiometry (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., 2008). The sampling strategy

691

of the cruise was settled to follow drifting sediment trap and to perform high frequency CTD casts during the cruise (minimum interval between 2 casts 3 h) 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., 2008).

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 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  $\mu$ M as NaNO<sub>3</sub>+1  $\mu$ M as NH<sub>4</sub>Cl), inorganic phosphorus (P: 0.25  $\mu$ M as NAH<sub>2</sub>PO<sub>4</sub>) or glucose (G: 10  $\mu$ M 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, and 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 trip-

692

licates showed a good reproducibility and suggest that factors of increase more than x1.5 are highly significant (Pinhassi et al., 2006; Van Wambeke et al., 2008).

## 2.2 Physico-chemical parameters

Dissolved organic carbon (DOC) by HTOCO 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 Auto-analyser® as described in Marty et al. (2008). Nitrate (abbreviated as NO<sub>3</sub> 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 (±0.003) μM, 0.02 (±0.01) μM for NO<sub>3</sub> and SRP, respectively. Depths 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

693

<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 thermostated 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  $K_m$  (Michaelis constant which reflects enzyme affinity for MUF-P) by fitting the data using a non-linear regression on the following equation:

$$V = V_{max}S/(K_m + S)$$

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 Mevel et al. (2008). Bacterial production was measured using the <sup>3</sup>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 trichloroacetic 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

694

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  $2.3 \text{ nmol l}^{-1} \text{ h}^{-1}$ ). It is thus possible that such high rates were underestimates. Variability between duplicates was, on average, 6% and the blank represented a mean of 7% of the signal.

## 5 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  $3 \mu\text{m}$ -pore-size filters (47 mm, Nuclepore) to remove most eukaryotic organisms and to prevent clogging of the final filter. Bacterial cells were concentrated onto  $0.22 \mu\text{m}$ -pore-size filters (47 mm, PC Nuclepore) and stored in 2 ml Eppendorf tubes at  $-20^\circ\text{C}$  until extraction. Frozen filters were cut with sterilized scissors into small strips and  $840 \mu\text{l}$  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  $37^\circ\text{C}$  after adding  $50 \mu\text{l}$  of freshly prepared lysosyme solution ( $20 \text{ mg ml}^{-1}$ ), and a second incubation at  $55^\circ\text{C}$  for 40 min after adding  $100 \mu\text{l}$  of 10% sodium dodecyl sulfate and  $10 \mu\text{l}$  of proteinase K ( $20 \text{ mg ml}^{-1}$ ).

Six hundred  $\mu\text{l}$  of lysate was treated with  $10 \mu\text{l}$  of a  $100 \text{ mg ml}^{-1}$  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  $400 \mu\text{l}$  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

695

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).

## 5 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  $12.8 \pm 5.3 \text{ m}$  depth between 17 September and 11 October. The stratification partially disrupted after this date as seen on  $Z_m$  depth which started to deepen on 12 October (Fig. 1, down to 40 m) after two successive strong wind events (11 and 16 October, Andersen et al., 2008).

### 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 phosphocline ( $Z_p$ ) as well as sharper gradient of concentration along  $Z_p$ . For instance at 70 m means concentrations of soluble reactive phosphorus (SRP) switched from  $0.10 \pm 0.05 \mu\text{M}$  and  $0.10 \pm 0.06 \mu\text{M}$  at C1 and C2 to  $0.28 \pm 0.09 \mu\text{M}$  and  $0.22 \pm 0.05 \mu\text{M}$  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

696

mean concentration of SRP did not change within  $Z_m$ , with concentrations at the lower range of sensitivity ( $0.030 \pm 0.017 \mu\text{M}$ : 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 between 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  $\text{NO}_3$  switched from  $3.9 \pm 1.1 \mu\text{M}$  and  $2.9 \pm 1.1 \mu\text{M}$  at C1 and C2 to  $7.2 \pm 1.1 \mu\text{M}$  and  $5.9 \pm 1.2 \mu\text{M}$  at C3 to 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  $\text{NO}_3$  within  $Z_m$  (data from 0–10 m layers) were:  $0.030 \pm 0.034$ ,  $0.038 \pm 0.033$ ,  $0.045 \pm 0.017$  and  $0.092 \pm 0.049$  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  $0.1 \mu\text{M}$ .

The  $\text{NO}_3$  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 ( $60 \pm 6$  m and N/P  $83 \pm 30$  during C1,  $62 \pm 10$  m and N/P  $78 \pm 24$  during C2,  $p > 0.05$  for both comparisons). At C3, the peak was at  $46 \pm 6$  m (N/P  $116 \pm 79$ ) and was at  $47 \pm 9$  m during C4 (N/P  $145 \pm 103$ ). 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  $0.9 \pm 1.0$ ,  $1.6 \pm 1.6$ ,  $0.9 \pm 0.5$  and  $4.6 \pm 3.8$  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,

697

$p > 0.05$ ), due to the high sd values, in turn, related to the very low concentration (close to the detection limits) of  $\text{NO}_3$  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:  $25 \pm 4$ ,  $19 \pm 4$ ,  $28 \pm 6$  and  $26 \pm 6 \text{ ng C l}^{-1} \text{ h}^{-1}$  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 the 3 first cycles (means of data from 5 m:  $3.0 \pm 0.3$ ,  $2.9 \pm 0.5$ , and  $3.2 \pm 0.4 \text{ nmol MUF-P hydrolysed l}^{-1} \text{ h}^{-1}$  for cycles C1, C2, and C3, respectively) and decreased significantly only during C4 ( $2.4 \pm 0.3 \text{ nmol MUF-P hydrolysed l}^{-1} \text{ h}^{-1}$ , 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:  $3.9 \pm 1.1$ ,  $4.6 \pm 1.5$ ,  $4.0 \pm 0.8$  and  $3.7 \pm 0.5 \text{ nmol MCA-leu hydrolysed l}^{-1} \text{ h}^{-1}$  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  $4.4 \pm 0.2$ ,  $5.3 \pm 1.2 \text{ nmol MUF-palmitate hydrolysed l}^{-1} \text{ h}^{-1}$  during C1 and C2, respectively to  $1.6 \pm 0.1$  and  $2.0 \pm 0.4 \text{ nmol MUF-palmitate hydrolysed l}^{-1} \text{ h}^{-1}$  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 phosphatase activity showed high variability

698

ity at all time scales (Fig. 3).  $V_m$  ranged 1.8 to 4.0 nmol l<sup>-1</sup> h<sup>-1</sup>,  $K_m$  22 to 124 nM and turnover time TT ( $K_m/V_m$  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  $K_m$  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 ( $0.69 \pm 0.02$ ) and lipase ( $0.56 \pm 0.08$ ), 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  $0.97 \pm 0.04$ , not statistically different than 1) varied in the same proportions than BP on the range of activities tested. Aminopeptidase and lipase per unit BP (nmole ng C<sup>-1</sup>) were indeed higher for a range of BP less than 10 ng C l<sup>-1</sup> h<sup>-1</sup> ( $0.41 \pm 0.29$  nmole ng C<sup>-1</sup> for  $0.19 \pm 0.09$  nmole ng C<sup>-1</sup>, ANOVA,  $p < 0.001$  or aminopeptidase,  $0.25 \pm 0.21$  nmole ng C<sup>-1</sup> for  $0.11 \pm 0.08$  nmole ng C<sup>-1</sup>, ANOVA,  $p < 0.005$  for lipase). In contrast, phosphatase activity per unit BP remained constant throughout the range of BP (mean  $\pm$  sd:  $0.12 \pm 0.09$  nmole ng C<sup>-1</sup>).

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,

699

Fig. 4c). Considering total counts, the slope of log-log regressions were significantly higher than 1 for phosphatase ( $1.97 \pm 0.10$ ) and aminopeptidase ( $1.57 \pm 0.05$ ). Thus, phosphatase and aminopeptidase activities per cell were significantly lower for a range of abundance lower than  $4 \times 10^5$  cells ml<sup>-1</sup> (mean  $\pm$  sd:  $1.4 \pm 1.6$  amol cell<sup>-1</sup> h<sup>-1</sup> for  $3.9 \pm 1.9$  amol cell<sup>-1</sup> h<sup>-1</sup>, ANOVA,  $p < 0.0001$  for phosphatase,  $3.9 \pm 2.8$  amol cell<sup>-1</sup> h<sup>-1</sup> for  $6.4 \pm 2.2$  amol cell<sup>-1</sup> h<sup>-1</sup>, ANOVA,  $p < 0.0001$  for aminopeptidase). Lipase activity per cell was stable within the range of abundances encountered (mean sd  $3.3 \pm 2.7$  amol cell<sup>-1</sup> h<sup>-1</sup>). 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).

### 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  $\pm$  sd:  $1.8 \pm 0.9$ ), excluding the experiences made at 5 m depth at the beginning of the cruise ( $\times 6$  on 18 September) and at 80 m depth at the end of the cruise ( $\times 9$  and  $\times 46$  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  $\times 1.9$ ,  $\times 1.2$  and  $\times 1.7$  compared to the control for N, G and P respectively, Fig. 5a) whereas addition of NPG simultaneously could stimulate leucine incorporation rates up to  $\times 53$  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  $\times 3.7$  and  $\times 4.4$ . For 4 and 7 October experiments, the P alone stimulation factor were the highest obtained during the cruise ( $\times 15$  and  $\times 5.6$ , respectively, Fig. 6). When the mixed layer started to deepen, after 12 October, the stimulation factors after NPG amendments decreased ( $\times 2.6$  to  $\times 10$  compared to  $\times 15$  to  $\times 50$  before), and stimulation of leucine incorporation rate after addition of P alone was still higher than the control ( $\times 1.7$  to  $\times 8.6$ ). At 5 m depth, addition of nitrate+ammonium alone

700

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 phosphocline from 76 m (21 September) to 57 m (7 October), whereas the depth of the nitracline was more or less constant (49 m and 47 m, respectively, Table 1). This resulted in higher stocks of SRP integrated from the surface to 80 m on 7 October (6 for 4.6 mmole P m<sup>-2</sup>). 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<sub>3</sub>/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 difference 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 leucine incorporation rates (×53 and ×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).

701

### 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) on 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<sup>-1</sup> h<sup>-1</sup>). 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 commu-

702



nity 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 ( $\times 5.6$ ,  $469 \text{ pmol l}^{-1} \text{ h}^{-1}$ ).

## 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 phosphocline 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  $\text{NO}_3/\text{SRP}$  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  $\text{NO}_3/\text{SRP}$  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

703

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 1:16 may theoretically lead to  $\text{NO}_3/\text{SRP}$  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 ( $50 \mu\text{M}$  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  $12 \text{ nmole l}^{-1} \text{ h}^{-1}$ ; TT 14 h) than during this survey. Higher  $V_{\text{max}}$  of phosphatase were obtained in coastal areas in NW Mediterranean Sea in spring (Thingstad et al., 2008, using a range of MUF-P concentration of 5–200 nM:  $V_{\text{max}} 28 \text{ nmole l}^{-1} \text{ h}^{-1}$ ) but on the same range as ours in eastern ultra-oligotrophic Mediterranean Sea in May (Thingstad and Mantoura, 2005:  $2\text{--}5 \text{ nmole l}^{-1} \text{ h}^{-1}$ ). For higher concentrations of analog substrate used by some other authors (Sala et al 2001:  $200 \mu\text{M}$ , Zoppini et al., 2005:  $125 \mu\text{M}$ ), 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. Bourguet et al. (2003) reported lipase rates (with  $12.5 \text{ nM}$

704

MUF-oleate as substrate) in the range  $0.9\text{--}5.8\text{ nmol l}^{-1}\text{ h}^{-1}$ , in a New-Caledonia lagoon. Goutx et al. (unpublished results) measured lipase activities in the range  $5\text{--}7\text{ nmoles l}^{-1}\text{ h}^{-1}$  using  $30\text{ }\mu\text{M}$  saturating MUF-oleate concentrations in the  $0\text{--}60\text{ m}$  layer in western Mediterranean including the DYFAMED site. Using also MUF-oleate analog substrate ( $20\text{ }\mu\text{M}$ ), Martinez et al. (1996) reported lipase activities per cell ranging 3 orders of magnitude ( $0.2\text{--}584\text{ amol cell}^{-1}\text{ h}^{-1}$ ) on pure cultures and  $20\text{--}70\text{ amol cell}^{-1}\text{ h}^{-1}$  in coastal Californian waters, i.e. higher values than we obtained at DYFAMED site (mean sd  $3.3\pm 2.7\text{ amol cell}^{-1}\text{ h}^{-1}$ ). 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- $\beta$ -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

705

(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 ( $80\text{--}150\text{ m}$ ) 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 (Fukuda et al., 2000) and 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  $5\text{ m}$  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 on the total count. 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 ( $>3\text{ }\mu\text{m}$  fraction) whereas aminopeptidase was more associated to free living bacterial fraction ( $0.2\text{--}3\text{ }\mu\text{m}$ ). In our study, attached bacteria were nevertheless probably more efficient to work in low abundance condi-

706

tion (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 reference therein), 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, Mével et al., 2008). Some inverse relationships between BP and aminopeptidase have been obtained (trophic gradient in Caribbean 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), stoichiometry of nutrients, chloroplast lipids

707

(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 incorporation 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 turnover time of phosphatase 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  $\text{nmol l}^{-1} \text{h}^{-1}$ , range of TT 9–47 h), compared to 0.6–12.6  $\text{nmol l}^{-1} \text{h}^{-1}$  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. Nevertheless, P limitation 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. The apparent severity of limitation of P is thus related to the availability of the second direct limiting factor after P (Pinhassi et al., 2006). What we show here during this monthly scale is that, even in stable stratifi-

708

cation conditions when P is frequently the primary factor limiting bacterial production, small scale physical changes (i.e. winds) can rapidly alter the stoichiometry 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  
5 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 stoichiometry of nutrients and factors limiting BP.

#### 10 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  
15 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  
25 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

709

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  
5 samples on 21 September 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  
15 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  
25 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).

710

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.

711

## 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 stoichiometry 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.

*Acknowledgements.* We specifically acknowledge the contribution of our friend Valérie Andersen who was chief scientist during the Dynaproc2 cruise and passed away before she could participate in the valorisation of her oceanographic campaign. We are grateful to John Dolan and Madeleine Goutx for helpful comments on the manuscript. This work was supported by PROOF grant provided by the CNRS-INSU and was part of the PECHE project "Production and Exportation of Carbon: control by heterotrophic organisms at small time scales".

## References

- Agis, M., Granda, A., and Dolan, J. R.: A cautionary note: examples of possible microbial community dynamics in dilution grazing experiments, *J. Exp. Mar. Biol. Ecol.*, 341, 176–183, 2007.
- 5 Andersen, V., Goutx, M., Prieur, L., and Dolan, J. R.: Short-scale temporal variability of physical, biological and biogeochemical processes in the NW Mediterranean Sea: an introduction, *Biogeosciences Discuss.*, 5, 5233–5251, 2008, <http://www.biogeosciences-discuss.net/5/5233/2008/>.
- Béthoux, J. P. and Copin-Montégut G.: Biological fixation of atmospheric nitrogen in the Mediterranean Sea, *Limnol. Oceanogr.*, 31, 1353–1358, 1986.
- 10 Boguea, G., Jean, N., Jamet, J. L., Jamet, D., and Richard, S.: Seasonal changes in phosphatase activities in Toulon Bay (France), *Mar. Environ. Res.*, 61, 1–18, 2006.
- Bourguet, N., Torréton, J. P., Arondel, V., Galy, O., and Goutx, M.: Specific and sensitive radiometric assay for measurement of real microbial lipase activities in marine water samples, *Appl. Environ. Microb.*, 69, 7395–7400, 2003.
- 15 Bourguet, N., Goutx, M., Ghiglione, M., Pujo-Pay, M., Mével, G., Momzikoff, A., Mousseau, L., Guigue, C., Garcia, N., Raimbault, P., Pete, R., Oriol, L., and Lefèvre, D.: Lipid biomarkers and bacterial lipase activities as indicators of organic matter and bacterial dynamics in contrasted regimes at the Dyfamed site, NW Mediterranean, *Deep-Sea Res.-Pt. II*, doi:10.1016/j.dsr2.2008.11.034, 2008.
- 20 Bouvier, T., Del Giorgio, P., and Gasol, J.: A comparative study of the cytometric characteristics of high and low nucleic acid bacterioplankton cells from different aquatic ecosystems, *Environ. Microbiol.*, 9, 2050–2066, 2007.
- Chrost, R. J. and Rai, H.: Ectoenzyme activity and bacterial secondary production in nutrient-impo-  
25 verished and nutrient-enriched freshwater mesocosms, *Microb. Ecol.*, 25, 131–150, 1993.
- Diaz, F., Raimbault, P., Garcia, N., and Moutin, T.: Early phosphorus limitation during spring in the Gulf of Lions, *Mar. Ecol.-Prog. Ser.*, 211, 51–62, 2001.
- Donachie, S. P., Christian, J. R., and Karl, D. M.: Nutrient regulation of bacterial production and ectoenzyme activities in the subtropical North Pacific Ocean, *Deep-sea Res.-Pt. II*, 48, 1719–1732, 2001.
- 30

713

- Duhamel, S., Gregori, G., Van Wambeke, F., Mauriac R., and Nedoma, J.: A method for analysing phosphatase activity in aquatic bacteria at the single cell level using flow cytometry, *J. Microbiol. Meth.*, 269–278, 2008.
- Dyhrman, S.: Ectoenzymes in *Prorocentrum minimum*, *Harmful algae*, 4, 619–627, 2005.
- 5 Eilers, H., Pernthaler, J., and Amann, R.: Succession of marine bacteria during enrichments: a close look at cultivation-induced shifts, *Appl. Environ. Microb.*, 66, 4634–4640, 2000.
- Fiala, M., Cahet, G., Jacques, G., Neveux, J., and Panouse, M.: Fertilisation des communautés phytoanctoniques: I. Cas d'un milieu oligotrophique: Méditerranée nord-occidentale, *J. Exp. Mar. Biol. Ecol.*, 24, 151–163, 1976.
- 10 Fukuda, R., Sohrin, Y., Saotome, N., Fukuda, H., Nagata, T., and Koike, I.: East-west gradient in ectoenzyme activity in the subarctic Pacific: Possible regulation by zinc, *Limnol. Oceanogr.*, 45, 930–939, 2000.
- Ghiglione, J. F., Larcher, M., and Lebaron, P.: Spatial and temporal scales of variation in bacterioplankton community structure in NW Mediterranean Sea, *Aquat. Microb. Ecol.*, 40, 229–240, 2005.
- 15 Ghiglione, J. F., Palacios, C., Marty, J. C., Mével, G., Labrune, C., Conan, P., Pujo-Pay, M., Garcia, N., and Goutx, M.: Role of environmental factors for the vertical distribution (0–1000 m) of marine bacterial communities in the NW Mediterranean Sea, *Biogeosciences*, 5, 1751–1764, 2008, <http://www.biogeosciences.net/5/1751/2008/>.
- 20 Goutx, M., Guigue, C., Aritio, D., Ghiglione, J., Pujo-Pay, M., and Andersen, V.: Short term variability of dissolved lipid classes during summer to autumn transition in the Ligurian sea (NW Mediterranean), *Biogeosciences Discuss.*, 6, 27–69, 2009, <http://www.biogeosciences-discuss.net/6/27/2009/>.
- 25 Haldeman, D. L., Amy, P. S., White, D. C., and Ringelberg, D. B.: Changes in bacterial recoverable from subsurface volcanic rock samples during storage at 4°C, *Appl. Environ. Microb.*, 60, 2697–2703, 1994.
- Hoppe, H. G.: Significance of exoenzymatic activities in the ecology of brackish water: measurement by means of methylumbelliferyl-substrates, *Mar. Ecol.-Prog. Ser.*, 11, 299–308, 1983.
- 30 Hoppe, H. G., Giesenhausen, H. C., and Gocke, K.: Changing patterns of bacterial substrate decomposition in a eutrophication gradient, *Aquat. Microb. Ecol.*, 15, 1–13, 1998.

714

- Kirchman, D. L.: Leucine incorporation as a measure of biomass production by heterotrophic bacteria, in: *Handbook of methods in aquatic microbial ecology*, edited by: Kemp, P. F., Sherr, B. F., Sherr, E. B., and Cole, J. J., Lewis, 509–512, 1993.
- Kirchman, D. L., Dittel, A., Findlay, S., and Fischer, D.: Changes in bacterial activity and community structure in response to dissolved organic matter in the Hudson River, New York, *Aquat. Microb. Ecol.*, 35, 243–257, 2004.
- Krom, M. D., Kress N., and Brenner, S.: Phosphorus limitation of primary production in the eastern Mediterranean Sea, *Limnol. Oceanogr.*, 36, 424–432, 1991.
- Mc Gill, D. A.: The relative supplies of phosphate, nitrate and silicate in the Mediterranean Sea, *ICES Marine Symposium*, 18, 767–744, 1965.
- Martinez, J. and Azam, F.: Aminopeptidase activity in marine chroococcoid cyanobacteria, *Appl. Environ. Microb.*, 59, 3701–3707, 1993.
- Martinez, J., Smith, D. C., Steward, G. F., and Azam, F.: Variability in ectohydrolytic enzyme activities of pelagic marine bacteria and its significance for substrate processing in the sea, *Aquat. Microb. Ecol.* 10, 223–230, 1996.
- Marty, J. C., Garcia, N., and Raimbault, P.: Phytoplankton dynamics and primary production under late summer conditions in the NW Mediterranean Sea, *Deep-Sea Res. Pt. I*, 55, 1131–1149, 2008.
- Marty, J. C., Chiaverini, J., Pizay, M. D., and Avril, B.: Seasonal and interannual dynamics of nutrients and phytoplankton pigments in the western Mediterranean Sea at the DYFAMED time-series station (1991–1999), *Deep-Sea Res. Pt. II*, 49, 1965–1985, 2002.
- Massana, R., Pedros-Alió, C., Casamayor, E. O., and Gasol, J. M.: Changes in marine bacterioplankton phylogenetic composition during incubations designed to measure biogeochemically significant parameters, *Limnol. Oceanogr.*, 46, 1181–1188, 2001.
- Mével, G., Vernet, M., Goutx, M., and Ghiglione, J. F.: Seasonal to hour variation scales in abundance and production of total and particle-attached bacteria in the open NW Mediterranean Sea (0–1000 m), *Biogeosciences*, 5, 1573–1586, 2008, <http://www.biogeosciences.net/5/1573/2008/>.
- Misic, C., Castellano, M., Ruggieri, N., and Covazzi Harriague, A.: Variations in ectoenzymatic hydrolytic activity in an oligotrophic environment (Southern Tyrrhenian Sea, W Mediterranean), *J. Mar. Syst.*, 73, 123–137, 2008.
- Misic, C. and Fabiano, M.: Ectoenzymatic activity and its relationship to chlorophyll-a and bacteria in the gulf of Genoa (Ligurian Sea, NW Mediterranean), *J. Mar. Syst.*, 60, 193–206,

715

- 2006.
- Moutin, T. and Raimbault, P.: Primary production, carbon export and nutrients availability in western and eastern Mediterranean sea in early summer 1996 (MINOS cruise), *J. Mar. Res.*, 33–34, 273–288, 2002.
- Muyzer, G., Brinkhoff, T., Nübel, U., Santegoeds, C., Schäfer, h., and Wawer, C.: Denaturing gradient gel electrophoresis (DGGE) in microbial ecology, in: *Molecular microbial ecology manual*, edited by: Akkermans, A. D. L., van Elsas, J. D., and de Bruijn, F. J., Kluwer Academic Publishers, Dordrecht, The Netherlands, 3.4.4, 1–27, 1998.
- Pinhassi, J., Gómez-Consarnau, L., Alonso-Sáez, L., Sala, M., Vidal, M., Pedrós-Alió, C., and Gasol, J.: Seasonal changes in bacterioplankton nutrient limitation and their effects on bacterial community composition in the NW Mediterranean Sea, *Aquat. Microb. Ecol.*, 44, 241–252, 2006.
- Raimbault, P., Slawyk, G., Coste, B., and Fry, J.: Feasibility of using an automated colorimetric procedure for the determination of seawater nitrate in the 0 to 100 nM range: Examples from field and culture, *Mar. Biol.*, 104, 347–351, 1990.
- Rath, J., Schiller, C., and Herndl, G. J.: Ectoenzyme activity and bacterial dynamics along a trophic gradient in the Caribbean Sea, *Mar. Ecol.-Prog. Ser.*, 102, 89–96, 1993.
- Rosso, A. L. and Azam, F.: Proteolytic activity in coastal oceanic waters: depth distribution and relationship to bacterial populations, *Mar. Ecol.-Prog. Ser.*, 41, 231–240, 1987.
- Sala, M. M., Karner, M., Arin, L., and Marrasé, C.: Measurement of ectoenzyme activities as an indication of inorganic nutrient imbalance in microbial communities, *Aquat. Microb. Ecol.*, 23, 301–311, 2001.
- Sala, M. M., Peters, F., Gasol, J. M., Pedrós-Alió, C., Marrasé, C., and Vaqué, D.: Seasonal and spatial variations in the nutrient limitation of bacterioplankton growth in the northwestern Mediterranean, *Aquat. Microb. Ecol.*, 27, 47–56, 2002.
- Schäfer, h., Servais, P., and Muyzer, G.: Successional changes in the genetic diversity of a marine bacterial assemblage during confinement, *Arch. Mikrobiol.*, 173, 138–145, 2000.
- Smith, D. C. and Azam, F.: A simple, economical method for measuring bacterial protein synthesis rates in sea water using 3H-Leucine, *Marine Microbial Food Webs*, 6, 107–114, 1992.
- Suzuki, M. T.: Effect of protistan bacterivory on coastal bacterioplankton diversity, *Aquat. Microb. Ecol.*, 20, 261–272, 1999.
- Taylor, G. T., Way, J., Yu, Y., and Scranton, M.: Ectohydrolase activity in surface waters of the Hudson river and western Long Island Sound estuaries, *Mar. Ecol.-Prog. Ser.*, 263, 1–15,

716

- 2003.
- Thingstad, T. and Mantoura, R.: Titrating excess nitrogen content of phosphorus-deficient eastern Mediterranean surface water using alkaline phosphatase activity as a bio-indicator, *Limnol. Oceanogr.-Meth.*, 3, 94–100, 2005.
- 5 Thingstad, T. F., Zweifel, U. L., and Rassoulzadegan, F.: P limitation of heterotrophic bacteria and phytoplankton in the northwest Mediterranean, *Limnol. Oceanogr.*, 43, 88–94, 1998.
- Tréguer, P. and LeCorre, P.: Manuel d'analyse des sels nutritifs dans l'eau de mer, Utilisation de l'AutoAnalyser II Technicon, Univ. Bretagne Occidentale, Laboratoire de Chimie marine, Brest, France, 2nd edn., 1–110, 1975.
- 10 Unanue, M., Ayo, B., Azua, I., Barcina, I., and Iriberry, J.: Temporal variability of attached and free-living bacteria in coastal waters, *Microb. Ecol.*, 23, 27–39, 1992.
- Van Wambeke, F., Christaki, U., Giannakourou, A., Moutin, T., and Souvemerzoglou, K.: Longitudinal and vertical trends of bacterial limitation by phosphorus and carbon in the Mediterranean Sea, *Microb. Ecol.*, 43, 119–133, 2002.
- 15 Van Wambeke, F., Bonnet, S., Moutin, T., Raimbault, P., Alarcón, G., and Guieu, C.: Factors limiting heterotrophic bacterial production in the southern Pacific Ocean, *Biogeosciences*, 5, 833–845, 2008, <http://www.biogeosciences.net/5/833/2008/>.
- Zobell, C. E. and Anderson, D. Q.: Observations on the multiplication of bacteria in different volumes of stored sea water and the influence of oxygen tension and solid surfaces, *Biol. Bull.*, 71, 324–342, 1936.
- 20 Zohary, T. and Robarts, R. D.: Experimental study of microbial P limitation in the eastern Mediterranean, *Limnol. Oceanogr.*, 43, 387–395, 1998.
- Zoppini, A., Puddu, A., Fazi, S., Rosati, M., and Sist, P.: Extracellular enzyme activity and dynamics of bacterial community in mucilagenous aggregates of the northern Adriatic Sea, *Sci. Total Environ.*, 353, 270–286, 2005.
- 25

**Table 1.** Synthesis of the criteria used to determine depths of mixed layer ( $Z_m$ ) phosphacline ( $Z_p$ ) and nitracline ( $Z_N$ ).

Criterion	mixed layer $Z_m$	phosphacline $Z_p$	nitracline $Z_N$
Depth for which the difference between density excess (for $Z_m$ ), and nitrate ( $Z_N$ ) phosphate (for $Z_p$ ) concentration is more or equal to	0.05 kg m <sup>-3</sup>	0.01 μM	0.5 μM
	0.1 kg m <sup>-3</sup>	0.05 μM	1 μM
Depth for which the vertical gradient is more or equal to	0.02 kg m <sup>-3</sup>	0.05 mol m <sup>-4</sup>	0.5 mmol m <sup>-4</sup>
	0.01 kg m <sup>-3</sup>	0.1 mmol m <sup>-4</sup>	1 mmol m <sup>-4</sup>



**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.

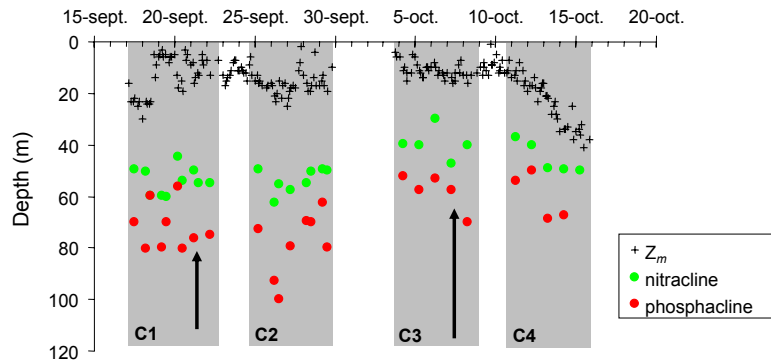
		Units	21 September	6 October
$Z_m$	Depth	m	13	15
	mean temperature	°C	22.2	20.9
P stocks	Phosphacline depth	m	76	57
	Integrated 0–80 m	mmole m <sup>-2</sup>	4.6	6
N stocks	Nitracline depth	m	49	47
	Integrated 0–80 m	mmole m <sup>-2</sup>	137	211
Leu inc rates	Integrated 0–80 m	μmol m <sup>-2</sup> h <sup>-1</sup>	2.2	1.1
APase	Integrated 0–80 m	μmol m <sup>-2</sup> h <sup>-1</sup>	225	108
AMPase	Integrated 0–80 m	μmol m <sup>-2</sup> h <sup>-1</sup>	254	189

719

**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: nmole l<sup>-1</sup> h<sup>-1</sup> for ectoenzymatic activities, ng C l<sup>-1</sup> h<sup>-1</sup> for bacterial production, ml<sup>-1</sup> for abundances. \* Plot corresponding Fig. 4a, \*\* Plot corresponding Fig. 4c.

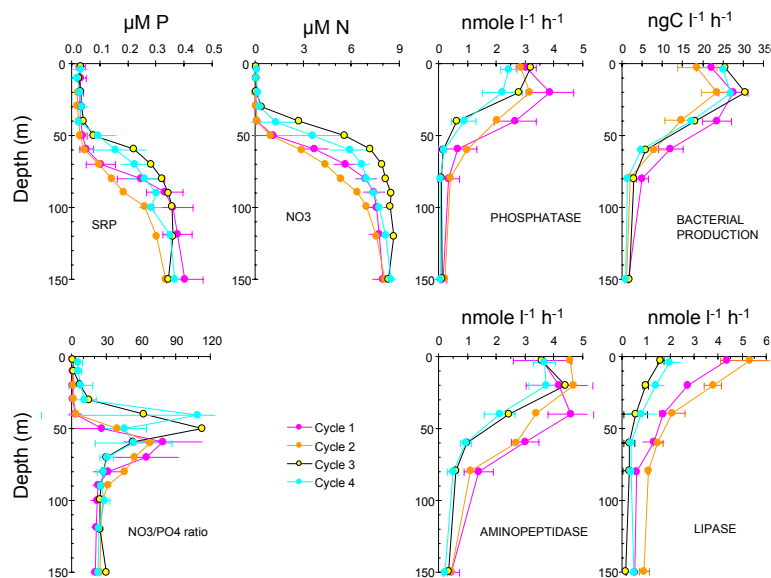
	Y intercept	Slope	$R$	$N$
BP				
Phosphatase*	-1.015±0.040	0.974±0.040	0.856	220
Aminopeptidase*	-0.365±0.024	0.691±0.023	0.890	222
Lipase*	-0.496±0.092	0.564±0.089	0.680	48
Total abundance				
Phosphatase**	-11.175±0.558	1.976±0.100	0.800	220
Aminopeptidase	-8.534±0.274	1.575±0.049	0.906	222
Lipase	-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

720



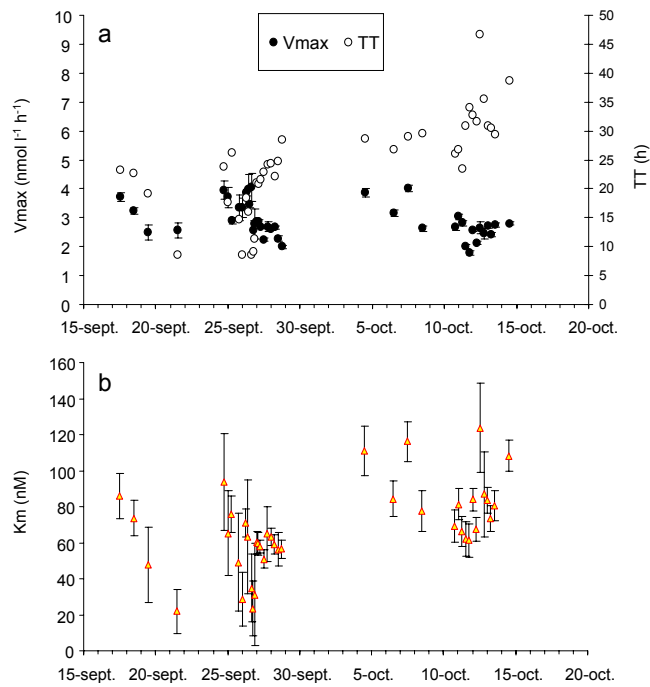
**Fig. 1.** Evolution of mixed layer depth ( $Z_m$ ) phosphacline ( $Z_p$ ) and nitracline ( $Z_n$ ) from 17 September to 15 October, during DYNAPROC cruise, when the ship was at the fixed point DYFAMED station. Shaded areas shows period of high frequency sampling of CTDs casts, defined as cycle 1 (C1, 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 Fig. 5).

721



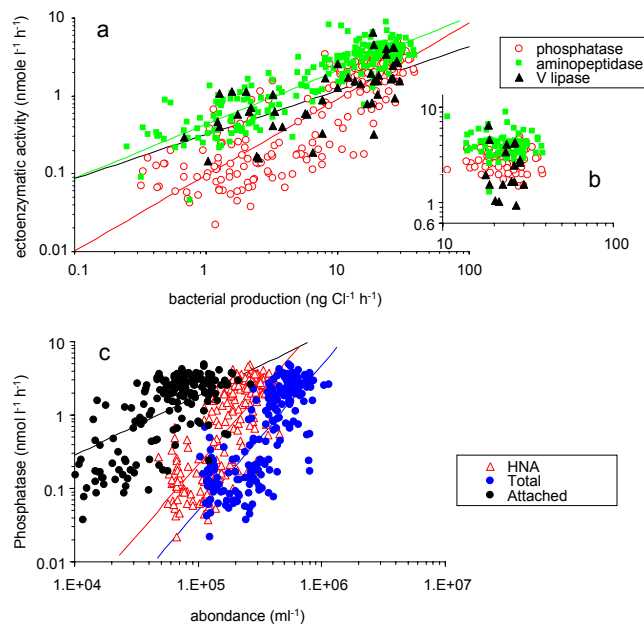
**Fig. 2.** Mean vertical distributions of nutrients (nitrate, phosphate, nitrate to phosphate ratio), heterotrophic bacterial production, and ectoenzymatic activities (phosphatase, aminopeptidase, lipase) along 0–150m water column during cycles C1, C2, C3 and C4. Errors bars (standard deviations of 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 site were made.

722



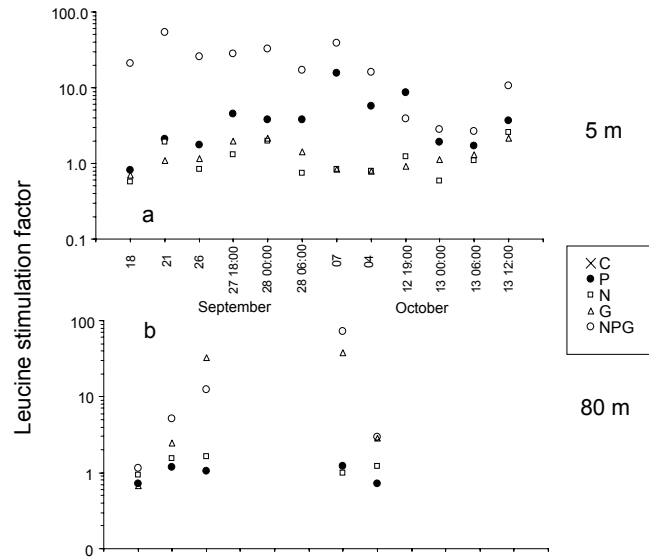
**Fig. 3.** Evolution of kinetic parameters determined from concentration kinetics realized on phosphatase activity at 5 m depth. **(a)** Maximum velocities ( $V_m$ , nmole MUF-P hydrolyzed  $l^{-1}h^{-1}$ ), and turnover time ( $TT=K_m/V_m$ , h); **(b)** half saturation constant ( $K_m$ , nM). Bars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

723



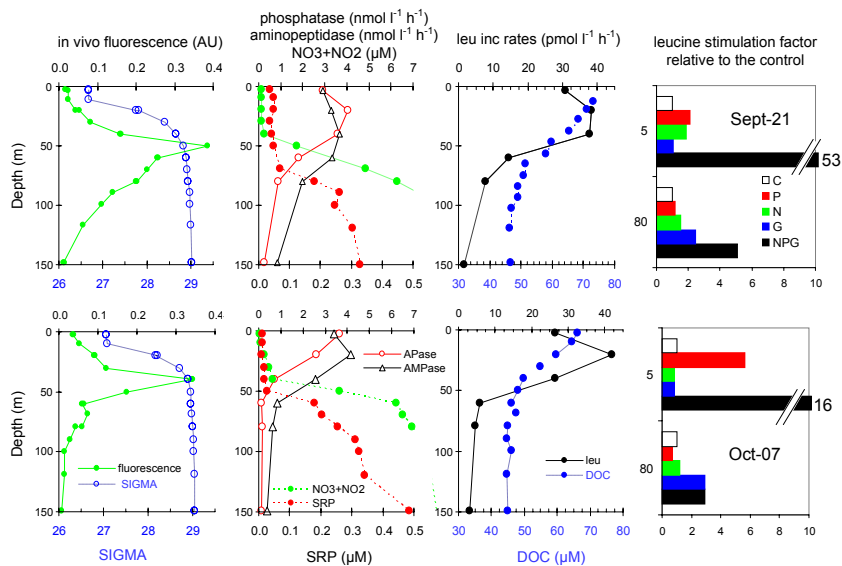
**Fig. 4.** Relation between **(a)** ectoenzymatic activities (phosphatase, aminopeptidase and lipase) versus bacterial production and **(b)** phosphatase alkaline activity versus total abundance, attached bacterial abundance and HNA-group. All data set 0–150 m is presented. The smaller graph in **(a)**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

724



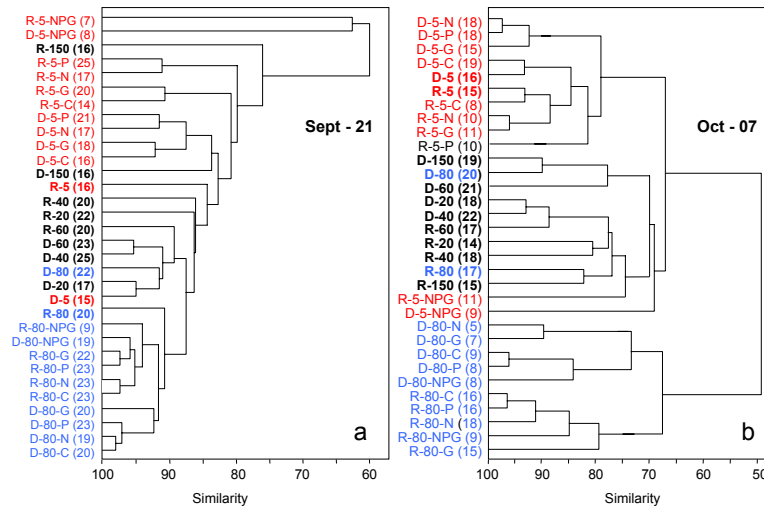
**Fig. 5.** Factor of stimulation of leucine incorporation rates after 24 h addition of phosphate (P), nitrate+ammonium (N), glucose (G) and 4 elements (NPG), compared to the control unamended (C), i.e. for C the value is set to 1. **(a)** enrichment experiments made at 5 m depth, **(b)** at 80 m.

725



**Fig. 6.** Detailed information on vertical distributions of density, in vivo fluorescence, phosphate and nitrate concentrations, dissolved organic carbon concentration, 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).

726



**Fig. 7.** Cluster analysis dendrogram based on 16S 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 and 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.