Alkaline phosphatase activity related to phosphorus stress of microphytoplankton in different trophic conditions.

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Running title: Microphytoplankton adaptation to P-stress

Abstract

The northern Adriatic (NA) is a favorable basin for studying the adaptive strategies of plankton to a variety of conditions along the steep gradients of environmental parameters over the year. Earlier studies identified phosphorus (P)-limitation as one of the key stresses within the NA that shape the biological response in terms of biodiversity and metabolic adjustments. A wide range of reports supports the notion that P-limitation is a globally important phenomenon in aquatic ecosystems. In this study P stress of marine microphytoplankton was determined at species level along a trophic gradient in the NA. In P-limitation all species with considerable contributions to the diatom community expressed alkaline phosphatase activity (APA), compared to only a few marginal dinoflagellate species. Nevertheless, APA expressing species did not always dominate the phytoplankton community, suggesting that APA is also an important strategy for species to survive and maintain active metabolism outside of their mass abundances. A symbiotic relationship could be supposed for diatoms that did not express APA themselves and probably benefited from APA expressed by attached bacteria. APA was not expressed by any microphytoplankton species during the autumn when P was not limiting, while most of the species did express APA during the P-limitation. This suggests that APA expression is regulated by orthophosphate availability. The methods employed in this study allowed the microscopic detection of APA for each microphytoplankton cell with simultaneous morphologic/taxonomic analysis. This approach uncovered a set of strategies to compete in P-limited conditions within the marine microphytoplankton community. This study confirms the role of P-limitation as a shaping factor in marine ecosystems.

1. Introduction

Growing evidence suggests that phosphorus (P) is frequently the limiting nutrient in coastal systems (Thingstad et al., 1993; 1998) and oligotrophic oceans (Cotner et al., 1997; Karl and Yanagi, 1997). Furthermore, it is believed that the open ocean, far from the continental inputs of nutrients, should evolve towards P-limited conditions due to N_2 fixation, while P is a non-renewable limiting nutrient (Tyrrell, 1999). The rise in N_2 fixation and the simultaneous disappearance of P stocks observed in the subtropical North Pacific Ocean between 1989 and 2004 is an illustration of this phenomenon (Karl et al., 2001, Karl, 2007). Therefore, the importance of alkaline phosphatase activity (APA) with regard to the transformation and turnover of organic compounds in marine environments has been investigated with growing attention (for review see Hoppe, 2003; Yamaguchi and Adachi, 2010).

A number of studies in the northern Adriatic (NA) evidenced that this region is currently P-limited (citations reported in the following text). This shallow (up to 50 m) coastal sea is characterized by significant freshwater input, mainly from the Po River. Although in the Po River waters both nitrogen (N) and P concentrations (total nitrogen 263 μmol L^{-1} and total phosphorus 4.8 μmol L^{-1}; Cozzi and Giani, 2011) are more than one order of magnitude higher than in the NA waters, the inorganic N:P molar ratio (about 84:1; Cozzi and Giani, 2011) provides a strongly unbalanced N versus P supply for phytoplankton requirements (presumed balanced N:P=16:1; Redfield *et al.*, 1963). Earlier studies in this area showed that organic phosphorus concentrations markedly exceeded orthophosphate (PO_4) concentrations, representing an important source of P for microbial communities (Ivančić and Degobbis, 1987). A significant contribution to the pool of dissolved P in the NA could be due to the release of dissolved DNA by nanoflagellates grazing on nucleic acid-rich bacterial biomass (Turk et al., 1992). These authors reported that during stratified conditions a significant part

of the P demand by the autotrophs may be sustained by the rapid turnover of DNA-mediated release of inorganic phosphorus. The importance of dissolved organic phosphorus (DOP) in the area during periods of P-limitation was evidenced also by a recent mesocosm experiment (Malfatti et al., 2014). However, the authors pointed out that not all DOP is directly hydrolysable by alkaline phosphatase (AP) and multiple hydrolase activities were available with potential to transform various organic matter pools into molecules accessible to AP and other hydrolases that are the terminal step in P regeneration.

Bioassays based on selective nutrient additions and nutrient molar ratios identified P-limitation of the NA (Maestrini et al., 1997 and citation therein). Enzyme assays with soluble substrate showed that extracellular AP is important in providing P for phytoplankton growth, particularly during blooms induced by freshwater imported nutrients (Ivančić et al., 2009; 2010). However, although measurements with soluble substrate allowed detection of the phytoplankton P status, they provided only the APA of the whole community and did not yield information on the individual species. Furthermore, it was not possible to mark the exact location of the enzymatic activity in the cells or to determine the contribution of each species to the overall enzyme signal.

In the present study an AP substrate with insoluble fluorogenic product, termed ELF (Enzyme Labelled Fluorescence), was used to investigate how the APA status varies among phytoplankton species at two locations along the trophic gradient. This method yields a stable, highly fluorescent precipitate at the site of enzyme activity and thus has the capability to determine the APA status of individual cells (González-Gill et al., 1998). Hence, it can give insights into the mechanisms of P nutrition of phytoplankton in natural waters. In the previous study it was found that low PO_4 concentrations can modify the structure of plankton communities and constrain the phytoplankton distribution (Ivančić et al., 2012) as also reported for other areas (Egge, 1998; Moutin et al., 2005; Mackey et al., 2007). In addition P-

limitation can affect phytoplankton abundance and composition, viral and prokaryotic abundance, microbial activity and organic matter production and utilization (Larato et al., 2010). However, not all microorganisms are equally affected. By comparing estimated taxon-specific C:P utilization rates with particulate C:P ratios in P-replete and P-deplete cultures it was shown that different phytoplankton groups experienced different degrees of P stress in the same nutrient environment (Casey et al., 2009).

In this study, investigations are concentrated on microphytoplankton as it is often the dominating component of the phytoplankton community biomass in the NA (Center for Marine Research, unpubl. data) and it is taxonomically well defined. Microphytoplankton also seems to play the major role in benthic pelagic coupling in the area (Pfannkuchen et al., 2009; 2010). This study aims to answer the questions: do all microphytoplankton species unequivocally contribute to the bulk APA? Or perhaps, are there alternative strategies to cope with the gradients of P-availability in the NA? Another covered topic is the behavior in the planktonic community of (i) species which do follow the observed bulk APA and (ii) species which follow alternative strategies to cope with P-limitation.

2. *Methods*

2.1. Sampling strategy

Measurements were carried out at two stations (SJ101, RV001) in the northern Adriatic (Fig. 1) during eight cruises performed from March to November 2013. These stations were specifically chosen as they are in areas with different nutrient regimes. SJ101 (bottom depth 32 m), situated in the western area, is usually under riverine nutrient pressure while RV001 (bottom depth 27 m), situated in the eastern coastal area, is only intermittently experiencing freshwater influence. Further, SJ101 is usually under more direct freshwater nutrient influence, while freshwater reaching RV001 is already impoverished from nutrients.

97 Fig. 1

98 During all cruises sea temperature and salinity were determined with an SBE25
99 conductivity-temperature-depth probe (Sea-Bird Electronics, Washington, USA). Samples for
100 analysis of nutrients, bulk APA, and phytoplankton were collected with 5 L Niskin bottles at
101 three depths (surface i.e. 0-1 m, 10 m, and 2 m above the bottom). Samples for the
102 determination of single cell APA (ELF assay) were collected by vertical tow of the
103 phytoplankton net (pore size 20 μm) from 10 to 0 m depth (i.e. upper waters). Data for the
104 daily Po flow mean measured at Pontelagoscuro, Italy, were kindly supplied by Assessorato
105 Programmazione, Pianificazione e Ambiente of the Emilia Romagna region (Italy).

106 *2.2. Analytical protocol*

107 Inorganic nutrient analyses were performed onboard, immediately after sample
108 collection, using methods described earlier (Strickland and Parsons, 1972; Ivančić and
109 Degobbis, 1984). Samples for total dissolved phosphorus were filtered (Whatman GF/F,
110 precombusted at 500 ºC) and stored in autoclavable polypropylene tubes at -30 ºC. In the
111 laboratory ashore analyses were performed using a wet combustion oxidation method with
112 persulphate (Menzel and Corwin, 1965). DOP was calculated by subtracting PO_4 from the
113 total dissolved phosphorus. Dissolved inorganic nitrogen (DIN) was calculated as the sum of
114 nitrate, nitrite and ammonia.

115 Determination of APA was performed aboard the research vessel immediately after
116 sample collection. Sea water (5 mL per one sample) was filled into plastic syringes directly
117 from samplers and filtered through a 200 μm mesh (to remove mesozooplankton), 3 μm
118 (polycarbonate Nuclepore filter, Whatman) and 0.22 μm filters (mixed cellulose esters
119 Millipore membrane filter, Millex-GS). All filtrations were performed manually and gently
120 applying light pressure using manual Millipore filter units. Determined activity in the 0.22 μm
121 filtrate was very low and in upper waters often below the detection limit, thus indicating that

gentle filtration did not cause cell rupture. Filters of 3 μm were preferred (passing through both bacteria and picocyanobacteria) instead of 2 μm at which an unknown part of picocyanobacteria is retained. The nanophytoplankton fraction was retained on the 3 μm filter as confirmed by microscopic inspection. The phytoplankton APA was calculated by subtracting activity in the 3 μm from the activity in the 200 μm filtrate.

All APA measurements were performed using the fluorogenic substrate analogue methyllumbelliferyl-phosphate (MUF-P) dissolved in methylcellosolve and diluted with water immediately before addition, following the procedure of Hoppe (1983). Aliquots of 5 mL of all the filtrates, in duplicate, were used for APA measurements. The final concentration of the substrate in the samples was 50 μmol L^{-1} (Ivančić et al., 2009). Incubation of the samples was performed in dark in baths filled with water from the sampling depths, i.e. at *in situ* temperature. Fluorescence was measured immediately after substrate addition and after ~1h of incubation using a Turner Designs-700 fluorometer with excitation at 365 nm and emission at 460 nm. APA (nmol L^{-1} h^{-1}) was calculated as the difference between those measurements divided by the incubation time after calibration of the fluorometer with methyllumbelliferone, the product of MUF-P degradation. Additionally, in the 200 μm filtrate, single measurements were performed using various MUF-P concentrations: from 0.5 μmol L^{-1} to 50 μmol L^{-1}. Those measurements were used to determine the half saturation constant (K_m) and maximum activity (V_{max}) of the Michaelis-Menten kinetics, employing the Wolf-Hanes linearization. P turnover time (T) was estimated by the K_m:V_{max} ratio (Labry et al., 2005).

Single cell APA was detected utilizing the ELF®97 Endogenous Phosphatase Detection Kit (E6601) (Thermo Fisher Scientific, Waltham, USA). Cells were fixed in 3.5% buffered (pH 8) formaldehyde at 4 ºC for 30 min and not permeabilized with detergents as suggested by the ELF-kit manufacturer. Afterwards, cells were rinsed in sterile seawater and subjected to microscopic analysis. ELF substrate was diluted 20 fold in detection buffer and

147 mixed with fixed cells to reach a final dilution of 40 fold, directly prior to microscopic
148 examination. Chloroplast autofluorescence was recorded at 580-600 nm (555 nm excitation)
149 and ELF signal at 550-555 nm (405 nm excitation) on a Zeiss (Oberkochen, Germany) LSM
150 (laser scanning microscope) 700 setup. Nominal thickness of optical sections under confocal
151 conditions was 1.2 µm. Thresholding signal intensity and three dimensional signal
152 reconstructions allowed to identify cell surface associated signal after the analysis of axial
153 cross sections and correction for signal point spread functions (ZEN black, Zeiss). When an
154 ELF positive cell was found, the species was determined and 99 additional intact cells of the
155 same species were examined for ELF signal. Cells with leaking cytoplasm or without
156 chloroplasts were disregarded. Each cell was tallied as either positive or negative for ELF
157 labelling on the basis of the presence or absence of the fluorescent ELF®97 precipitate. Cells
158 were regarded as ELF positive as soon as an ELF signal (signal intensity above the threshold
159 as determined from the negative controls without addition of substrate) was located on the cell
160 surface. The average standard error for triplicate counts for 100 cells using the ELF®-97
161 labelling technique was determined to be 3% (Dyhrman and Palenik, 1999), and a similar
162 error is expected in the present study. The respective figures show tangential optical sections
163 best representing cell surface associated ELF signal.

164 Samples for the determination of phytoplankton composition and relative abundance
165 were filtered through a 200 μm mesh to remove zooplankton, and filtrates were preserved
166 with formaldehyde solution (2% final concentration) buffered with sodium acetate. After 38 h
167 sedimentation of 50 mL of filtrate, cell counts were performed on an inverted Axiovert 200
168 microscope (Zeiss GmbH) following the Utermöhl method (1958). During counting,
169 phytoplankton cells were attributed to microplankton or nanoplankton fractions, based on
170 observed cell dimensions (Sieburth et al., 1978) and counted at 200 X and 400 X
171 magnifications, respectively. Phytoplankton cells were identified at the lowest possible

taxonomical rank. Diatoms and dinoflagellates within microphytoplankton were determined on the level of species.

Comparison of the contribution of ELF labelled organisms in microphytoplankton communities with bulk APA in fraction > 3 μm was tested using least square linear regression and principal component factor analysis (PCA). One-way ANOVA was performed in order to test if there was a difference between the contribution of ELF labelled species in P-limited and P-repleted conditions. The conditions of normal distribution were tested with the Shapiro-Wilk test.

3. Results

3.1. Hydrological conditions, nutrient status and phytoplankton community composition

From March to the end of June the Po River flow was high (on average 3023 $m^3 s^{-1}$) with maximal impulses up to 7120 $m^3 s^{-1}$ in May (Fig. 2A). At the end of June the Po flow decreased to typical summer values and remained relatively constant until the end of October (in average 1070 $m^3 s^{-1}$), with frequent small impulses (up to 1437 $m^3 s^{-1}$). From the end of October the Po flow increased up to 2743 $m^3 s^{-1}$ at the end of November.

Fig. 2

In March the entire water columns at both stations were cold (9.78–10.65 ºC) and almost homogenous (Fig. 2B). During April–August, temperatures gradually increased (13.21–24.67 ºC at the surface and 10.05–17.70 ºC at the bottom) with the establishment of thermal stratification at both stations, being the sharpest between the depths of 12 and 20 m. From September a progressive cooling of the surface waters (21.84–14.15 ºC; Fig. 2B) started a progressive mixing in the water columns. At the end of November temperature of the water columns was homogenous.

195 At SJ101 freshwater influence, extending down to 10-15 m, was considerable during
196 the whole studied period (surface salinity 15.90-36.54) (Fig. 2C). At RV001, freshwater
197 influence was detected during May-October (surface salinity 35.82-37.00) extending down to
198 5-15 m, while in March-April and November it was low (surface salinity 37.21-37.54; Fig.
199 2C). In deeper layers of both stations more saline water was observed during the whole
200 investigation period (salinity 37.20-38.41).

201 PO_4 concentrations at RV001 were exceptionally low (< 0.03 μmol L^{-1}; Fig. 3A; S1A)
202 in the entire water column. At the surface of SJ101 somewhat higher PO_4 concentrations were
203 found during freshets in early spring and autumn (March-April, October-November; 0.07-0.17
204 μmol L^{-1}), while during the summer values were exceptionally low (< 0.03 μmol L^{-1}). At this
205 station a moderate accumulation of PO_4 in the bottom waters started with the onset of
206 stratification in May and persisted during the summer and autumn (0.05-0.16 μmol L^{-1}; Fig
207 S1A). DOP concentrations (0.10-0.59 μmol L^{-1}) always exceeded those of PO_4 (Fig. 3A; S1B)
208 at both stations. Lower DOP concentrations were characteristic for summer and autumn,
209 while during spring they were markedly higher.

210 Fig. 3

211 During summer, DIN concentrations (0.77-1.18 μmol L^{-1}) in upper waters of both
212 stations (Fig. 3A, S1C) were markedly lower than in spring and autumn (1.75-4.28 and 2.57-
213 76.15 μmol L^{-1} at RV001 and SJ101, respectively), with the highest values during the spring
214 freshets at SJ101. At SJ101, a moderate accumulation of DIN was observed in the bottom
215 waters during the summer (up to 5.88 μmol L^{-1}), while at RV001 no significant variation with
216 depth was observed (S1C).

217 At the surface and 10 m depth at both stations DIN was present in marked surplus with
218 respect to PO_4 (average inorganic N:P ~ 200). In bottom waters they were generally more
219 balanced for phytoplankton requirements (average inorganic N:P ~ 21).

The phytoplankton abundance was in the range of ~ 10^4-10^6 cell L^{-1} (Fig. 3B; S2). The highest abundances were found in the surface waters and generally decreased with depth. The highest abundances were found from March to May in upper waters of SJ101 (up to 2.0 x 10^6 cell L^{-1}), generally for an order of magnitude higher than at RV001. From July to November the abundances were generally similar at both stations (5.2 x 10^4-4.8 x 10^5 cell L^{-1}). In the bottom waters, phytoplankton abundance (2.8 x 10^4-1.5 x 10^5 cell L^{-1}) was lower than in upper waters and without noteworthy changes during the year or by station (Fig. S2C).

Generally, the phytoplankton community at both stations was dominated by the microphytoplankton in the upper waters, while the nanophytoplankton was dominating at the bottom (Fig. 3B; S2). Diatoms were generally much more abundant than dinoflagellates. In the microphytoplankton, diatoms and dinoflagellates were the two most diverse and abundant groups, while other groups accounted for < 3% of the respective community. During spring (March-April) the community composition in upper waters differed between the two stations (Fig. 3B). Microphytoplankton, comprised of almost only diatoms, strongly predominated at SJ101 (91%), while at RV001 domination of nanophytoplankton (75-98%) was recorded. In May-November the phytoplankton community composition was rather similar at both stations. During May and July nanophytoplankton dominated in the community, especially at SJ101 (60-80%). Dinoflagellates during this period reached their maximal contribution at both stations (10-22% and 6-16% at RV001 and SJ101, respectively). During August-November diatoms strongly predominated (generally 70-90%), while dinoflagellates accounted for a minor part of the community (0.2-4.6%).

3.2. APA and P turnover time

In upper waters bulk APA gradually increased from March to high summer values (Fig. 4A; S3; up to 362.8 and 2916 nmol L^{-1} h^{-1}, at RV001 and SJ101, respectively). During these months, values at SJ101 were constantly higher than at RV001, especially in March

when APA at RV001 was exceptionally low (< 10 nmol L^{-1} h^{-1}). In autumn (October-November) a decrease in APA was observed (18.8 and 53.8 nmol L^{-1} h^{-1} at SJ101 and RV001, respectively). In upper waters most of bulk APA was observed in the fraction > 3 μm (phytoplankton fraction; generally 70-90%), except in November when most of the activity was found in the fraction < 3 μm (Fig. 4A). At the bottom, APA was generally for an order of magnitude lower than in upper waters (Fig. S3C).

Fig. 4

APA-mediated PO_4 turnover time (T) at the bottom was generally far above 5 h (up to 135 h; 4B), indicating that phytoplankton was not P-limited (Nausch et al., 2004; Xu et al., 2008). On the contrary, in upper waters T was generally below 5 h, with exceptionally low values in August at the surface of SJ101 (about 1 min), indicating P-limitation. In surface waters T > 5 h were observed only in March at RV001 (about 63 h), then in October and November at both stations (17-257 h), indicating that P-limitation did not occur.

ELF signal was found localized around associated bacteria (Fig. 4Ca), diffused across the cell surface (Fig. 4C b), in irregular shaped structures on the cell surface (Fig. 4C c), in regular shaped islands on the cell surface (Fig 4C d), in small aggregations across the cell surface (Fig. 4C e,f), diffusely associated with chloroplasts but on the cell surface (Fig. 4C g) and in regular shaped cell surface islands associated to chloroplast (Fig. 4C h).

In the microphytoplankton community a variety of diatoms showed a positive ELF signal (Table 1). The lowest contribution of ELF positive species in diatom community (about 15-38%) was found during spring at RV001 (Fig. 5A,B).

Fig. 5

In March, during the water column mixing, only few species in relatively low abundance (*Chaetoceros constrictus*, *Cerataulina pelagic*a, *Thalassiosira* sp.), and in April

the dominant *Chaetoceros circinalis*, showed ELF signal at RV001 (Fig. 5A,B). *Chaetoceros brevis* was not ELF labelled, but bacteria attached to it showed strong ELF signal (Fig. 5A). Neither the dominant species *Bacteriastrum furcatum* in March nor the co-dominant *Cerataulina pelagica* in April were ELF labelled. Contrary, during the spring freshets at SJ101 the dominant *Skeletonema marinoi*, as well as the major part of diatom species (up to 99.5% in diatom community), showed ELF signal (Fig. 5A,B). The co-dominant *Chaetoceros socialis* was ELF positive in April, but not in March when bacteria attached to it had a strong positive signal. In May the diatom community was strongly dominated by ELF labelled species (about 83-91%) at both stations (Fig. 5C). During summer the maximal number of ELF labelled species was found (Fig. 6). The dominant species were ELF labelled, with exception of *Chaetoceros* sp. in August at SJ101. However, at both stations, during summer a variety of species with relatively high contribution to the diatom community did not show any ELF signal.

Fig. 6

A variety of species with minor contribution and no detectable ELF signal ("Other not ELF labelled", Figs. 5, 6) were found at both stations during all sampling terms (Table 1).

Dinoflagellates were scarcely ELF labelled (Table 2). The labelled species made up 3-10% of the dinoflagellate community in June and August, but generally labelled species contributed less than 2% to the dinoflagellate community. Dinoflagellates in total generally contributed < 10% to the microphytoplankton abundance.

In October no ELF signal was detected in any microphytoplankton species.

The contribution of ELF positive species to the diatom community was strongly correlated with the bulk APA in the > 3 μm fraction (phytoplankton APA; $p=0.009$), in contrast to species from the dinoflagellates community ($p=0.538$). Principal Component

Analysis (Fig. 7) also indicated a tight relationship between the contribution of ELF positive diatoms and phytoplankton APA. In addition, positive relations with nitrate and temperature, and inverse relations with PO_4, DOP and salinity were found. The contribution of ELF positive species to the dinoflagellate community was loosely related with phytoplankton APA, but showed strong positive relationship with temperature. ANOVA showed that during P-limitation the contribution of ELF positive species in both diatom and dinoflagellate communities was significantly higher than during period when P was not limiting ($p<0.001$ and $p=0.014$, respectively).

Fig. 7

4. Discussion

The seasonal development of bulk APA in 2013 was similar to those described in earlier studies (Ivančić et al., 2009; 2010; 2012). As observed earlier, during periods of high APA, DOP was consumed, and higher temperature and freshwater nitrate input incited, while PO_4 repressed APA expression. This strongly indicates a seasonally reoccurring pattern of APA and that DOP is an important source of P in the region. Maximal hydrolysis rates and P-limitation were found in upper water column where single cell APA in microphytoplankton was identified using the ELF assay. This assay identifies which members of the phytoplankton community express APA, offering substantially enhanced resolution along the size fractionation. This method is being increasingly used to identify single cell APA in field populations (Dyhrman et al., 2002; Lomas et al., 2004; Dyhrman and Ruttenberg, 2006; Ou et al., 2006; Girault et al., 2013).

The distribution of APA across different species was highly variable between sites and seasons. The contribution of ELF positive species to the diatom community generally related with the phytoplankton APA, and a variety of labelled species were observed during P-limitation (Table 1).

318 During spring freshets, the western area (SJ101) provided conditions with high
319 nutrient availability in which diatoms dominated the phytoplankton community. However,
320 high bulk APA and very fast APA-mediated PO_4 turnover time (hereinafter termed P
321 turnover) implied a severe P-limitation. In such conditions ELF labelled species represented
322 up to 99.7% of the diatom community. The dominant species *Skeletonema marinoi* uses APA
323 to create an advantage in the competition for organic P sources. It appears safe to suggest, that
324 expressing APA is a successful strategy for diatoms to compete for organic P under the
325 influence of riverine nutrient input, since at that time they reached the highest abundance
326 during the year.
327 Markedly different phytoplankton strategies of P acquisition were found at the eastern
328 area (RV001)**.** In this area, the cyclonic circulation (Artegiani et al., 1997) advects the water
329 from the more southern parts of the Adriatic Sea, with low nutrient content and
330 microphytoplankton abundances (Viličić et al., 2009; Zavatarelli et al., 1998). Contrary to the
331 western area, at the time microphytoplankton community in this area does not experience
332 pronounced fluctuations of nutrient availability. In March low bulk APA and exceptionally
333 high P turnover time (about 63 h) showed that PO_4 was not limiting phytoplankton growth.
334 The water column was mixed and a modest phytoplankton biomass, mainly
335 nanophytoplankton, developed on PO_4 regenerated during the winter. In the diatom
336 community only few larger species did already express APA (*Chaetoceros constrictus*,
337 *Cerataulina pelagica* and *Thalassiosira* sp.), indicating them being P-stressed, or even P-
338 limited since their abundance was low. *C. pelagica* in April did not show any APA signal,
339 indicating that expression of APA in March was due to P-stress/limitation. Probably the other
340 two species follow the same pattern. These larger species have probably a higher half
341 saturation constant of PO_4 uptake (K_m) than the other present species. Larger cells require
342 more nutrients for growth, implicating higher K_m (Eppley et al., 1969) and consequently may

343 be at a disadvantage in nutrient uptake competition compared to smaller phytoplankton. Oh et
344 al. (2010) reported that K_m might be an important index of nutrient affinity as well as a
345 threshold for APA induction. In fact, in oligotrophic conditions found in March at the eastern
346 area, the dominating smaller species *Bacteriastrum furcatum* (60% in diatom community) did
347 not express APA. Probably a lower K_m of PO_4 uptake and hence an adaptation for success in
348 more oligotrophic conditions, allowed growth of *B. furcatum* without the use of APA. This
349 species co-dominated the diatom community expressing APA in May under P-limited
350 conditions. Consequently, in this species APA regulation according to environmental
351 conditions is supposed. In April P-limiting conditions were observed also at the eastern site
352 and the dominant *Chaetoceros circinalis* expressed APA to thrive on DOP. However, a
353 variety of diatoms contributed considerably to the overall diatom abundance (about 60%)
354 without utilizing APA. At the time overall oligotrophic conditions were observed at the
355 eastern site. Hence most of the diatoms did not compete for DOP due to low nutrient
356 availability, but probably slowed down their metabolisms, as supported by their exceptionally
357 low abundances.
358 From May on phytoplankton communities at both sampling sites share a common
359 history. Typically, at that time of the year the general circulation transports water from the
360 western coast towards the eastern coast, where the current turns northwards (Russo et al.,
361 2005; Lyons et al., 2007), considerably reducing the influence of southern Adriatic waters at
362 the eastern area. In such circumstances, microphytoplankton developed near the western coast
363 under considerable influence of riverine nutrient input, subsequently reach the eastern area.
364 Consequently, both sampling sites show microphytoplankton communities under the
365 influence of unbalanced nutrient input. At the western site, species experienced the
366 unbalanced nutrient input directly, and under nutrient rich conditions they grew to larger
367 numbers exhausting PO_4. Consequently, water spreading toward the east was poor in PO_4, but

368 enriched in organic matter produced at the western area. In both cases P-limitation occurred,
369 as confirmed by very fast P turnover (down to 1 min). In such strong P-limited conditions
370 diatoms reacted by expressing APA to sustain (May, July with low abundance), or even to
371 further develop their abundance and activity (August, September, high abundance). As soon
372 as these species again reach the river plume (closed circulation), they will benefit immediately
373 (without lag phase due to dormancy or very low abundances) from the rich nutrient inputs.
374 Additionally, diatoms are known for longer generation times, and hence they would profit
375 from the capability of P pool formation to compete with faster growing species.
376 Despite of general P-limiting conditions during the summer, a variety of species that
377 significantly contributed, or even co-dominated the diatoms community did not express APA.
378 Except for *Chaetoceros* sp., the other species were found to express APA under at least some
379 conditions. This indicates that those species were not P-limited when found ELF negative.
380 They probably either thrived on intracellular P pools (most likely for species observed at the
381 western site) or had alternative mechanisms to overcome P-limitation (particularly at the
382 eastern site). Possible alternatives indicated by obtained results are (i) the use of PO_4 liberated
383 by free/attached enzymes from different species and (ii) the production of non-phospholipids
384 (Ivančić et al., 2012; Van Mooy et al., 2009). During P-limitation, *Chaetoceros socialis* and
385 *Chaetoceros brevis* did not show APA themselves when bacteria attached to them showed a
386 strong APA signal. Since *C. socialis* was found able to use DOP when P is limiting, it is
387 possible that associated bacteria provided P for its growth. Nevertheless, it is not possible to
388 exclude, that *C. socialis* (found during freshets at the western area), deposited P in
389 intracellular pools while it was abundantly available and that at the sampling time the
390 intracellular availability of P did not yet set on the expression of APA. Phytoplankton
391 accumulate P in response to pulses in P supply (Andersen et al., 1991; Tantanasarit et al.,
392 2013; Wasmund et al., 2014) and stored P can ensure numerous divisions (Silkin et al., 2013).

393 P accumulation in the phytoplankton community of the NA was observed in earlier
394 experiments (Ivančić et al., 2004). Furthermore, phospholipid:non-phospholipid ratio at the
395 investigated sites during the summer (P-limitation) was markedly lower than during
396 November (not P-limitation) (B. Gašparović, unpubl. data). This indicates a preferential
397 synthesis of non-phospholipids during P-limitation, as already reported for the NA (Ivančić et
398 al., 2012).
399 At the investigated sites a variety of species have not yet been found ELF labelled
400 (Table 1). Except for *Chaetoceros* sp., they were not successful during P-limitation,
401 contributing < 3% to the diatom community. This observation again fosters the hypothesis,
402 that APA is a necessary prerequisite for diatom species to maintain larger abundances during
403 P-limited conditions.
404 After the onset of vertical mixing in October, bulk APA decreased being minimal in
405 November when water columns were completely mixed. In these months long P turnover (up
406 to 257 h) showed that phytoplankton was not P-limited. During the vertical mixing none of
407 the microphytoplankton species expressed APA. Many of the most abundant species in this
408 period (*Lioloma pacificum*, *Bacteriastrum hyalinum*, *Rhizosolenia fragilissima*, *Chaetoceros*
409 *socialis*, *Thalassionema nitzschioides*, *Rhizosolenia imbricata*, *Skeletonema marinoi*,
410 *Cyclotella* sp.) on the contrary did express APA during the P-limitation in summer. That
411 means there is so far no evidence for APA to be expressed constitutively. APA is rather
412 expressed only when P is limiting, while under other conditions APA is down regulated or not
413 expressed. It seems that during this period the constant supply of PO_4 from the bottom
414 prevented P-limitation, as observed earlier (Ivančić, et al., 2012).
415 In the case of dinoflagellates, the contribution of ELF positive species to the community
416 was low regardless of high or low hydrolytic rates or establishment of P-limited conditions.
417 Dinoflagellates expressed APA rarely, and if so, those species had really low abundances

(Table 2). Even in May and July, when dinoflagellates approached diatom abundances, only marginal species (< 8% in dinoflagellate community) expressed APA. This indicates that dinoflagellates in the NA do not employ AP as a means for reaching large abundances. Dinoflagellates did not reach large abundances at the sampled stations, as usual for the investigated area (Godrijan et al., 2013). In contrast to our findings, in other areas a high percentage of dinoflagellates were ELF labelled as reported for the Sargasso Sea (Lomas et al., 2004), Oregon coastal area (Dyhrman and Ruttenberg, 2006), the China Sea (Ou et al., 2006) and subtropical North Pacific (Girault et al., 2013). Some of the reported species in the mentioned areas were identified also in the present study in the NA. Consequently, a low contribution of ELF labelled dinoflagellate species in the respective community in the NA probably does not reflect their inability to employ AP, but rather a so far unidentified limitation or possibility, prevent the certain dinoflagellate species from expressing APA. Their reported capability of heterotrophy might make the expression of extracellular APA redundant (Jeong et al., 2010). In fact, the Oregon coast field data indicate that cell-specific APA of dinoflagellates may not be strongly P regulated, and unlike for diatoms, the presence of cell-specific APA does not support an interpretation of P stress for dinoflagellates (Dyhrman and Ruttenberg, 2006).

5. Conclusions and future directions

Overall, in P-limited conditions all species with considerable contributions to the diatom community, with only a few exceptions, expressed APA, while only few dinoflagellate species expressed APA. Diatoms often dominated the phytoplankton community, showing APA to be seemingly a very important prerequisite for their success in the NA. APA-expressing species do not necessarily dominate the phytoplankton community, since species in very low relative abundances that nevertheless did express APA were found. This suggests that APA is also an important strategy for species to survive and maintain

443 active metabolic state outside of their mass abundances or blooms. This allows them to
444 immediately react to short term nutrient availability (e.g. riverine input). This feature appears
445 to be very beneficial in a complex and fast changing environment with gradients of nutrient
446 availability and point sources for nutrient input. Findings of co-dominating species in the
447 diatom community that did not express APA, let presume that those species store P
448 intracellularly, especially at the western site (close to the Po plume), and at the sampling time
449 still lived on those pools. Other species did not express APA themselves, however bacteria
450 attached to those cells did show APA. For these species a symbiotic relationship could be
451 supposed, where the larger host diatom cell makes use of APA expressed by the attached
452 bacteria.

453 Further research should address strategies for successful competition of phytoplankton
454 in P-limiting conditions. Such strategies are likely to be found globally. From a variety of
455 possible phytoplankton strategies we propose here 4 of them (Fig. 8) that in the light of results
456 presented herein, authors′ experiences and earlier work (Gašparović et al., 2013; Godrijan et
457 al., 2013; Ivančić et al., 2004; 2012; Viličić et al., 2013) appear to be the most plausible.

458 Fig. 8

459 Strategy 1: Efficient PO_4 uptake. K_m for PO_4 uptake should be lower than PO_4 concentration.
460 Species dominate in oligotrophic conditions without APA when P is not limiting. Cell
461 division time is not essential but rather endurance and uptake efficiency. Such species use
462 APA for survival or bloom formation/prolongation after unbalanced nutrient input.
463 Strategy 2: Sustaining abundances and activity during P-limitation with APA. Fast bloom
464 formation when nutrients become available due to sustained abundances and active state. The
465 absence of APA in oligotrophic conditions might be due to limitation with other resources.
466 Strategy 3: Sustaining abundances or bloom formation on P-pools or/and production of non-
467 phospholipids. Species co-dominating in P-limited conditions without APA.

Strategy 4: Symbiotic relationship with bacteria. Species that co-dominate in P-limited conditions without APA, but with attached bacteria that do express APA. When experiencing eutrophic riverine input those species grow fast and co- dominate the community. Under oligotrophic conditions those species are moderately abundant but attached bacteria are virtually absent or do not express APA.

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661

Table 1. ELF detection of extracellular alkaline phosphatase activity in microphytoplankton species at SJ101 and RV001 during 2013. ELF positive species (+), ELF negative species (-) and ELF positive species where the signal is located at attached bacteria (a).

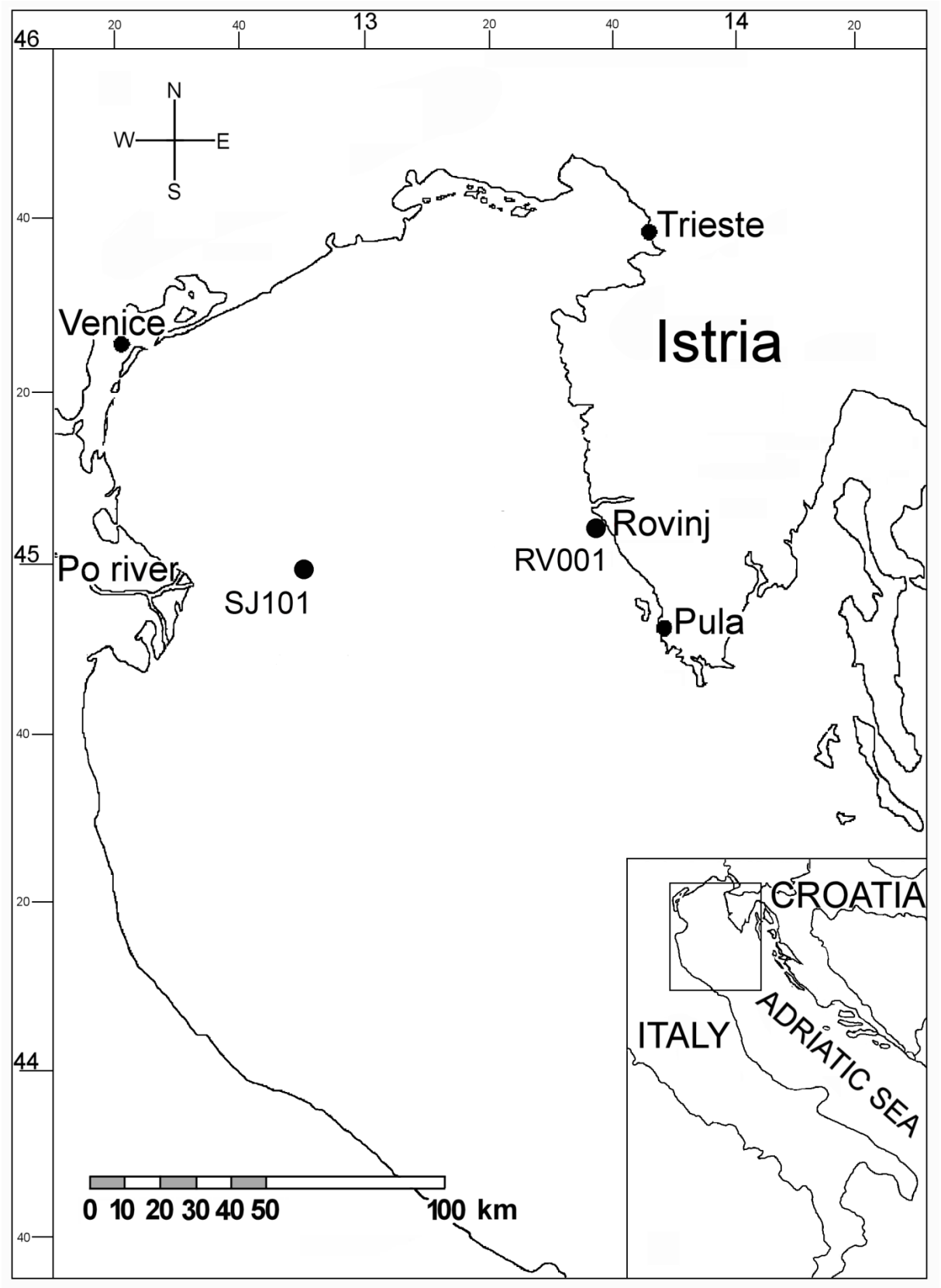
Species DIATOM	ELF	DINOFLAGGELATE	ELF
Asterionella formosa	-	*Alexandrium minutum*	-
Asteromphalus heptactis	-	*Ceratium candelabrum*	-
Bacteriastrum furcatum	+	*Ceratium extensum*	-
Bacteriastrum hyalinum	+	*Ceratium falcata*	-
Bacteriastrum jadranum	-	*Ceratium furca*	+
Bacteriastrum mediterraneum	-	*Ceratium furca* var. *eugrammum*	-
Cerataulina pelagica	+	*Ceratium fusus*	-
Chaetoceros affinis	-	*Ceratium hexacanthum*	-
Chaetoceros brevis	a	*Ceratium pavillardi*	-
Chaetoceros circinalis	+	*Ceratium symmetricum*	-
Chaetoceros concavicornis	-	*Ceratium trichoceros*	+
Chaetoceros constrictus	+	*Ceratium tripos*	-
Chaetoceros curvisetus	-	*Dinophysis caudata*	+
Chaetoceros dadayi	-	*Diplopsalis complex*	-
Chaetoceros danicus	+	*Goniaulax polygramma*	-
Chaetoceros diversus	+	*Goniaulax* sp.	-
Chaetoceros lorenzianus	-	*Goniodoma acuminatum*	+
Chaetoceros rostratus	-	*Gymnodinium* sp.	-
Chaetoceros socialis	+/a	*Gyrodinium fusiforme*	-
Chaetoceros sp.	-	*Gyrodinium* sp.	-
Chaetoceros tetrastichon	-	*Hermesinium adriaticum*	+
Chaetoceros tortissimus	-	*Heterocapsa* sp.	-
Chaetoceros vixvisibilis	+	*Kofoidinium velelloides*	-
Chaetoceros wighami	-	*Minuscula bipes*	-
Chateoceros contortus	+	*Oxyphysis oxytoxoides*	+
Coscinodiscus oculus iridis	-	*Oxytoxum caudatum*	-
Coscinodiscus sp.	-	*Oxytoxum sceptrum*	-
Cyclotella sp.	+	*Oxytoxum variabile*	-
Cylindrotheca closterium	-	*Phalacroma biceps*	-
Dactyliosolen mediterraneus	-	*Phalacroma rotundatum*	-
Diploneis bombus	-	*Podolampas elegans*	-
Eucampia cornuta	+	*Prorocentrum compressum*	-
Guinardia flaccida	+	*Prorocentrum micans*	-
Guinardia striata	-	*Prorocentrum minimum*	-
Hemiaulus hauckii	+	*Prorocentrum triestinum*	-
Leptocylindrus danicus	+	*Protoperidinium brochii*	-
Leptocylindrus minimus	-	*Protoperidinium conicum*	-
Licmophora sp.	-	*Protoperidinium diabolus*	+

Lioloma pacificum	+	*Protoperidinium divergens*	-
Navicula sp.	-	*Protoperidinium leonis*	-
Nitzschia incerta	-	*Protoperidinium ovum*	-
Paralia sulcata	-	*Protoperidinium pallidum*	+
Pleurosigma sp.	+	*Protoperidinium paulseni*	-
Pseudonitzschia spp.	+	*Protoperidinium pyriforme*	-
Rhizosolenia alata f. *gracillima*	+	*Protoperidinium solidicorne*	-
Rhizosolenia alata var. *indica*	-	*Protoperidinium steinii*	-
Rhizosolenia calcar-avis	+	*Protoperidinium tuba*	-
Rhizosolenia fragilissima	+	*Pseliodinium vaubanii*	-
Rhizosolenia imbricata	+	*Pyrocystis lunula*	-
Rhizosolenia robusta	-	*Scripsiella* sp.	-
Rhizosolenia sp.	-	*Torodinium* sp.	-
Rhizosolenia stolterfothii	-		
Skeletonema marinoi	+		
Striatella unipunctata	-		
Thalassionema nitzschioides	+		
Thalassiosira angulata	-		
Thalassiosira sp.	+		
Thalassiothrix frauenfeldii	-		

Table 2. ELF positive dinoflagellates and their contribution to the dinoflagellate abundance at SJ101 and RV001 during 2013.

Species	Contribution (%)
Ceratium furca	0.54-6.99
Ceratium trichoceros	0.33-1.72
Dinophysis caudata	0.76
Goniodoma acuminatum	0.64
Hermesinium adriaticum	0.76-8.17
Oxyphysis oxytoxoides	0.64
Protoperidinium diabolus	0.33-0.64
Protoperidinium pallidum	0.81

750 Figures



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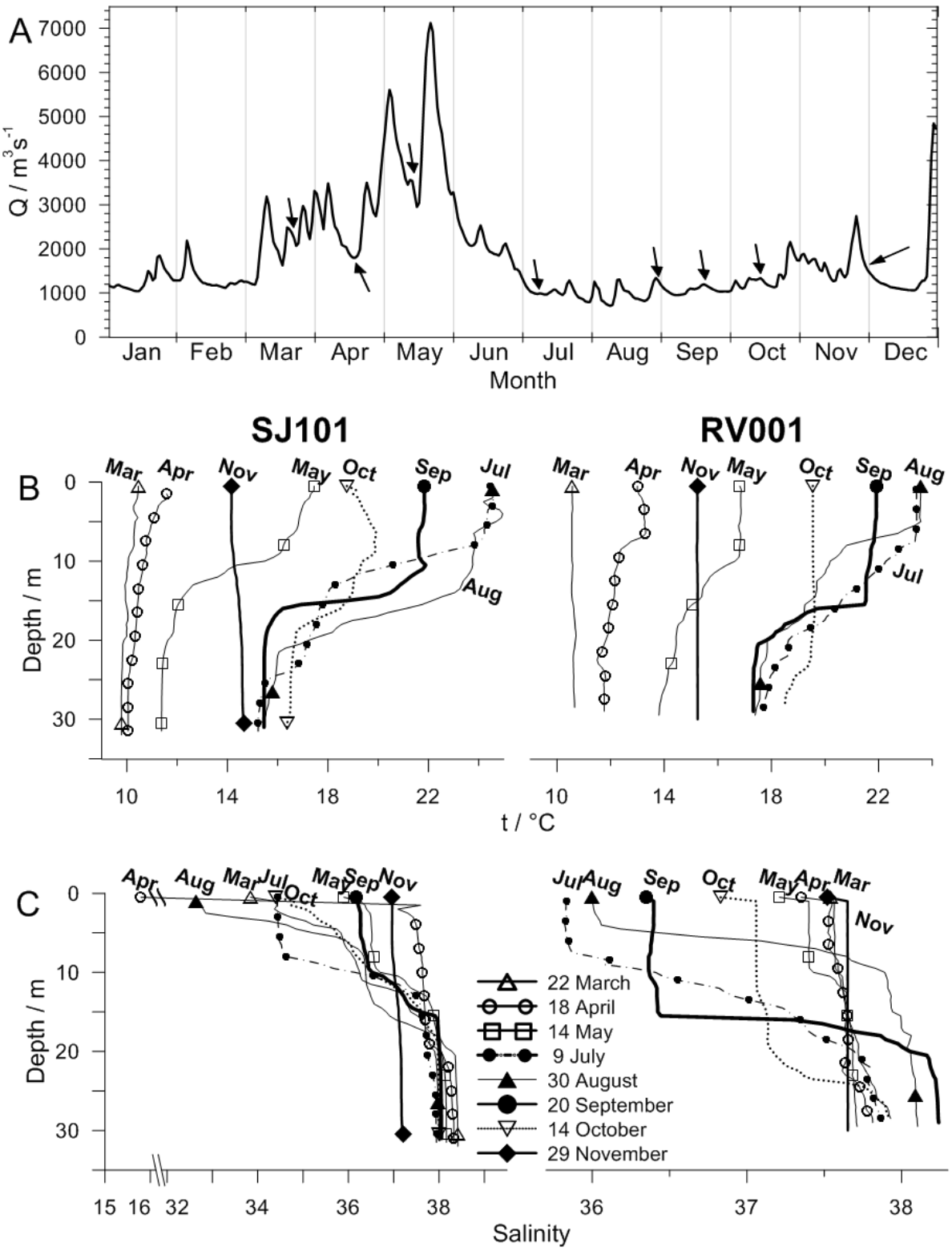
752 Fig. 1. Research area and sampling stations in the northern Adriatic Sea.



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754 Fig. 2. (A) Daily mean of the Po River discharge rate (Q) with cruise dates denoted by arrows.

755 (B) Temperature (t) and (C) salinity profiles at SJ101 and RV001 during 2013.

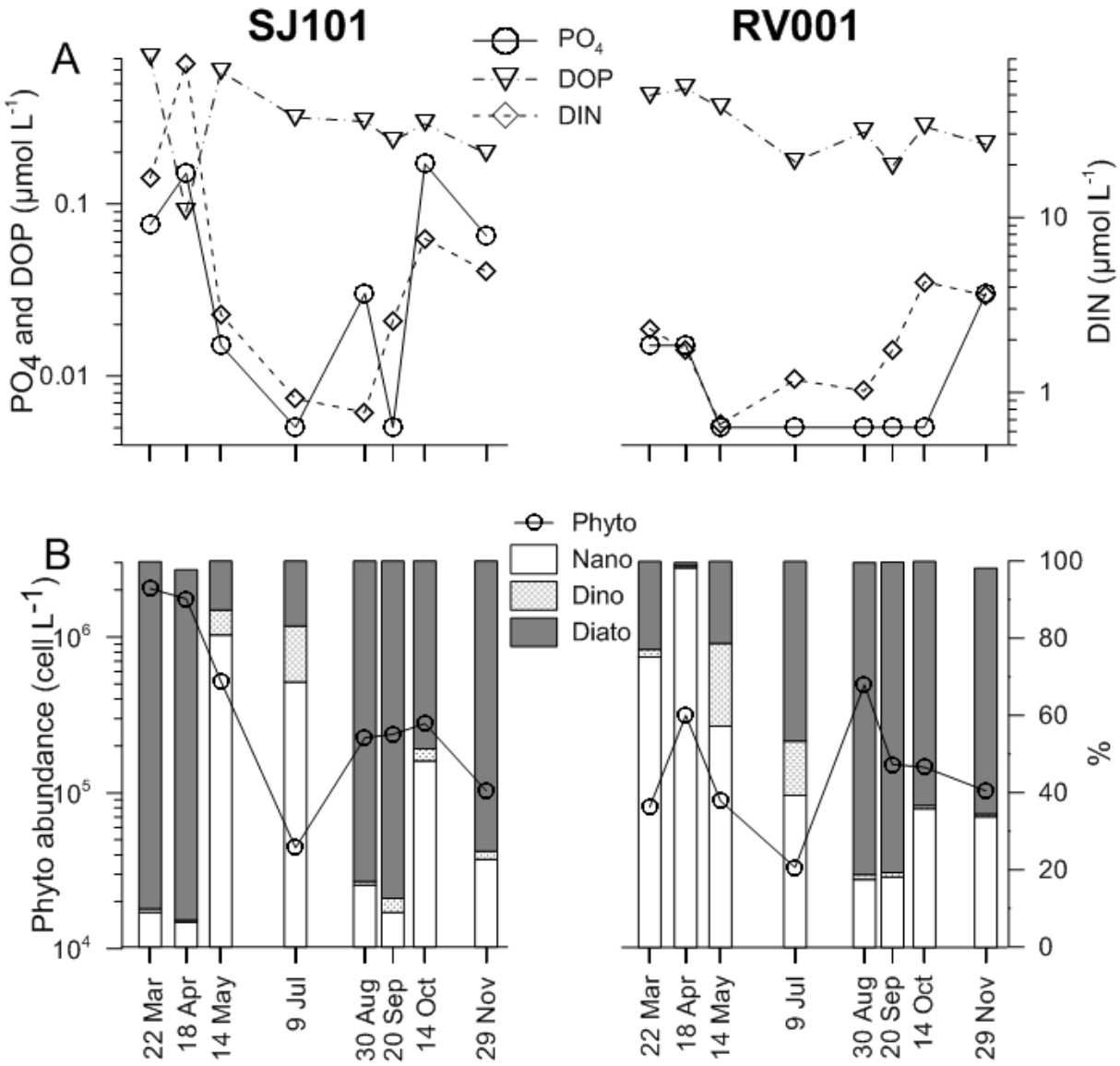
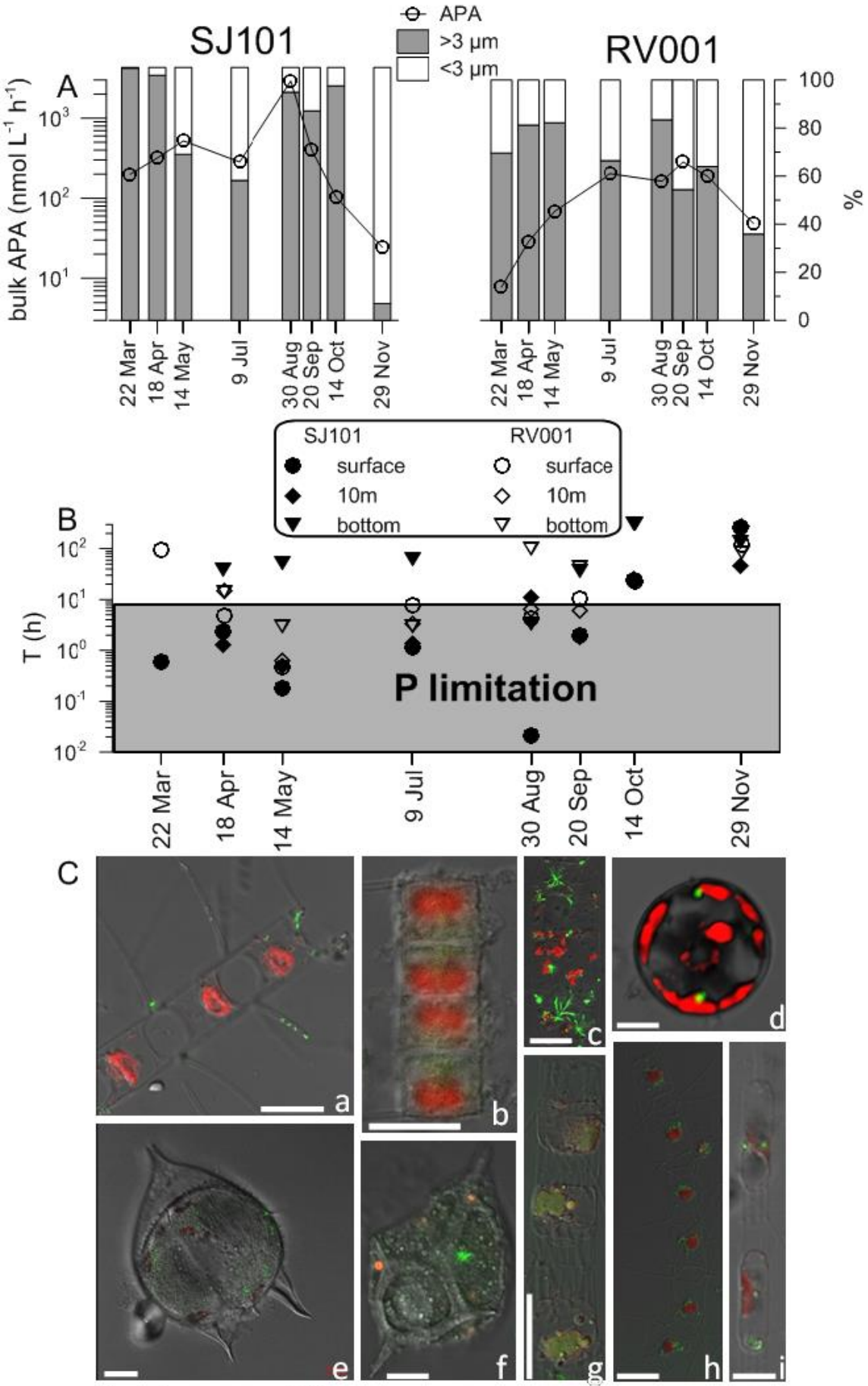


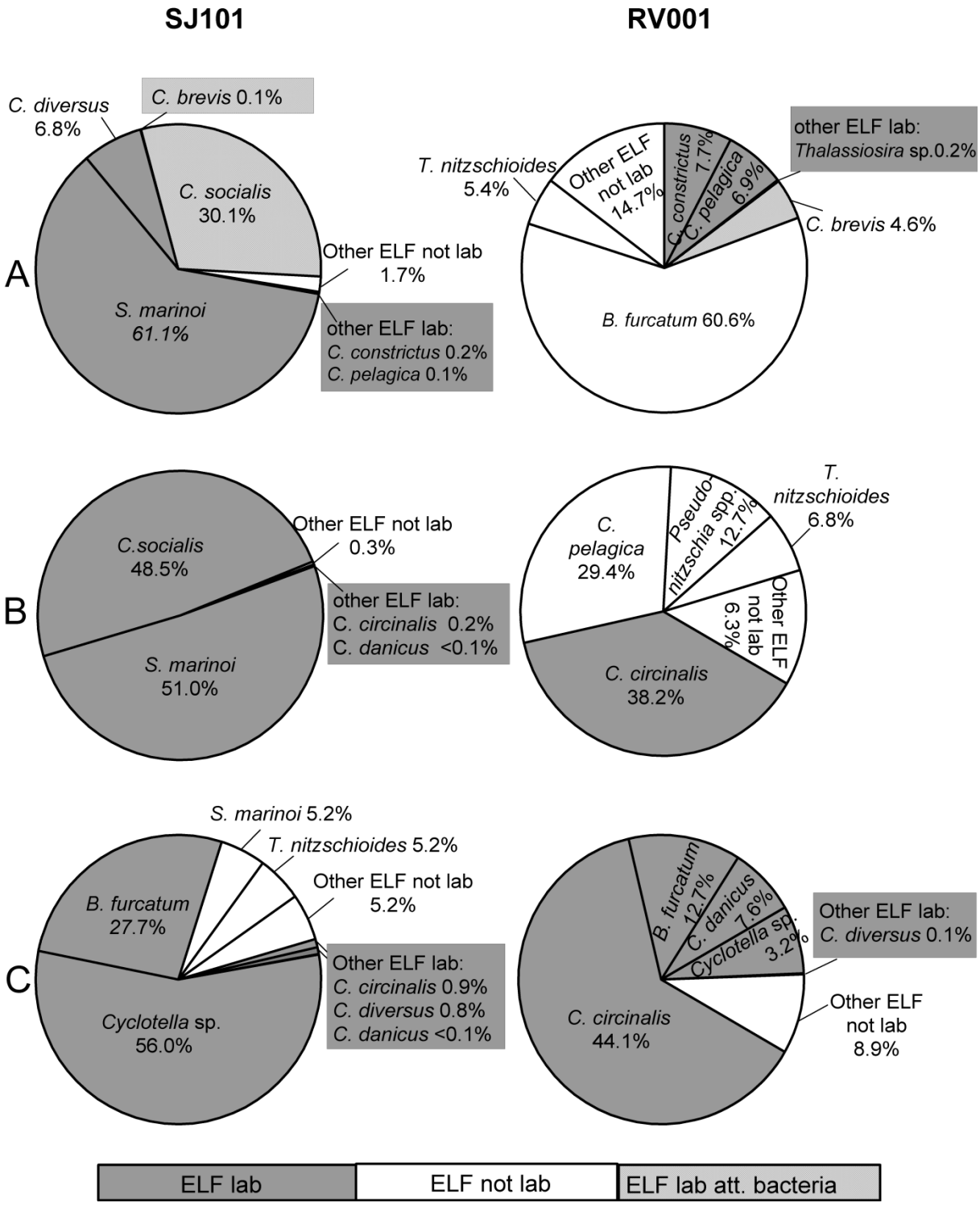
Fig. 3. (A) Concentrations for orthophosphate (PO_4; circle), dissolved organic phosphorus (DOP; triangle), dissolved inorganic nitrogen (DIN; diamond), and (B) phytoplankton abundance (phyto) and community composition at surface of SJ101 and RV001 during 2013.



Scale bars represent 10 µm.

761 Fig. 4. (A) Bulk APA, contribution of > 3 μm and < 3 μm fractions in the total activity at the
762 surface, and (B) APA-mediated turnover time at SJ101 and RV001 during 2013. P-limitation
763 is denoted by grey area. (C) Chloroplast fluorescence in red, ELF-mediated signal for APA in
764 green. *Chaetoceros brevis* APA with bacteria attached to setae (a). *Chaetoceros* sp. APA
765 diffused on the cell surface, dislocated from the chloroplasts (b). *Cerataulina pelagica* APA
766 on elongated, branching aggregations on the cell surface (c). *Thalassiosira* sp. APA in 2 small
767 aggregations per valve face, dislocated from the chloroplasts (d). *Protoperidinium pyriforme*
768 APA in microaggregations across the cell surface (e). *Hermesinum adriaticum* APA diffused
769 across the cell surface (f). *Skeletonema marinoi* APA on the cell surface where chloroplasts
770 are located (g). *Skeletonema marinoi* APA in few islands on the cell surface and dislocated
771 from chloroplasts (i). *Chaetoceros socialis* APA in concentrated islands on the cell surface
772 adjacent to chloroplasts (h). Scale bars represent 10 μm.

773



774

775 Fig. 5. Contribution (%) of ELF labelled (ELF lab) and ELF not labelled (ELF not lab)
776 species in diatom community, as well as contribution of species with labelled attached
777 bacteria (att. bacteria ELF lab) in the upper waters (0-10 m) at SJ101 and RV001 in (A)
778 March (B) April and (C) May 2013.

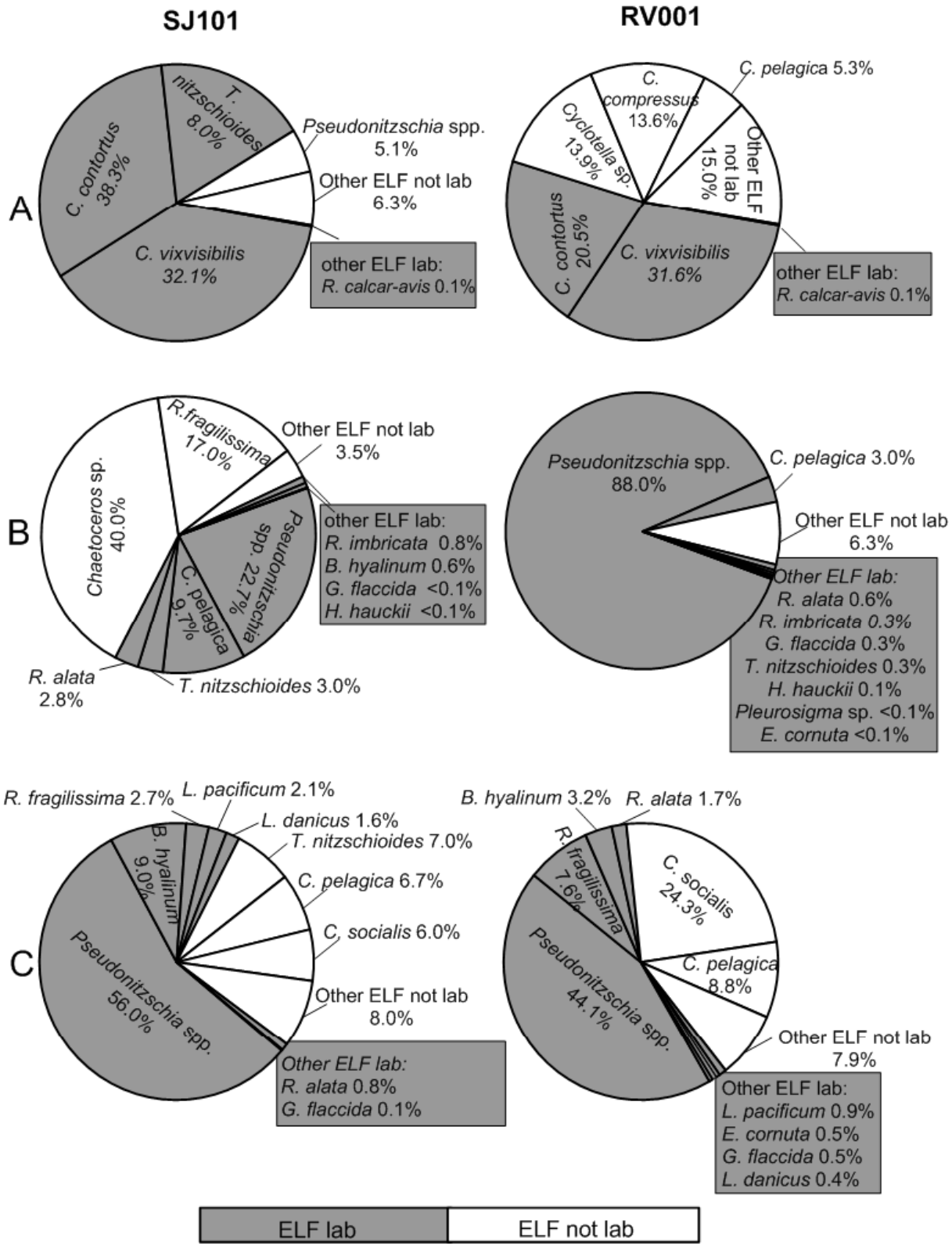
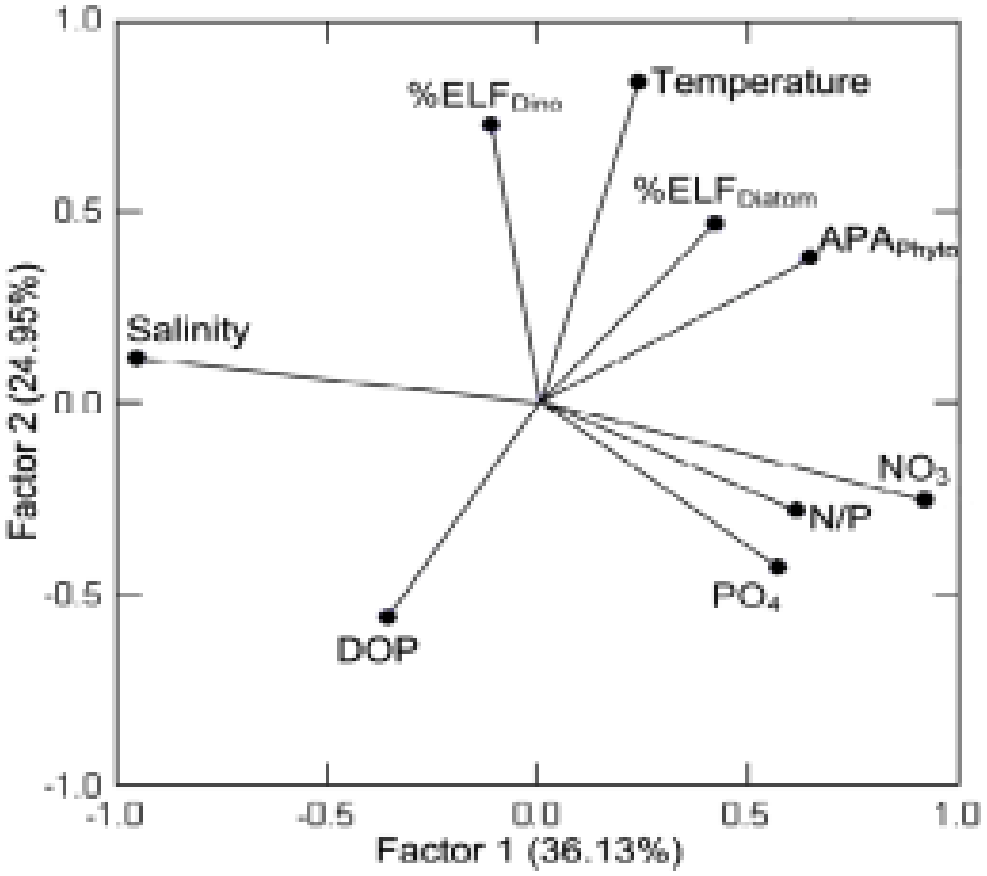


Fig. 6. Contribution (%) of ELF labelled (ELF lab) and ELF not labelled (ELF not lab) species in diatom community in the upper waters (0-10 m) at SJ101 and RV001 in (A) July (B) August and (C) September 2013.



783

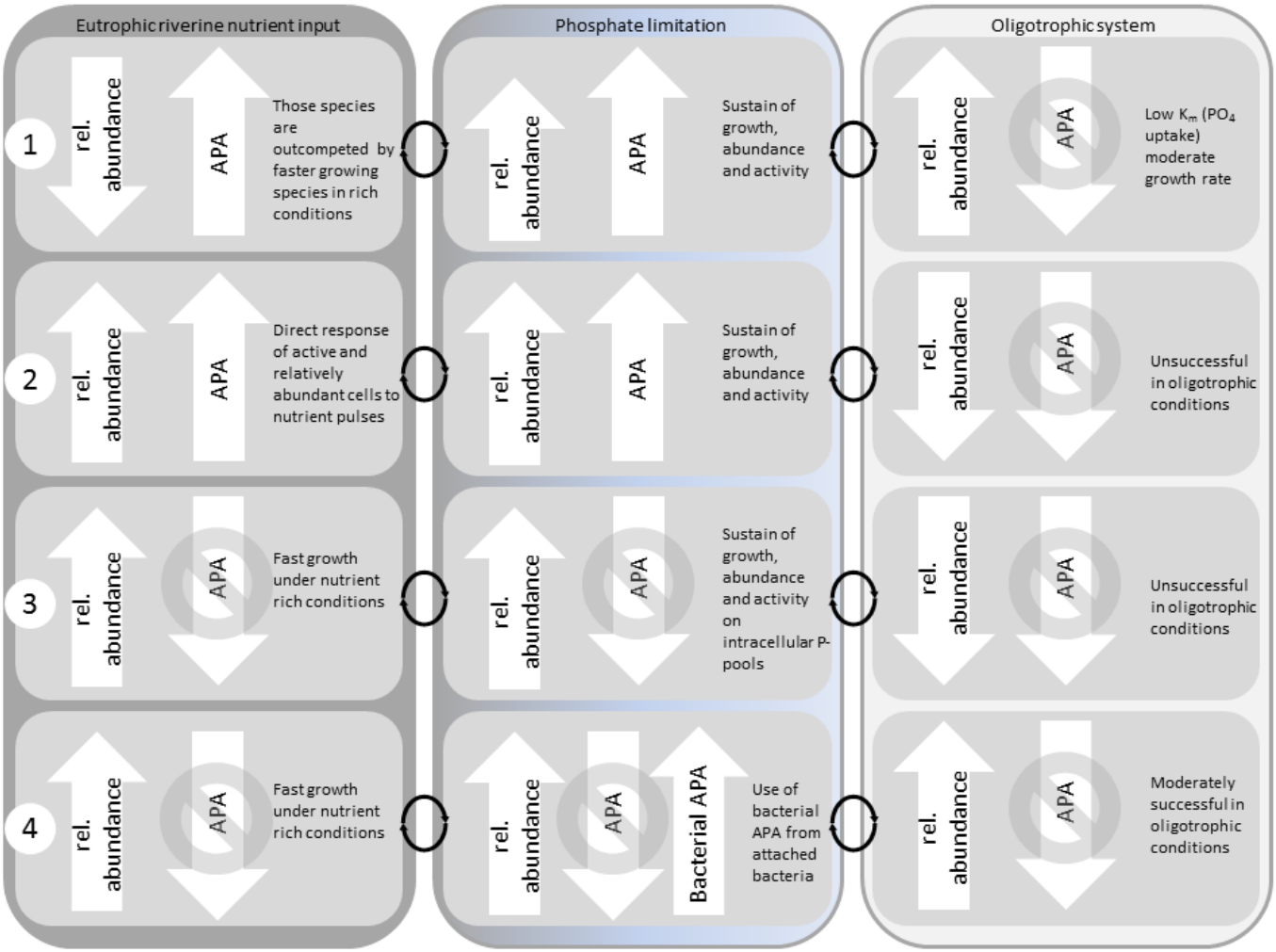
784 Fig. 7. Principal Component Analysis of measured variables: temperature, salinity,

785 orthophosphate (PO_4), nitrate (NO_3), dissolved organic phosphorus (DOP), inorganic N/P

786 ratio, contribution of ELF positive species in diatom community ($\%ELF_{Diatom}$), contribution of

787 ELF positive species in dinoflagellate community ($\%ELF_{Dino}$) and APA in fraction > 3µm

788 (APA_{Phyto}).

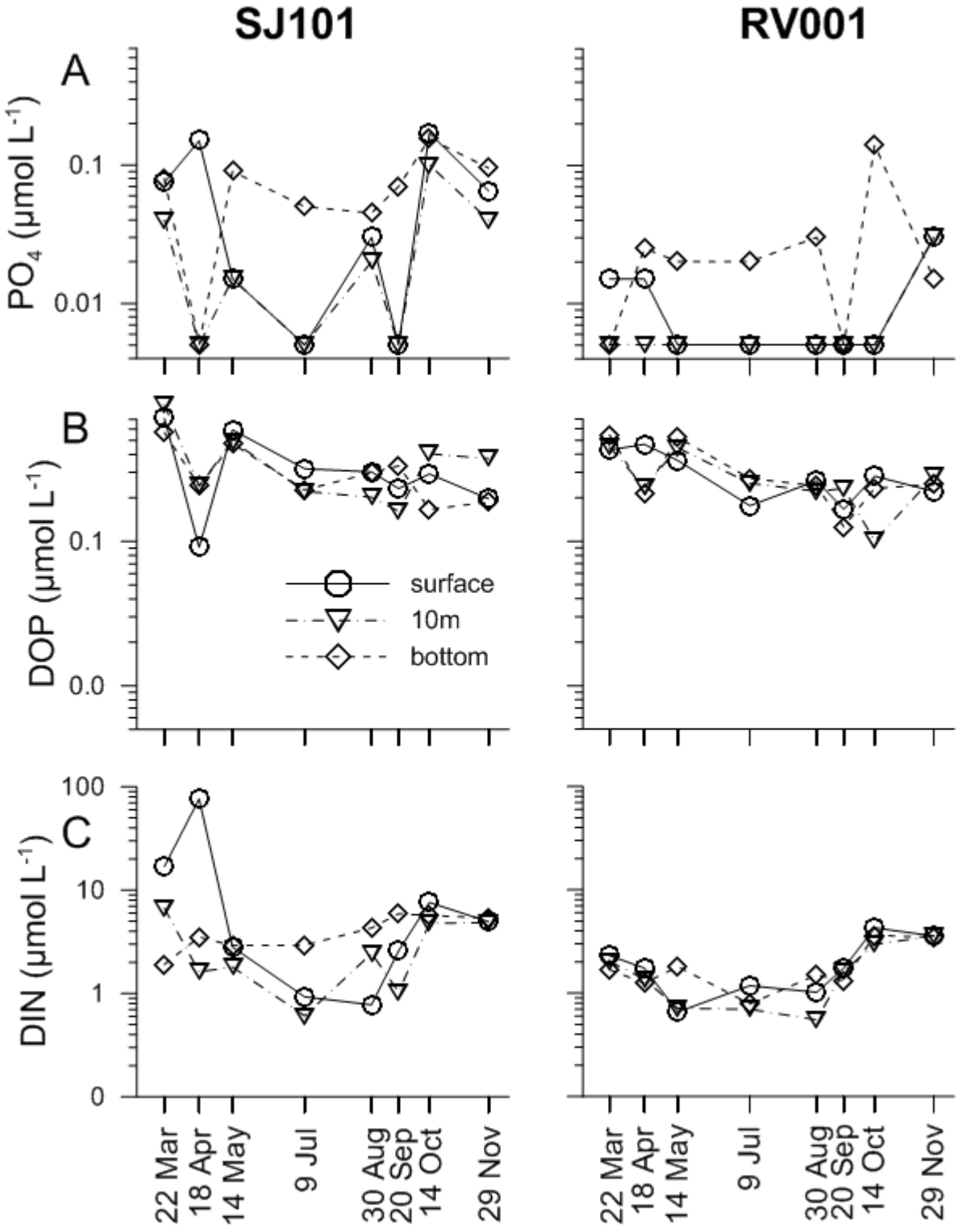


789

790 Fig. 8. Four identified strategies for successful competition in the NA. Within one row the
791 figure shows how one strategy addresses the different trophic conditions that plankton species
792 face when following the current systems in the NA (as shown by circular arrows). Although
793 both areas (SJ101 and RV001) can be P-limited, they completely differ in trophic conditions:
794 prevalently eutrophic at SJ101 and more oligotrophic on RV001.

795

796 Supplementary figures



797

798 Fig. S1. Concentrations for (A) orthophosphate (PO_4), (B) dissolved organic phosphorus

799 (DOP) and (C) dissolved inorganic nitrogen (DIN) at SJ101 and RV001 during 2013.

800

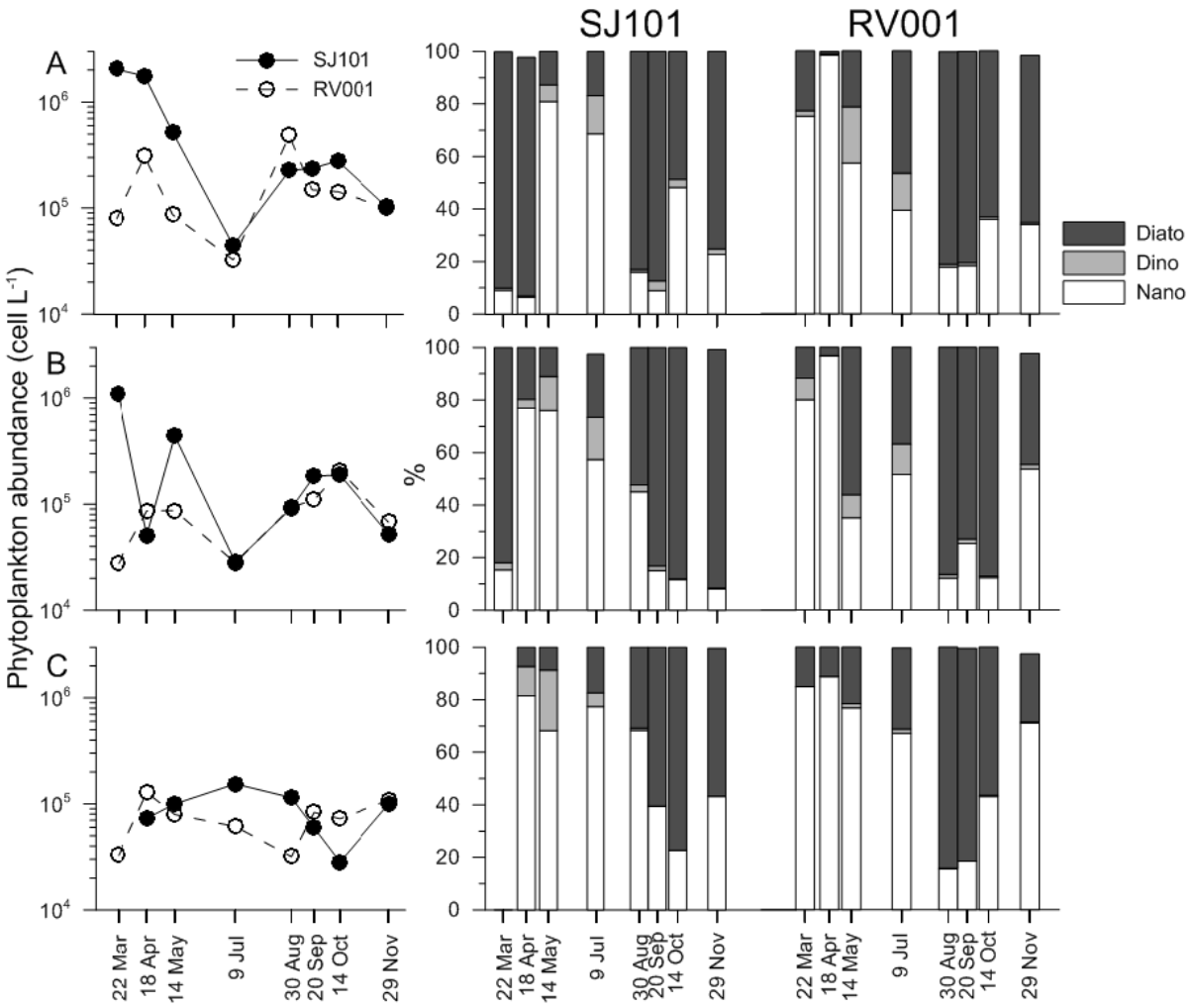
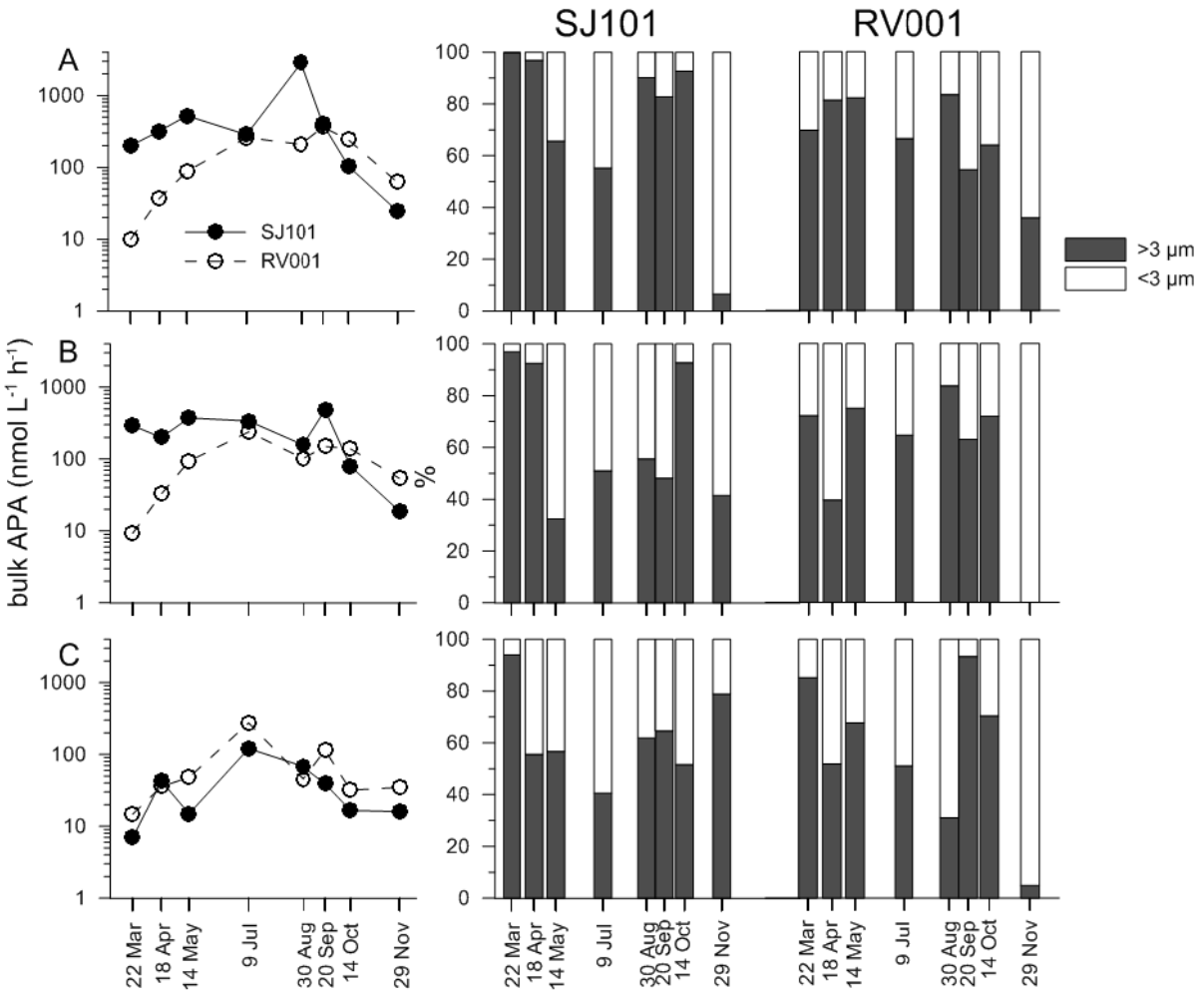


Fig. S2. Phytoplankton abundance (Phyto abundance) and contribution of diatoms, dinoflagellates and nanophytoplankton in phytoplankton community at (A) surface, (B) 10 m depth and (C) bottom at SJ101 and RV001 during 2013. In March it was not possible to count nanophytoplankton at the bottom of SJ101 (due to the high quantity of detritus), and the relative contributions were not calculated.



807

808 Fig. S3. Bulk APA and contribution of > 3 µm and < 3 µm fractions in the total activity at (A)

809 surface, (B) 10 m depth and (C) bottom at SJ101 and RV001 during 2013.

810