PHYTOPLANKTON AND BACTERIA ALKALINE PHOSPHATASE ACTIVITY IN THE NORTHERN ADRIATIC SEA

Ingrid Ivančić[*a], Dragica Fuks[a], Tomislav Radić[b], Daniel M. Lyons[a], Tina Šilović[a], Romina Kraus[a], Robert Precali[a]

[a] Center for Marine Research, Ruđer Bošković Institute, G. Paliaga 5, 52210 Rovinj, Croatia

[b] Institute for Adriatic Crops and Karst Reclamation, Put Duilova 11, 21000 Split, Croatia

Key words: APA, nutrients, DOP, phytoplankton, bacteria, northern Adriatic

∗ Corresponding author: Ingrid Ivančić, tel. 00385 52 804 743; fax. 00385 52 813 496; email ingrid@cim.irb.hr

ABSTRACT

The importance of bacterial, phytoplankton and dissolved alkaline phosphatase activity (APA) in the northern Adriatic was investigated during 2006. In upper waters total APA increased from early spring (0.05-0.08 µmol l^{-1} h^{-1}) to late spring (up to 4.64 µmol l^{-1} h^{-1}) and remained relatively high during the summer (0.46-0.71 µmol l^{-1} h^{-1}), due to an increase in specific phytoplankton (up to 30 µmol µg C^{-1} h^{-1}) and bacterial APA (up to 23 µmol µg C^{-1} h^{-1}). Activity of free enzymes was not important. During late spring and summer both communities exploited dissolved organic phosphorus although, taking into account biomass, phytoplankton activity usually dominated over bacterial. In autumn an extra P supply from deeper waters drastically reduced phytoplankton APA, though not bacterial APA, in upper waters. Probably in these months bacteria degrading phytoplankton produced organic matter were P limited. In deeper waters APA was low and mainly due to the activity of free enzymes.

1. INTRODUCTION

The northern Adriatic is characterised by significant freshwater input, mainly from the Po River. In Po River waters the average total phosphorus and total nitrogen concentrations in the 1999-2002 period (5.16 µmol l^{-1} and 285.71 µmol l^{-1}, respectively; Milan et al., 2003), were more than an order of magnitude higher than in northern Adriatic waters (Giani, 2003). Consequently, riverine waters increase nutrient content in seawater leading to high microbial activity and eutrophication of the western area of the northern Adriatic (Gilmartin and Revelante, 1983; Gilmartin et al., 1990; Karner et al., 1992). However, in river waters inorganic nitrogen concentrations (DIN) were markedly higher than orthophosphate (PO_4) concentrations, resulting in a strongly unbalanced N/P atomic ratio (about 100/1; Milan et al., 2003) for microbial requirements (balanced N/P=16/1 for phytoplankton, 9/1 for bacteria; Redfield et al., 1963; Goldman et al., 1987). As a consequence P limitation is expected in productive northern Adriatic waters influenced by freshwater. It was observed that in these waters organic phosphorus concentrations markedly exceeded PO_4 concentrations, representing an important source of P for microbial communities (Ivančić and Degobbis, 1987). In the presence of low PO_4 concentrations microbes can induce extracellular alkaline phosphatase (AP), which enables them to use organic phosphorus esters as a source of this element (see Hoppe, 2003). A study carried out during 2004 showed that in the concerned area AP was important for providing P for microbial growth, particularly during phytoplankton blooms

induced by freshwater imported nutrients (Ivančić et al., 2009). Alkaline phosphatase activity (APA) studies in the marine environment have been focused mainly on algae and have rarely dealt with bacterial activities even though bacteria are also known to have significant APA (Martinez and Azam, 1993; Labry et al., 2005). Recently, Zaccone et al. (2002) showed that most of the bacterial strains in the northern Adriatic are capable of expressing APA.

The objectives of the present paper are to evaluate the importance of phytoplankton and bacteria in expressing APA in one of the most productive areas of the northern Adriatic. In this area where inorganic N supply greatly exceeds inorganic P supply, productivity depends on the ability of microorganisms to obtain P from dissolved organic phosphorus (DOP). For this purpose total APA and parameters characterising microbial communities were measured seasonally at two stations mostly influenced by Po River runoff. Phytoplankton and bacterial APA were determined during blooms induced by freshwater imported nutrients, as well as during minimal external nutrient input in summer. Even if size fractionation by filtration does not completely separate groups of microorganisms (overlapping size) it does however give a useful indication as regards the major microorganisms contributing to APA.

2. MATERIAL AND METHODS

2.1. Sampling strategy

Measurements were carried out at two stations (SJ101, SJ108) in the northern Adriatic (Fig. 1) during 7 cruises performed from March to October 2006. These stations were specifically chosen as they are in one area permanently under riverine nutrients pressure, unlike other areas along the Rovinj-Po Delta profile which only intermittently experience freshwater influence. Further, these stations represent two different regimes where SJ108 is under direct freshwater nutrient influence while freshwater spreading toward SJ101 is more impoverished with nutrients. During all cruises conventional parameters (sea temperature, salinity, nutrients, DOP, chl *a*, bacteria and picocyanobacteria counting and phytoplankton determination) and total APA were measured at three depths within the water column (surface, 10 m, and 1 m from the bottom: 30 m). In addition, APA fractions were determined during blooms in different seasons (May, September and October), as well as during minimal phytoplankton biomass in June.

Fig. 1

2.2. Analytical protocol

Water samples were collected with 5 l PVC Niskin samplers. Temperature and salinity profiles were acquired during the downcasts of a Seabird SBE 25 CTD probe.

Inorganic nutrient analyses were performed onboard, on unfiltered water immediately after sample collection, using methods widely used in oceanography (Strickland and Parsons, 1972; Ivančić and Degobbis, 1984). Samples for total dissolved phosphorus were filtered (Whatmann GF/C, precombusted at 500 ºC) and stored in polyethylene tubes at -30 ºC. In the laboratory ashore analyses were performed using a chemical combustion method with persulphate (Menzel and Corwin, 1965). DOP was calculated by subtracting PO_4 from the total dissolved phosphorus. DIN was calculated as the sum of nitrate, nitrite and ammonia. The N/P ratio was calculated by linear regression between PO_4 and DIN.

Determination of APA was performed aboard the research vessel immediately after sample collection. Measurements were carried out in unfiltered water (total APA) and two pre-filtered fractions: <0.22 µm and <3 µm. Picocyanobacteria and heterotrophic bacteria overlap in size ranges, and for this reason filters of 3 µm were preferred (retaining bacteria and picocyanobacteria) instead of 1 µm, which do not retain all bacteria, or 2 µm at which an unknown part of picocyanobacteria is retained. The abundance of picoeukaryotes which are not retained on the 3 µm filters was three orders of magnitude lower than picocyanobacteria abundance (Fuks, unpublished data). The nanophytoplankton fraction was retained on the 3 µm filter, as confirmed by microscopic measurements. The 0.2-3 µm fraction contained heterotrophic bacteria and picocyanobacteria, mainly *Synechococcus* (Fuks, unpublished data), and is subsequently referenced in the text as the bacterial fraction. The >3 µm fraction contained nano- and microphytoplankton and is subsequently referenced as the phytoplankton fraction.

All APA measurements were performed in duplicate with a fluorogenic substrate analogue using methyllumbelliferyl-phosphate (MUF-P) dissolved in methylcellosolve and diluted with water immediately before addition following the procedure of Hoppe (1983). The final concentration of substrate in sample was 50 µmol l^{-1}. This concentration was chosen since it was observed that in seawater with various microbial activities saturation

with substrate was always achieved (Ivančić et al., 2009). In addition, kinetic parameters (half saturation constant K_m, maximum activity V_{max}) were determined at the surface at SJ108 in May, June and September using various MUF-P concentrations (0.5, 5, 10, 50, 100, 150, 200, 250 µmol l^{-1}). Incubation was performed in the dark in an insulated water bath using water collected from the same depth as the sample to maintain the *in situ* temperature and pH. Fluorescence was measured immediately after substrate addition and after ~1 h of incubation using a Turner TD-700 fluorometer with excitation at 365 nm and emission at 460 nm. APA was calculated as the difference between these two measurements divided by the incubation time after calibration of the fluorometer with methyllumbelliferone. Results are presented as the mean value of duplicates. K_m and V_{max} were calculated using Woolf-Hanes linearization. Although several methods are available, P turnover time (T_n) was estimated by the K_m/V_{max} ratio as, for example, in Labry et al. (2005). An alternative method for determining turnover time as described by Xu et al. (2008) gave nearly identical results when DOP concentration was used as the natural substrate concentration (organic phosphorus esters). However, results using the latter method are not reported here since DOP concentration is not always a good approximation of the natural substrate concentration which is not measured.

The samples for chl *a* determination (Strickland and Parsons, 1972) were filtered onboard through Whatmann GF/C filters and stored at -30 ºC. Extractions with 90% acetone and fluorometric analyses were performed in the onshore laboratory within a few days.

Samples for microphytoplankton determination (200 ml) were filtered through a 300 µm mesh plankton net to remove zooplankton, and filtrates were preserved with Lugol solution (2% final concentration) and buffered with sodium acetate. Microphytoplankton abundance and composition were determined in the filtrate at 200x magnification by a Zeiss inverted microscope after 40 hours of sedimentation of a 50 ml subsample using the Utermöhl (1958) settling technique.

Samples for bacteria abundance (BA) and picocyanobacteria abundance (CBA) were preserved with formaldehyde (2% final concentration) and stored at 4 °C. BA was determined by cell counting using an epifluorescence microscope after staining with 4',6-diamido-2-phenylindole (Porter and Feig, 1980). CBA was also determined by epifluorescence microscopy and distinguished by orange autofluorescence under green excitation (Takahashi et al., 1985).

Specific APA in different fractions was calculated as the ratio between APA and the carbon content in each respective fraction. BA was converted to carbon content by a conversion factor of 20 fg C/cell (Lee and Fuhrman, 1987) and CBA by a factor of 250 fg C/cell (Kana and Gilbert, 1987). Phytoplankton C content was obtained by converting chl *a* using a factor of 50 µg C/µg chl *a* (Antia et al., 1963) followed by the subtraction of C content in picocyanobacteria. The bacterial and phytoplankton C content calculated from the abovementioned factors are commonly used to calculate their respective specific APA (Sala et al., 2001, Nausch et al., 2004; Labry et al., 2004) and data presented here are comparable with literature data.

3. RESULTS

3.1. Hydrological conditions and nutrient status

In March the entire water column at both stations was cold (7.6-8.1 ºC) and homogenous (Fig. 2a). In the April-July period temperatures increased (16.9- 27.8 ºC at the surface and 8.7-13.4 ºC at the bottom) with the establishment of thermal stratification. During September cooling of the surface started mixing in the water column and in October a nearly homogenous layer extended down to 20 m (18.1-21.6 ºC) with increased values in the bottom waters (16.2-18.4 ºC; Fig. 2a). Haline stratification started from April and persisted during the entire investigated period, except at SJ101 in June (Fig. 2b). At SJ108 the Po River plume (salinity <36) was detected in April, May and September to a depth of about 2-6 m. At this station freshwater influence was weak in June, July and October (surface salinity 36.6-37.1; Fig. 2b). At SJ101 the Po River plume was detected in May, September and October to a depth of about 1-6 m, while in April, June and July freshwater influence was weak (surface salinity 36.4-37.9; Fig. 2b). At a depth of 10 m and below the contribution of riverine water at both stations was always low and salinity was >37.

Fig. 2

PO_4 concentrations were always low (0.00-0.07 µmol l^{-1}) in the entire water column at both stations, even in the Po River plume, except for higher values (0.23 µmol l^{-1}) at the bottom in October (Fig. 3a). DOP concentrations were always several times higher than PO_4 concentrations. In March and October DOP (generally 0.3 -0.5 µmol l^{-1}) was considerably higher than from April to July (generally 0.05-0.25 µmol l^{-1}) when somewhat higher values (up to 0.3 µmol l^{-1}) were periodically found at the bottom (Fig. 3b). Periods

with low DOP coincided with high APA, and vice versa (See next chapter). At the surface and 10 m depth DIN was present in surplus with respect to PO_4, resulting in a high N/P ratio (28/1; data not shown). At the surface high DIN concentrations (3.5-14.0 µmol l^{-1}) were found during freshets (April, May, September, October), while minimal values (0.58-1.18 µmol l^{-1}) were found during minimal freshwater influence (June and July; Fig. 3c). At 10 m depth values were lower than at the surface, but showed basically the same trend (Fig. 3c). At the bottom minimal DIN concentrations were found during June and July (generally about 1 µmol l^{-1}), and maximal during October (up to 8.68 µmol l^{-1}). In these waters DIN and PO_4 were generally balanced for microbial requirements (N/P 9/1; data not shown).

Fig. 3

3.2. Microbial biomass evolution and total APA

The Po River plume was observed in April, May and September at SJ108, and in May, September and October at SJ101. In these waters freshwater imported nutrients increased phytoplankton biomass (102-667 µg C l^{-1}) while during the period of low freshwater influence phytoplankton biomass in the surface layer was markedly lower (about 1-24 µg C l^{-1}; Fig. 4a). Bacterial and picocyanobacterial biomass in the surface layer was not affected by freshwater, being generally higher in the July-October period (29-60 µg C l^{-1}) than in the first part of the year (typically 14-18 µg C l^{-1}; Fig. 4a). During blooms, phytoplankton biomass markedly exceeded bacterial and picocyanobacterial biomass, while in other situations they were either similar or bacterial and picocyanobacterial biomass exceeded that of phytoplankton. The highest total APA (2.59-4.64 µmol l^{-1} h^{-1}) was observed during phytoplankton blooms, except at SJ101 in September and October when the increase in surface phytoplankton biomass did not result in increased total APA (Fig 4a). During June and July, as well as in October at SJ108, periods characterised by low phytoplankton biomass, surface total APA (0.28-0.71 µmol l^{-1} h^{-1}) was markedly lower. Minimal surface total APA (0.05-0.08 µmol l^{-1} h^{-1}) was found in March when the water column was mixed, even though the microbial biomass was not lower than during the summer months (Fig. 4a).

Fig. 4

The phytoplankton blooms did not extend to intermediate waters (10 m depth) where phytoplankton biomass was generally low (6-34 µg C l^{-1}) and seasonal changes

much less pronounced (Fig. 4b). In these waters bacterial and picocyanobacterial biomass was also generally lower (8-38 µg C l^{-1}) than at the surface, though usually higher than that of phytoplankton (Fig. 4b). In these waters total APA was lower than in upper waters (Fig. 4b). The lowest APA was found in March and at SJ101 also in October (down to 0.02 µmol l^{-1} h^{-1}). In other months APA was generally 0.2-0.5 µmol l^{-1} h^{-1}.

At the bottom phytoplankton biomass (14-40 µg C l^{-1}) was somewhat higher, while bacterial and picocyanobacterial biomass had similar ranges as in intermediate waters (Fig. 4c). In these waters phytoplankton biomass was generally similar to those of bacteria and picocyanobacteria. Total APA was lower than in upper waters and generally <0.15 µmol l^{-1} h^{-1} (Fig. 4c).

3.3. APA fractions

The overlap of the size spectra of bacteria, picocyanobacteria and phytoplankton could affect their APA estimation. However, in surface and intermediate waters the ratios APA in 0.2-3 µm fraction/total APA and APA>3 µm fraction/total APA were significantly correlated (r^2=0.579, p<0.001) with the ratios bacterial biomass/total biomass and phytoplankton biomass/total biomass, respectively. This suggests that APA in the 0.2-3 µm fraction represented the bulk of bacterial and picocyanobacterial, while APA in the >3 µm fraction the bulk of phytoplankton, activity. Calculations were not made for the bottom layer where APA was not related to microbial biomass, but mostly due to free enzymes.

At the surface enzymatic activity was usually due to microbes, while activity of free enzymes was negligible (generally 0.0-4.2% of total activity; Fig. 5a). During the phytoplankton bloom in May practically all surface activity at both stations was due to the phytoplankton fraction (93.0-94.3%). In other months activity of the bacterial fraction also became important (30.8-47.9%), and in September (at SJ101) and October (at SJ108) most of the enzymatic activity was due to this fraction (64.8-79.1%; Fig. 5a). At 10 m depth the contribution of free enzymes (7-20%) was higher than at the surface, though still much less important than that of the microbial fraction (Fig. 5b). In this layer the contribution of phytoplankton to total APA was generally lower than at the surface, while the bacterial contribution increased (Fig. 5b). At both stations in May and at SJ101 in June, most of the activity was due to the phytoplankton fraction (about 49-66%), while in September and October, and also in June at SJ108, the contribution of the bacterial

fraction was more important (52.8-91.0%, Fig. 5b). At the bottom APA was mostly due to free enzymes (54-100%, Fig. 5c), except at SJ101 in June when the low activity was mostly due to bacteria.

Fig. 5

3.4 Specific APA and composition of the microbial communities

At the surface and in intermediate waters both phytoplankton and bacterial specific APA were high in May and June (7.64->30 nmol µg C^{-1} h^{-1} and 5.83-23.76 nmol µg C^{-1} h^{-1}, respectively; Fig. 6a,b). It should be noted that due to the very low phytoplankton biomass in June there was greater uncertainty in the calculation of specific phytoplankton APA, hence these data were simply denoted as >30 nmol µg C^{-1} h^{-1}. In September and October specific phytoplankton APA dropped to low values (1.11-4.52 nmol µg C^{-1} h^{-1}), while specific bacterial APA did not show such a drastic decrease in these months. At the surface specific bacterial APA in October was lower (3.78-6.07 nmol µg C^{-1} h^{-1}) than in other months, while in intermediate waters a decrease in October was observed only at SJ101 (Fig. 6b). Specific phytoplankton APA was generally higher at SJ101, while to the contrary specific bacterial APA was higher at SJ108.

Fig. 6

At the bottom the specific APA of both fractions was always low (generally <2 nmol µg C^{-1} h^{-1}; Fig 6a,b).

Since observed seasonal and spatial variation in specific phytoplankton and bacterial APA in the surface and intermediate waters could be due to changes in the respective communities' composition, data characterising communities composition were analysed and compared with changes in their respective specific APA. At both stations the micro- and nanophytoplankton contributions alternated in importance irrespective of high or low specific phytoplankton APA (Table 1). At both stations and in all months diatoms predominated in the microphytoplankton fraction, except in June at the surface at SJ101.

Table 1

Dominant species during the period of high specific phytoplankton APA (*Pseudo-nitzschia delicatissima*, *Nitzschiella* sp., *Skeletonema* sp.) were also abundant during the period of low specific phytoplankton APA (Table 2). During the period of high specific

phytoplankton APA species composition at the surface (Table 2) and in intermediate waters (data not shown) in June differed at the studied stations. However, in May at both stations *Pseudo-nitzschia delicatissima* strongly dominated in the microphytoplankton fraction. Further, in May the abundance of *Nitzschiella* sp was similar at the surface at both stations. Moreover, while in June at SJ101 *Prorocentrum triestinum* was the dominant species at the surface (Table 2) and *Pseudo-nitzschia delicatissima* in intermediate waters (data not shown), both of these species were however present in much higher abundance during the bloom in May at SJ108.

Table 2

The bacterial and picocyanobacterial contributions to the bacterial fraction biomass alternated in importance at both stations and no relation with the level of specific bacterial APA was observed (Table 3).

Table. 3

3.5. Kinetic parameters of APA

Total APA as a function of substrate concentration measured in May, June and September at the surface of SJ108 fitted the Michaelis-Menten model (Fig. 7). Activity increased up to a substrate concentration of 50 µmol l^{-1}, where V_{max} was reached, and then remained constant or slightly decreased. In May and September V_{max} (3.89 and 3.83 µmol l^{-1} h^{-1}, respectively) was similar and markedly higher than in June (0.54 µmol l^{-1} h^{-1}). The highest K_m was calculated for June (7.97 µmol l^{-1}), while in May and September (1.22 and 0.62 µmol l^{-1}, respectively) was significantly lower. Turnover time of phosphorus estimated from V_{max} and K_m was very short in May and September (0.31 and 0.16 h, respectively) and longer in June (14.79 h Fig. 7).

Fig. 7

DISCUSSION

The seasonal evolution of total APA during 2006 was similar to that found in 2004 (Ivančić et al., 2009) and 2005 (Ivančić, unpublished data). Furthermore a decrease of DOP concentrations as a result of high APA was again observed. This indicates that the same seasonal pattern of APA occurs year by year and that DOP is an important source of P in the region. In March 2006 the investigated area was not influenced by freshwater,

297 and hence the freshwater import of nutrients was minimal. At the time a modest
298 phytoplankton and bacterial biomass developed on nutrient reserves regenerated in the
299 water column during winter. Very low total APA, as well as specific APA (total APA/ total
300 microbial carbon content, 1.46-1.89 nmol µg C^{-1} h^{-1}; data not shown), showed that
301 microbes were not P limited. As a consequence of the microbial activity during early
302 spring, regenerated nutrients in the surface layer were consumed. The phytoplankton
303 blooms that developed in April and May in the low saline waters were stimulated with
304 freshwater imported nutrients. Since freshwater P supply is markedly lower when
305 compared to N supply (see Introduction), significant DIN remained in the water while PO_4
306 was exhausted and DOP became the largest reservoir of dissolved P available to
307 microbes. Consequently, a large increase of total APA (up to 40 times) occurred. As
308 temperature differences of about 10 °C were noted, APA would be expected to only
309 double (Petersson and Jansson, 1978). Therefore, temperature was considered to be a
310 less important factor, and the increased APA was the response of the microbial
311 communities to P limitation. APA was practically all due to microbial expression, and
312 activity of free enzymes was not important. A major part of the surface activity was due to
313 the phytoplankton fraction, mainly due to a markedly greater algal than bacterial biomass.
314 The dominance of algal APA during the blooms was also observed in the Bay of Biscay
315 (Labry et al., 2005). In the present study specific APA was high in both the phytoplankton
316 and bacterial fractions, indicating that both communities were P limited. At SJ108 P
317 turnover time in surface waters, estimated from AP kinetic parameters, was very short
318 (about 20 min). Such a calculated P turnover time could be used to compare the rate of P
319 recycling in different situations since it agrees with ^{33}P turnover times (Nausch et al., 2004;
320 Xu et al., 2008). Short AP mediated P turnover times (3-16 h) in P limiting conditions were
321 also found in many other areas, compared to much longer (<30-4585 h) in P repleted
322 conditions (Nausch et al., 2004; Labry et al., 2005; Xu et al., 2008).

323 In June and July the freshwater influence was weak and persistent stratification
324 hindered the replenishment of nutrients from deeper waters. Due to low external nutrient
325 input, phytoplankton biomass dropped to low values, while the decrease in bacterial and
326 picocyanobacterial biomass (smaller cells more efficiently use low nutrient concentrations)
327 was markedly less pronounced. In these conditions a marked decrease of surface total
328 APA was observed, mainly due to the decrease of phytoplankton APA, as shown by
329 fractionation in June. Surface APA was again practically all due to microbial expression,
330 although in contrast to May, in conditions of low phytoplankton biomass, bacteria

significantly contributed to total APA (30-44%). In June specific APA of both the phytoplankton and bacterial fractions was even higher than in May, suggesting strong P limitation. However, in conditions of low N supply this element could also limit microbial growth and this is probably the reason of markedly longer P turnover time in surface waters of SJ108 in June (about 15 h) compared to those during the bloom in May (about 20 min).

During autumn months total APA at the surface was low, irrespective of high or low microbial biomass, except for a high value in September in an area where a large microbial bloom was observed. Activity of free enzymes at the surface was not important. While bacteria significantly contributed to total APA during phytoplankton blooms (31-48%), their contribution was dominant in the absence of phytoplankton blooms (65-78%). The low specific phytoplankton APA found in autumn may be caused by a different percentage of phytoplankton actively expressing the enzyme due to less severe P limitation and/or by different phytoplankton species composition. However, inter-seasonal changes of size structure and composition of phytoplankton were not consistent with changes in specific phytoplankton APA. It is more probable that in autumn P less limited phytoplankton growth than during late spring and summer. In these months mixing of the water column started enriching upper waters with nutrients from deeper waters where they were regenerated in a close to balanced ratio for microbial requirements, as already observed during a previous study (Degobbis, 1990). Thus phytoplankton growth was stimulated not only by freshwater nutrients, but also by the nutrient flux from bottom waters. As a result PO_4 imported into the surface layer might be at higher concentrations and less deficient with respect to DIN than during spring and summer. Nausch (1998) found that PO_4 concentrations <0.2 µmol l^{-1} stimulated production of enzymes leading to very high specific APA, while at PO_4 concentrations between 0.2-1 µmol l^{-1} APA was a linear function of biomass and specific APA remained at the same level. Furthermore, a threshold for the regulatory function of PO_4 on APA was found at concentrations of 0.05-0.1 µmol l^{-1} (Labry et al., 2005; Sebastian et al., 2004). In the present study it was difficult to compare PO_4 concentrations and enzyme production since imported PO_4 uptake was very fast and its concentrations in water were usually below the detection limit. However, specific phytoplankton APA indicated highly stimulated enzyme production during late spring and summer, while during autumn APA was simply a function of biomass, and specific APA remained at the same low level. In contrast, specific APA of the bacterial fraction did not show a significant decrease during autumn. While it may be noted that

bacterial AP has a more complex function than that of phytoplankton, i.e. AP could also serve to provide C (see Hoppe, 2003), it is unlikely that bacteria were C limited. It is important to note that changes in specific bacterial APA were not related to the contribution of picocyanobacterial biomass to the total biomass of this fraction. It seems more probable that phytoplankton exhausted imported PO_4 and picocyanobacteria and bacteria were P limited. At the surface the highest specific APA in this fraction was observed at SJ108 during a large phytoplankton bloom in September. The high quantity of organic matter produced supported a large increase of bacteria. Most likely bacteria degrading the produced organic matter were P limited. During this bloom P turnover was fast (about 10 min) not only due to high bacterial activity, but also due to a high level of phytoplankton enzymes since phytoplankton biomass was very high.

In intermediate waters (10 m depth) total APA was markedly lower than in surface waters, in contrast to 2004 when activity at this depth did not differ noticeably from the surface (Ivančić et al., 2009). In 2006 Po River flow during spring and summer was considerably lower than in 2004 (unpublished data provided by Ministero dei Lavori Pubblici, Servizio Idrografico, Parma, Italy) and did not notably influence intermediate waters. As a consequence microbial biomass in these waters, especially phytoplankton, was low resulting in low total APA. In these waters the importance of free enzymes increased (9-30%), although the major part of APA was still due to microbes. In conditions where bacterial biomass was similar or somewhat higher than phytoplankton biomass, there was an increase of the bacterial contribution to enzyme production (up to 91%). Specific APA of both fractions followed the same pattern as in surface waters suggesting a similar seasonal evolution of P limitation in both communities.

A disproportion between specific phytoplankton APA and specific bacterial APA observed in surface and intermediate waters at the two stations was not related to differences in phytoplankton size and species composition or to the contribution of picocyanobacteria to the bacterial fraction, and was probably due to different nutrient content in those waters. Station SJ108 was generally better supplied with freshwater nutrients than SJ101. Phytoplankton superiority in PO_4 uptake was observed at high and bacterial superiority at low PO_4 concentrations (Thingstad et al., 1993; Xu et al., 2008). Therefore, at SJ108 phytoplankton probably took up most of the imported PO_4 and bacteria, degrading the phytoplankton produced matter, were even more P limited than phytoplankton. On the contrary, at SJ101 bacteria used low concentrations of PO_4 more efficiently and were less P limited than phytoplankton.

At the bottom total APA was low (generally <0.1 μmol l^{-1} h^{-1}). In these waters low enzymatic activity was due mainly to free enzymes (up to 100%), while particulate activity was low. As a consequence phytoplankton and bacterial enzymatic activity was usually lower than expected from their biomass and thus specific APA was always low (generally <1 μg C^{-1} h^{-1}) confirming that, in these waters where regeneration predominates over assimilation, microbes were not significantly P limited.

CONCLUSIONS

Both phytoplankton and bacteria use APA to obtain P, though with different seasonal patterns. During late spring phytoplankton blooms the major part of APA was due to the phytoplankton fraction, mainly due to markedly greater algal biomass than bacterial biomass. In summer, when phytoplankton and bacterial biomass were similar, both communities significantly contributed to total APA. Strong P limitation of both communities was observed during the stratification period when the phosphorus supply in upper waters depended on phosphorus recycling in these waters or freshwater input. A more balanced P supply during the mixing period drastically reduced phytoplankton, though not bacterial AP production in upper waters. In the mixing period the strongest P limitation of bacteria was observed during the massive phytoplankton bloom in September when they were degrading a high quantity of phytoplankton produced organic matter. In upper waters activity of free enzymes was not important, while in bottom waters enzymatic activity was due mainly to free enzymes.

ACKNOWLEDGEMENTS

The authors thank Dr. T. Đakovac, S. Dujmović, M. Buterer and the crew of RV “Vila Velebita” for help during sampling and analyses. Two anonymous reviewers are thanked for their constructive advice. This work is part of the scientific projects „Structure and physiology of microbial communities in northern Adriatic fronts“(098-0982705-2729), “Mechanism of long-term changes in the northern Adriatic ecosystem” (098-0982705-2731) and Project “Jadran” funded by the Ministry of Science, Education and Sport of the Republic of Croatia.

REFERENCES

Antia, N.J., McAllister, C.D., Parsons, T.R., Stephens, K., Strickland, J.D.H., 1963. Further measurements of primary production using a large-volume plastic sphere. Limnology and Oceanography 8,166-183.

Degobbis, D., 1990. A stoichiometric model of nutrient cycling in the northern Adriatic Sea and its relation to regeneration processes. Marine Chemistry 29, 235-293.

Giani, M. (Ed.), 2003. Rapporto Finale (Adriatico) Processi di Formazione delle Mucillagini nell'Adriatico e in Tirreno (MAT), Istituto Centrale per la Ricerca Scientifica e Tecnologica Applicata al Mare, Roma, 749 pp.

Gilmartin, M., Revelante, N., 1983. The phytoplankton of the Adriatic Sea: standing crop and primary production. Thalassia Jugoslavica 19,173-178.

Gilmartin, M., Degobbis, D., Revelante, N., Smodlaka, N., 1990. The mechanism controlling plant nutrient concentrations in the northern Adriatic Sea. Internationale Revue der gesamten Hydrobiologie und Hydrographie 75, 425-445.

Goldman, J. C., Caron, D. A., Dennet, M.R., 1987. Regulation of gross growth efficiency and ammonium regeneration in bacteria by substrate C:N ratio. Limnology and Oceanogaphy 32, 1239-1252.

Hoppe, H.G., 1983. Significance of exoenzymatic activities in the ecology of brackish water: measurements by means of methyllumbelliferyl-substrates. Marine Ecology Progress Series 11**,** 299-308.

Hoppe, H.G., 2003. Phosphatase activity in the sea. Hydrobiologia 493, 187-200.

Ivančić, I., Degobbis, D., 1984. An optimal manual procedure for ammonia analysis in natural waters by the indophenol blue method. Water Research 18, 1143-1147.

Ivančić, I., Degobbis, D., 1987. Mechanisms of production and fate of organic phosphorus in the northern Adriatic Sea. Maine Biology 94,117-125.

Ivančić, I., Radić, T., Lyons, D.M., Fuks, D., Precali, R., Kraus, R., 2009. Alkaline phosphatase activity in relation to nutrient status in the northern Adriatic Sea. Marine Ecology Progress Series 378, 27-35.

Kana, T.M., Gilbert, P.M., 1987. Effect of irradiance up to 2000 μE $m^{-1}s^{-1}$ on marine *Synechococcus* WH7803: I. Growth, pigmentation and cell composition. Deep-Sea Research 34, 479-495.

Karner, M., Fuks, D., Herndl. G., 1992. Bacterial activity along a trophic gradient. Microbial Ecology 24, 243-257.

Labry, C., Delmas, D., Herbland, A., 2005. Phytoplankton and bacterial phosphatase activities in relation to phosphate and DOP availability within the Gironde plume waters (Bay of Biscay). Journal of Experimental Marine Biology and Ecology 318, 213-225.

463 Lee, S., Fuhrman, J.A., 1987. Relationships between biovolume and biomass of naturally
464 derived marine bacterioplancton. Applied and Environmental Microbiology 53,
465 1298-1303.
466 Martinez, J., Azam, F., 1993. Periplasmic aminopeptidase and alkaline phosphatase
467 activities in marine bacterium:implications for substrate processing in the sea.
468 Marine Ecology Progress Series 92, 89-97.
469 Menzel, D.W., Corwin, N., 1965. The measurement of total phosphorus in seawater based
470 on the liberation of organically bound fractions by persulfate oxidation. Limnology
471 and Oceanography 10, 280-182.
472 Milan, C., Trentini, P., Ascanelli, M., Bignami, S., Barbieri, C., Libono, L., Malfato, M.G.,
473 2003. Valutazione dei carichi dei nutrienti del Po a Pontelagoscuro, in: Giani, M.
474 (Ed.), Rapporto Finale (Adriatico) Processi di Formazione delle Mucillagini
475 nell'Adriatico e in Tirreno (MAT). Istituto Centrale per la Ricerca Scientifica e
476 Tecnologica Applicata al Mare, Roma, pp. 727-749.
477 Nausch, M., 1998. Alkaline phosphatise activities and the relationship to inorganic
478 phosphate in the Pomeranian Bight (Southern Baltic Sea). Aquatic Microbial
479 Ecology 16, 87-94.
480 Nausch, M., Nausch, G., Wasmund, N., 2004. Phosphorus dynamics during the transition
481 from nitrogen to phosphate limitation in the central Baltic Sea. Marine Ecology
482 Progress Series 266, 15-25.
483 Petersson, K., Jansson, M., 1978. Determination of phosphatase activity in lake water-a
484 study of methods. Verhandlungen der Internationalen Vereinigung fűr
485 theoretische und angewandte Limnologie 20, 1226-1230.
486 Porter, K. G., Feig, Y. S., 1980. The use of DAPI for identifying and counting aquatic
487 microflora. Limnology and Oceanography 25, 943-948.
488 Redfield, A.C., Ketchum, B.H., Richards, F.A., 1963. The influence of organisms on the
489 composition of seawater, in: The Sea, vol 2. Interscience, New York, pp. 27-77.
490 Sala, M.M., Karner, M., Arin, L., and Marrasé, C. 2001. Measurement of ectoenzyme
491 activities as an indication of inorganic nutrient imbalance in microbial communities.
492 Aquatic Microbial Ecology, 23, 301-311.
493 Sebastian, M., Aristegui, J., Montero, M.F., Escanez, J., Niel, F.X., 2004. Alkaline
494 phosphatase activity and its relationship to inorganic phosphorus in the transition zone
495 of the North-western African upwelling system. Progress in Oceanography 62, 131-
496 150.

Strickland, J.D.H., Parsons, T.R., 1972. A practical handbook of seawater analysis. Bulletin of the Fisheries Research Board of Canada 167, 310 pp.

Takahashi, M.K., Kikuchi, T.R., Hara, Y., 1985. Importance of picocyanobacteria (unicellular blue-green algae) in the phytoplankton population of the coastal waters off Japan. Marine Biology 89, 63-69.

Thingstad, T.F., Skjodal, E.F., Bohne, R.A., 1993. Phosphorus cyclyng and algal-bacterial competition in Sandsfjord, western Norway. Marine Ecology Progress Series 99, 239-259.

Utermöhl, H., 1958. Zur Vervollkommnung der quantitativen Phytoplankton-Methodik. Internationale Vereinigung fur theoretische und angewandte limnologie. Mitteilung No.9, 17, 47-71.

Zaccone, R., Caruso, G., Cali, C., 2002. Heterotrophic bacteria in the northern Adriatic Sea: seasonal changes and ectoenzyme profile. Marine Environmental Research 54, 1-19.

Xu, J., Yin, K., He, L., Yuan, X., Ho, A.Y.T., Harrison, P.J., 2008. Phosphorus limitation in the northern South China Sea during late summer: Influence of the Pearl River. *Deep Sea Research* I 55, 1330-1342.

513 CAPTIONS

514 Fig. 1. Research area and sampling stations in the northern Adriatic Sea.

515 Fig. 2. (a) Temperature and (b) salinity profiles in March (○●), April (△▲), May (◇◆), June
516 (▽▼), July (▷▶), September (◁◀) and October (□■) at SJ101 (unfilled shapes and thin
517 line) and SJ108 (filled shapes and thick line) in 2006.

518 Fig. 3. (a) PO_4, (b) DOP and (c) DIN profiles in March (○●), April (△▲), May (◇◆), June
519 (▽▼), July (▷▶), September (◁◀) and October (□■) at SJ101 (unfilled shapes and thin
520 line) and SJ108 (filled shapes and thick line) in 2006.

521 Fig. 4. Seasonal evolution of phytoplankton (○, thin line) and bacterial and
522 picocyanobacterial (△, dashed line) biomass and total APA (◇, thick line) at (a) the surface,
523 (b) 10 m depth and (c) the bottom at sampling stations in 2006.

524 Fig. 5. Contribution of phytoplankton (grey), bacteria and picocyanobacteria (white) and free
525 enzymes (black) to total APA at (a) the surface, (b) 10 m depth and (c) the bottom at
526 sampling stations in 2006.

527 Fig. 6. (a) Specific phytoplankton and (b) bacterial APA at the surface (○, solid line), 10 m
528 depth (◇, dashed line) and the bottom (△, dash dot line) at sampling stations in 2006.

529 Fig. 7. Michaelis-Menten kinetics of total APA in May (●, thick line), June (△, dashed line)
530 and September (◇, thin line) at the surface at station SJ108. Values of V_{max} (µmol l^{-1} h^{-1}), K_m
531 (µmol l^{-1}) and T_n (h) are also reported.


13°
14° E
46° N
N
W
E
S
Trieste
Venice
Istria
Kvarner Bay
Rovinj
SJ101
45°
Po River
Pula
SJ108
CROATIA
ITALY
ADRIATIC SEA
44°
0 10 20 30 40 50
100 km

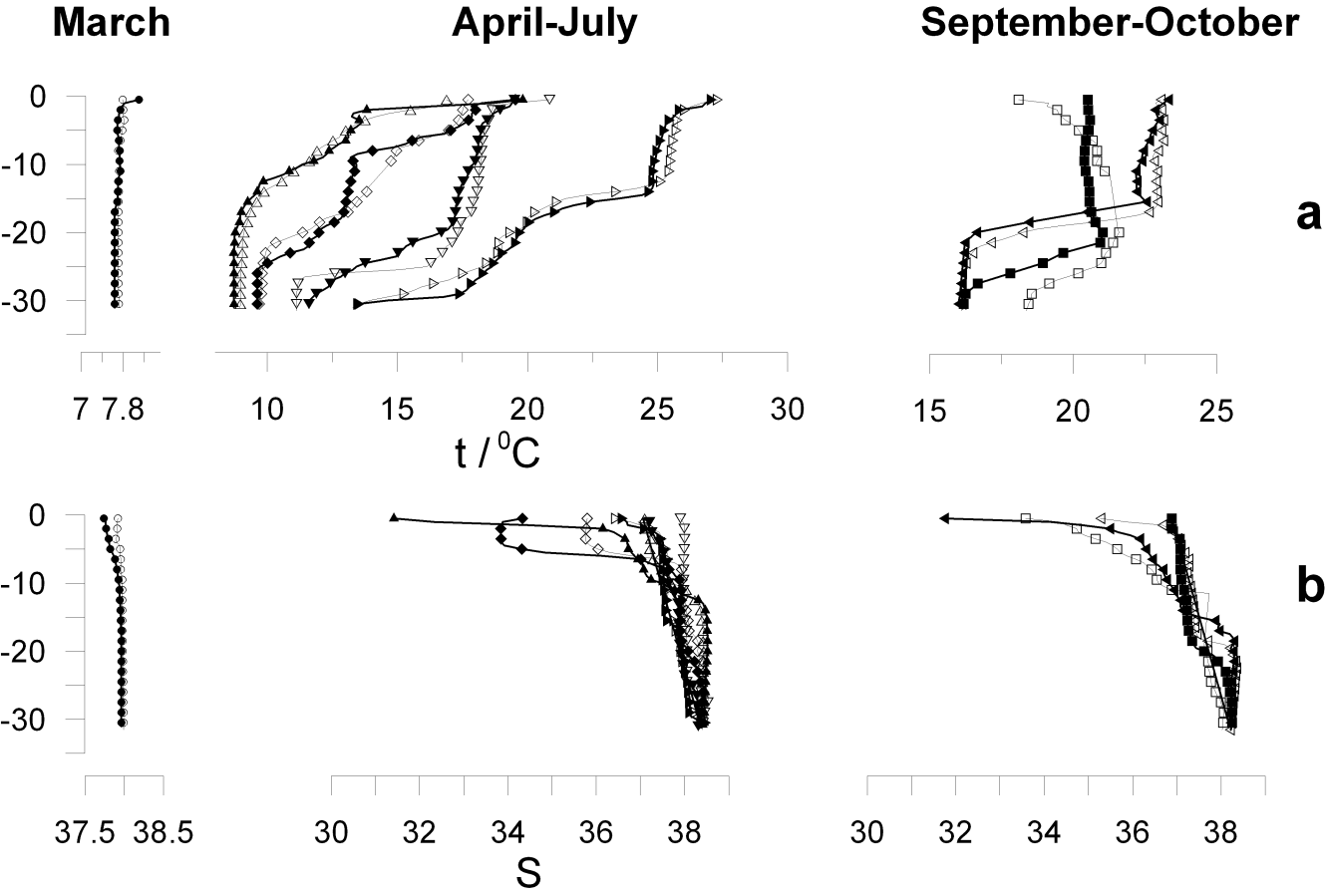
March
April-July
September-October
0
-10
-20
-30
7 7.8
10
15
20
25
30
15
20
25
t / ^{0}C
a
0
-10
-20
-30
37.5 38.5
30
32
34
36
38
30
32
34
36
38
S
b

Fig. 2

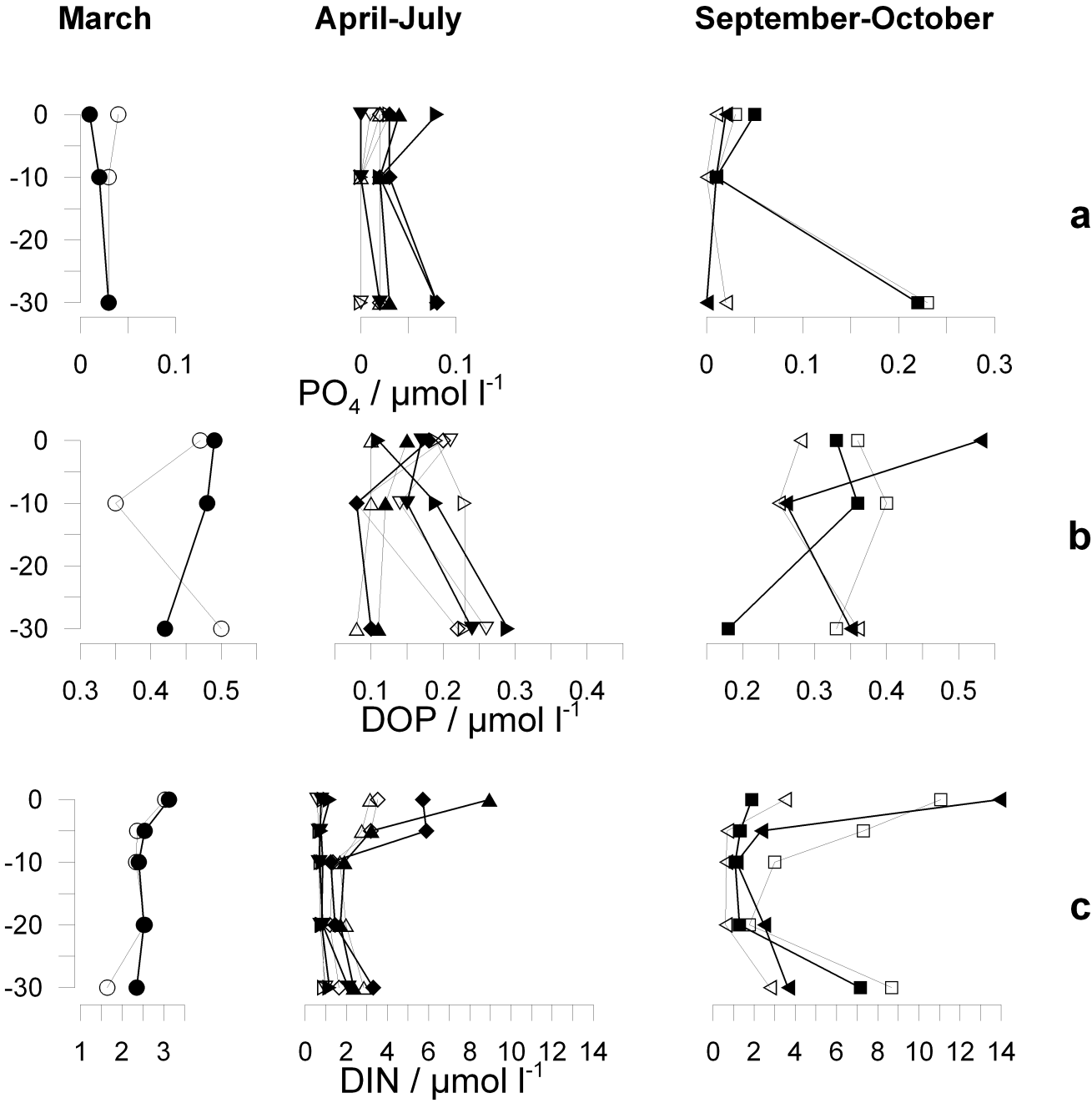
March
April-July
September-October
0
-10
-20
-30
0
0.1
0
0.1
0
0.1
0.2
0.3
PO4 / µmol l-1
a
0
-10
-20
-30
0.3
0.4
0.5
0.1
0.2
0.3
0.4
0.2
0.3
0.4
0.5
DOP / µmol l-1
b
0
-10
-20
-30
1
2
3
0
2
4
6
8
10
12
14
0
2
4
6
8
10
12
14
DIN / µmol l-1
c

Fig.3

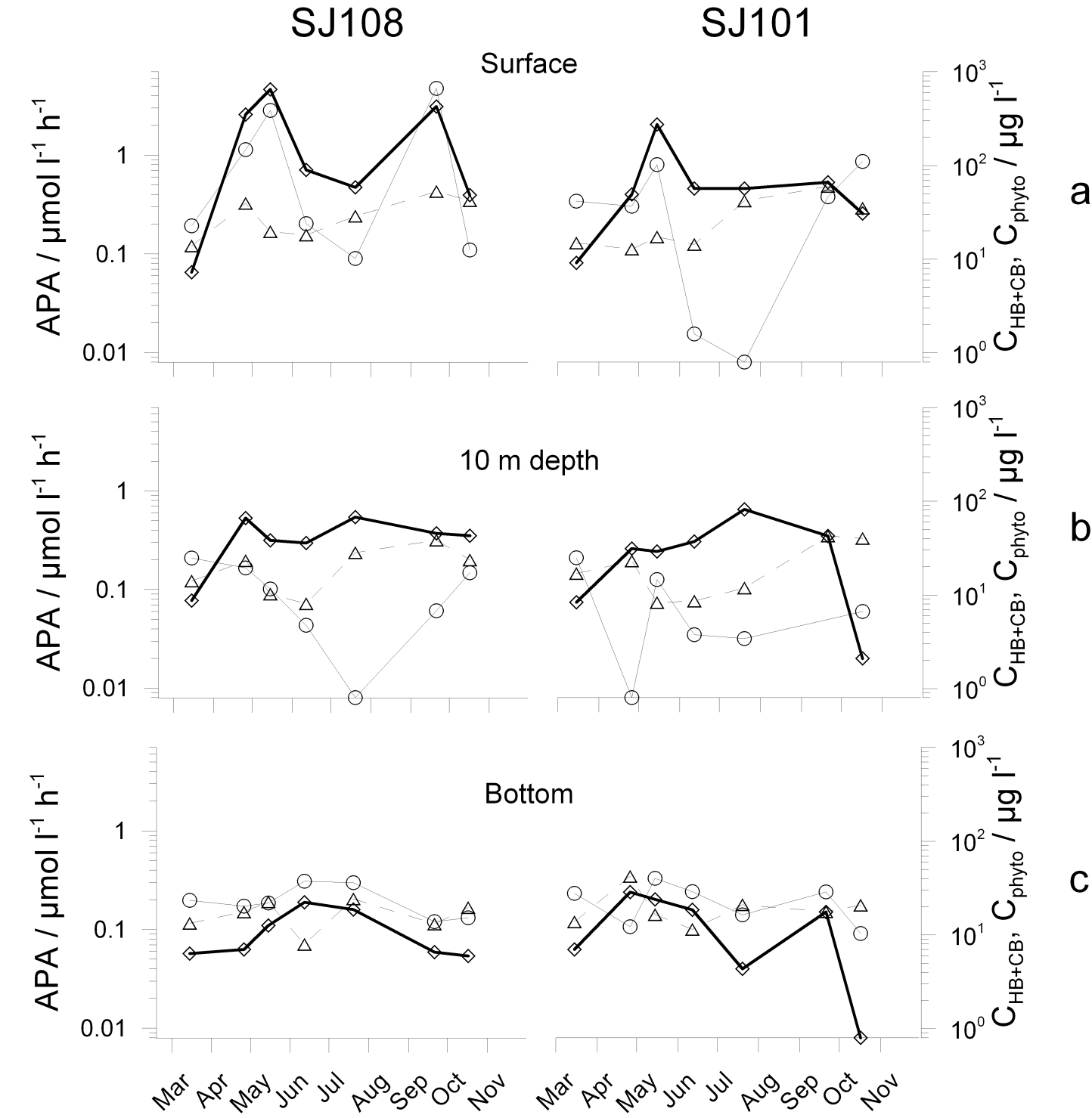
SJ108
SJ101
Surface
10 m depth
Bottom
APA / µmol l-1 h-1
CHB+CB, Cphyto / µg l-1
0.01
0.1
1
10^0
10^1
10^2
10^3
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
a
b
c

Fig.4

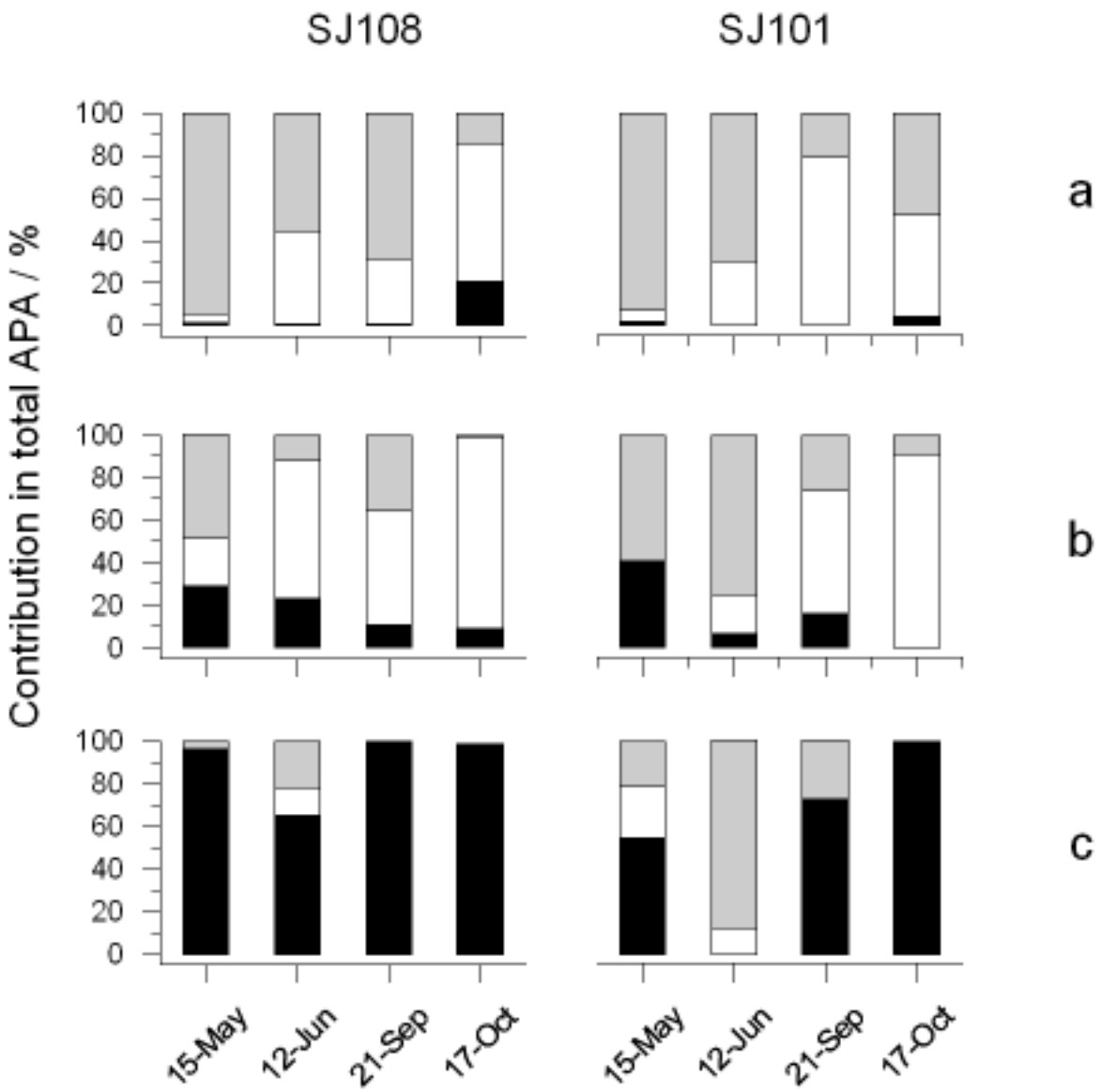
SJ108
SJ101
Contribution in total APA / %
100
80
60
40
20
0
a
b
c
15-May
12-Jun
21-Sep
17-Oct

Fig.5

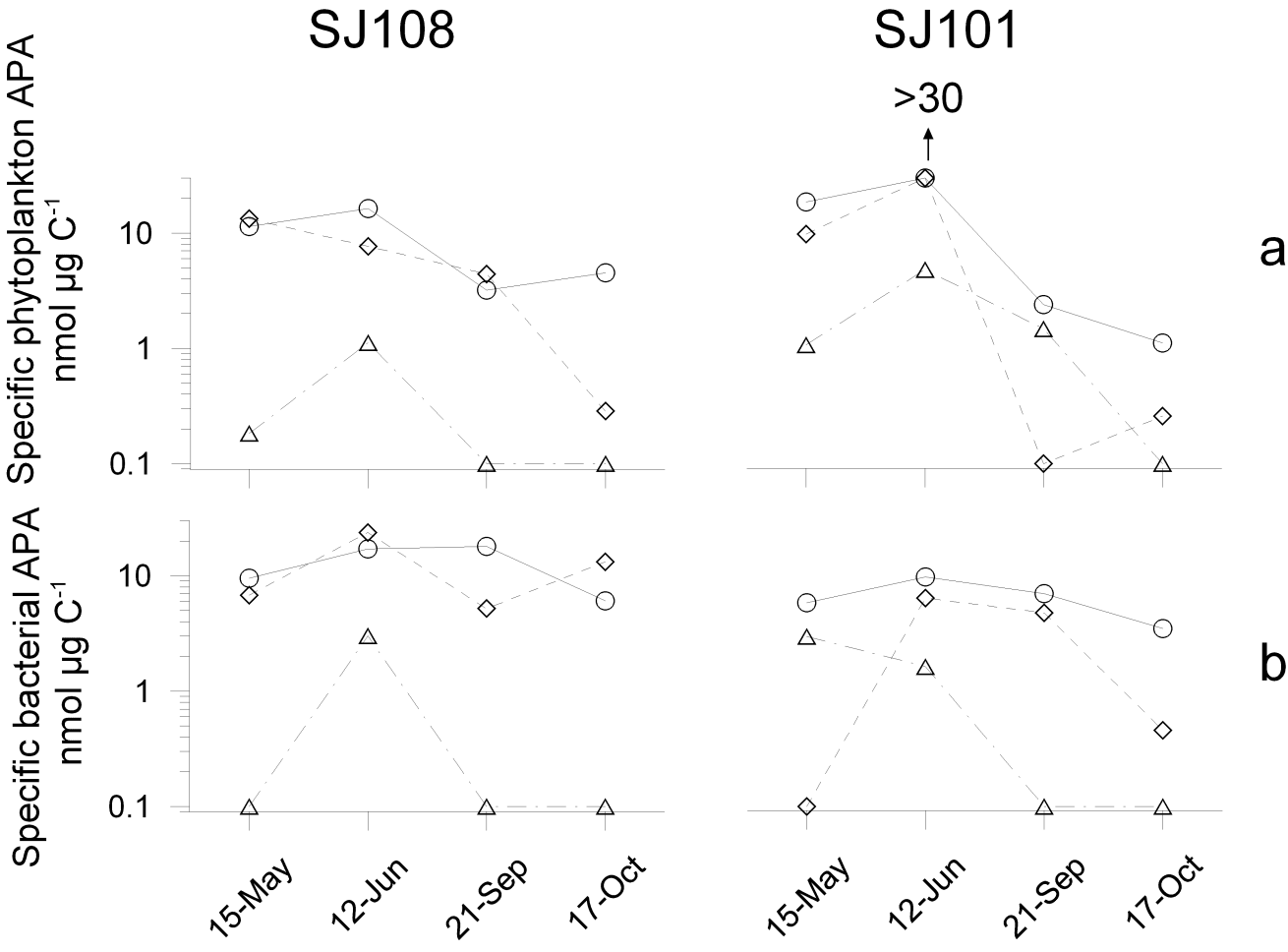
SJ108
SJ101
Specific phytoplankton APA
nmol µg C-1
Specific bacterial APA
nmol µg C-1
>30
10
1
0.1
a
b
15-May
12-Jun
21-Sep
17-Oct

Fig. 6

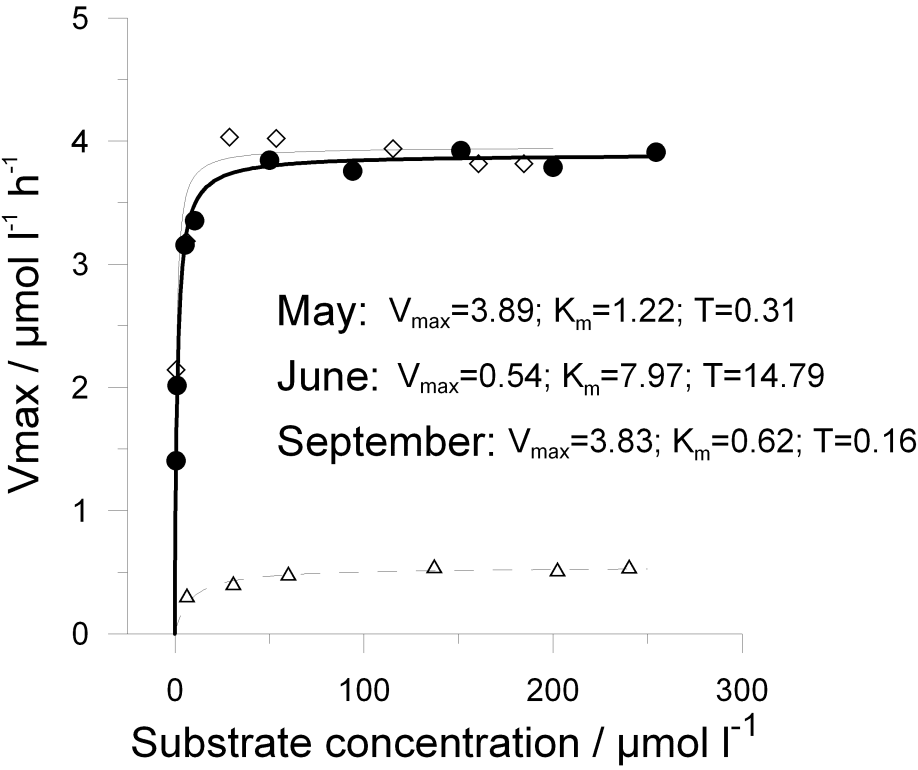
Vmax / µmol l-1 h-1
Substrate concentration / µmol l-1
May: Vmax=3.89; Km=1.22; T=0.31
June: Vmax=0.54; Km=7.97; T=14.79
September: Vmax=3.83; Km=0.62; T=0.16
0
1
2
3
4
5
0
100
200
300

Fig.7

Table 1. Mean values (0), standard error (SE) and number of data (N) for specific phytoplankton APA ($sAPA_{phyto}$) and specific bacterial APA ($sAPA_{bact}$). (a) Probability level (p) for differences between stratification and beginning of mixing periods at the surface and 10 m depth and (b) probability level for differences between stations at the surface and 10 m depths.

A	Stratification		Beg. of mix.		
	0 (SE)	N	0 (SE)	N	p
$sAPA_{phyto}$	17.10 (3.07)	82.03 (0.66)	8		0.000
$sAPA_{bact}$	9.91 (2.60)	87.27 (2.00)	8		1.000

B	SJ108		SJ101		
	0 (SE)	N	0 (SE)	N	p
$sAPA^*_{phyto}$	12.13 (1.80)	422.10 (4.91)		4	0.165
$sAPA_{bact}$	12.46 (2.37)	84.71 (1.16)	8		0.006

*data for the stratification period only

Table 2. Size composition of phytoplankton expressed as the % of micro fraction (Micro) in total chlorophyll *a* (chl *a*) and % of Diatoms in micro fraction during the period of high and low specific phytoplankton APA at the surface and 10 m depth.

		High specific APA			Low specific APA	
Station	Depth m	Date	Micro %	Diatoms %	Date	Micro %	Diatoms %
SJ108	0	15.05.06	64	60	21.09.06	48	98
	10		41	95		13	98
SJ101	0		85	98		15	95
	10		62	96		18	100
SJ108	0	12.06.06	53	99	17.10.06	24	98
	10		28	96		19	100
SJ101	0		33	25		54	100
	10		30	79		27	97

Table 3. List of the most abundant species of microphytoplankton at the surface during periods of high and low specific phytoplankton APA.

		High specific APA			Low specific APA	
Station	Date	Species	Cell l^{-1}	Date	Species	Cell l^{-1}
SJ108	15.05.06	*Pseudo-nitzschia delicatissima*	205860	21.09.06	*Chaetoceros* sp.	306900
		Prorocentrum balticum	102930		*Thalassiosira* sp.	244200
		Diplopsalis lenticula	18980		*Nitzschiella* sp.	244200
		Nitzschiella sp.	14600		*Asterionellopsis glacialis*	86900
		Prorocentrum triestinum	9460		*Pseudo-nitzschia delicatissima*	55000
		Chaetoceros sp.	8760		*Skeletonema* sp.	49500
		Prorocentrum micans	8760		*Diatoma* sp.	13200
		Glenodinium sp	5110		*Chaetoceros affinis*	7700
		Ceratium furca	5110		*Gyrodinium* sp.	6600
					Gonyaulax sp.	6600
					Cylindrotheca closterium	6600
SJ101		*Pseudo-nitzschia delicatissima*	273750		*Nitzschiella* sp.	52800
		Nitzschiella sp.	13140		*Asterionellopsis glacialis*	34600
		Bacteriastrum delicatulum	6570		*Leptocylindrus danicus*	15900
		Chaetoceros sp.	5110		*Pseudo-nitzschia delicatissima*	15400
					Chaetoceros sp.	9350
SJ108	12.06.06	*Skeletonema* sp.	8140	17.10.06	*Pseudo-nitzschia delicatissima*	51150
		Cerataulina pelagica	3700		*Chaetoceros* sp.	50600
		Prorocentrum balticum	2960		*Chaetoceros affinis*	13200
		Cyclotella sp.	2960		*Nitzschiella* sp.	9900
		Prorocentrum micans	2220		*Leptocylindrus danicus*	7700
					Cerataulina pelagica	7700
					Guinardia striata	6050
SJ101		*Prorocentrum triestinum*	2220		*Chaetoceros* sp.	649000
		Hemiaulus hauckii	1110		*Nitzschiella* sp.	101200
		Glenodinium sp.	1110		*Chaetoceros affinis*	53900
					Asterionellopsis glacialis	16500
					Chaetoceros costatus	6600

Table 4. Specific bacterial APA (bAPA) and % of picocyanobacteria (CB) in the bacterial fraction at the surface and 10 m depth.

Date	Station	Depth	bAPA	CB
		m	nmol µg C^{-1} h^{-1}	%
15.05.06	SJ108	0	9.55	26
		10	6.77	53
	SJ101	0	5.83	12
		10	0.10	58
12.06.06	SJ108	0	17.11	51
		10	23.76	30
	SJ101	0	9.75	35
		10	6.40	28
21.09.06	SJ108	0	17.94	27
		10	5.19	61
	SJ101	0	6.99	58
		10	4.77	55
17.10.06	SJ108	0	6.07	56
		10	13.27	40
	SJ101	0	3.78	25
		10	0.46	68