Ocean and Coastal Research

ORIGINAL ARTICLE

http://doi.org/ 10.1590/2675-2824071.22094eh
ISSN 2675-2824

Specific alkaline phosphatase activity as an indicator of phosphorus status in the plankton community of a highly stratified oligotrophic Ombla Estuary

Enis Hrustić[1,2*], Stijepo Ljubimir[1], Svjetlana Bobanović-Ćolić[1], Ingrid Ivančić[2]

[1] University of Dubrovnik - Institute for Marine and Coastal Research (Kneza Damjana Jude 12, 20000 Dubrovnik - PO box 83 - Croatia)
[2] Ruđer Bošković Institute - Center for Marine Research (Giordano Paliaga 5, 52210 Rovinj - Croatia)

* Corresponding author: enis.hrustic@unidu.hr

ABSTRACT

We evaluated the seasonal phosphorus (P) status of the plankton community in the Ombla Estuary (OE) by using its specific alkaline phosphatase activity (sAPA). Microphytoplankton (MICRO) indicated a substantially higher P deficiency than nanophytoplankton (NANO) and picoplankton (PICO). We found that the prolonged increase in the temperature of the surface estuarine water supported a notable growth of the dinoflagellate *Prorocentrum* spp. in late spring-early summer (June). In the summer (August), we found the maximum microphytoplankton sAPA (MICRO sAPA) (307.8 nmol µg C^{-1} h^{-1}) in the surface water, in which (84%) dinoflagellates predominated within MICRO with the maximum alkaline phosphatase activity (APA) in all size fractions, including free enzymes. Persistently low discharge of Ombla during summer-early autumn caused a transition from phosphorus- to potentially nitrogen-limited MICRO in the surface water in early autumn (October). Nutrient stress disappeared in winter, in which a significant amount of dissolved orthosilicate, dissolved inorganic nitrogen (DIN), and soluble reactive phosphorus (SRP) enriched the estuary via maximal river discharge and inflow of nutrient-rich coastal waters. MICRO (coccolithophorids and diatoms) had very low APA (surface water) and quantitatively undetectable APA (bottom water) in the nutrient-rich water column in January. This study shows a more significant impact of nutrient concentrations on MICRO than other size classes of the plankton community. Because of the similarity in seasonal hydrological features, we assume that the general pattern of switching from P- to N-limitation of phytoplankton growth also occurs in other highly stratified estuaries along the coastal karst of the eastern Adriatic Sea during the lowest river discharges and groundwater activities in summer-early autumn before the rainy season. This study indicates that a common highly stratified estuary on the eastern Adriatic coast can serve as a natural laboratory to explore connections between nutrient limitations and phytoplankton successions.

Keywords: Alkaline phosphatase, Nutrients, Estuaries, Phytoplankton, Adriatic Sea

INTRODUCTION

Orthophosphate (PO_4^{3-}) is a preferable source of phosphorus (P) for phytoplankton (Orret and Karl, 1987). Because of the relatively high ratio between dissolved inorganic nitrogen (DIN) and soluble reactive P (SRP, the usual measure of

Submitted: 13-Jul-2022
Approved: 13-Jul-2023

Editor: Rubens Lopes

PO_4^{3-}), including decreased P bioavailability in coastal freshwater discharges due to the increased sorption affinity of PO_4^{3-} toward the particles of Al- and Fe-oxyhydroxides (Jonge and Villerius, 1989; Spiteri et al., 2008), phytoplankton growth in estuaries is frequently P-limited. The bioavailability of P in estuaries is a complex topic involving the phosphate buffer mechanism (Froelich, 1988; Jonge and Villerius, 1989). Phytoplankton exposed to P deficiency activates the inductive synthesis of extracellular enzyme alkaline phosphatase (AP), involved in the break-up of the ester bond in phosphomonoesters (PME) (Torriani-Gorini, 1994). SRP concentrations constitute the main regulating factor for the expression of microbial alkaline phosphatase activity (APA) in aquatic systems. Phosphorus deficiency responses actually serve to maintain the levels of polyphosphates that are crucial to support cellular survival. When P stress is reached, degradation of polyphosphates dominates the P uptake and we find species-specific thresholds of polyphosphates that activate P deficiency responses (Li and Dittrich, 2019). Generally, heterotrophic bacteria (HBa) not only use APA to uptake PO_4^{3-} (Hoppe, 2003), but also to incorporate organic moieties after the break-up of PME, whereas phytoplankton APA has a more straightforward relation to a low availability of P (Li and Dittrich, 2019). Because of the possible positive linear correlation between APA and phytoplankton biomass in a range of relatively higher orthophosphate concentrations (Nausch, 1998), APA per biomass (Nedoma et al., 2006; Hrustić et al., 2011) or per particulate P (Tanaka et al., 2006), in both cases designated as specific APA (sAPA), better indicates P deficiency than only APA measurements as it mirrors the exponential production of AP when phytoplankton experiences P-limitation. A great body of research about the role of alkaline phosphatase activity in the aquatic environments has been recently summarized by Su et al. (2023).

Many coastal waters are N-limited but the Adriatic Sea is prevailingly oligotrophic and P-limited (Zavatarelli et al., 1998) and under a significant influence of the P-limited Eastern Mediterranean (Krom et al., 1991; 2005; Civitarese et al., 2010). Documented SRP concentrations in the Ombla Estuary (OE) in the South Adriatic Sea are below 0.2 µmol L^{-1} (Carić et al., 2012). According to Nausch (1998), this feature should help to elucidate clear differences in sAPA associated with the P status of the microbial community within a fine gradient of low SRP concentrations in the OE. This study shows our results on APA and sAPA as they relate to seasonal changes in P status in picoplankton (PICO), nanophytoplankton (NANO), and microphytoplankton (MICRO) of a highly stratified oligotrophic OE, a typical estuary on the eastern Adriatic coast. We discuss these issues by observing low microbial activity during high river discharge and great impact of coastal waters in winter, microbial bloom in late spring, relatively low microbial biomass with the highest APA and sAPA at low river discharge in summer, and a potential shift from P- toward N-limited growth of MICRO at the lowest river discharge in early autumn.

Study area and sampling strategy

The study was conducted in the middle OE (Figure 1) at "Ombla-2" station (depth of 17 m) with six bimonthly samplings (Table 1). February '11 and January '12, April '11 and June '11, August '11, October '11 refer to winter, spring, summer, autumn, respectively. We show our results for two characteristic OE layers, the surface water (0-1 m) exposed to some riverine influence and under the great impact of coastal surface water inflow and the more oligotrophic near-bottom marine water (15 m). River discharge considerably impacts the retention time of the water above the halocline and in the near-bottom layer of the OE (Carić et al., 2012). Surface water retention time fluctuates between two hours and two days at the highest and the lowest river discharge, respectively, whereas the retention time of the near-bottom water fluctuates between eight hours and eight days at the highest and the lowest river discharge, respectively (Carić et al., 2012). Rather than determined, the catchment area boundaries have been estimated from water budget analysis to be around 800–900 km^2 (Bonacci, 2001). The underground river Ombla flows only about 30 m above ground level before it connects with the estuarine water under a great influence of the coastal sea. The discharge rate of Ombla averages 26 m^3 s^{-1}

and varies from 2.3 to 112 m^3 s^{-1}. Sometimes, an abnormally high rise of the groundwater level can decrease outflow of the Ombla Spring by forcing overflow from the main spring catchment into catchments of surrounding springs (Bonacci, 2001). The tidal range in the OE is up to 30 cm. The upper reach of the estuary is about six m deep, whereas its lower reach, up to 25 m deep. Land use around OE, which is 4 km long, shows low intensity. The upper reach of the estuary has a yachting marina and a moderate population. This area receives no great impact of industrial or agricultural activities. Settlements around the estuary have a connection to the main sewage system of Dubrovnik, releasing wastewater 1.5 km off the Mala Petka Hill at the depth of 110 m toward the open sea and only occasionally some wastewater is discharged directly into the estuary. A detailed description of the OE and its adjacent coastal sea is available in Viličić et al. (1995) and Carić et al. (2012).

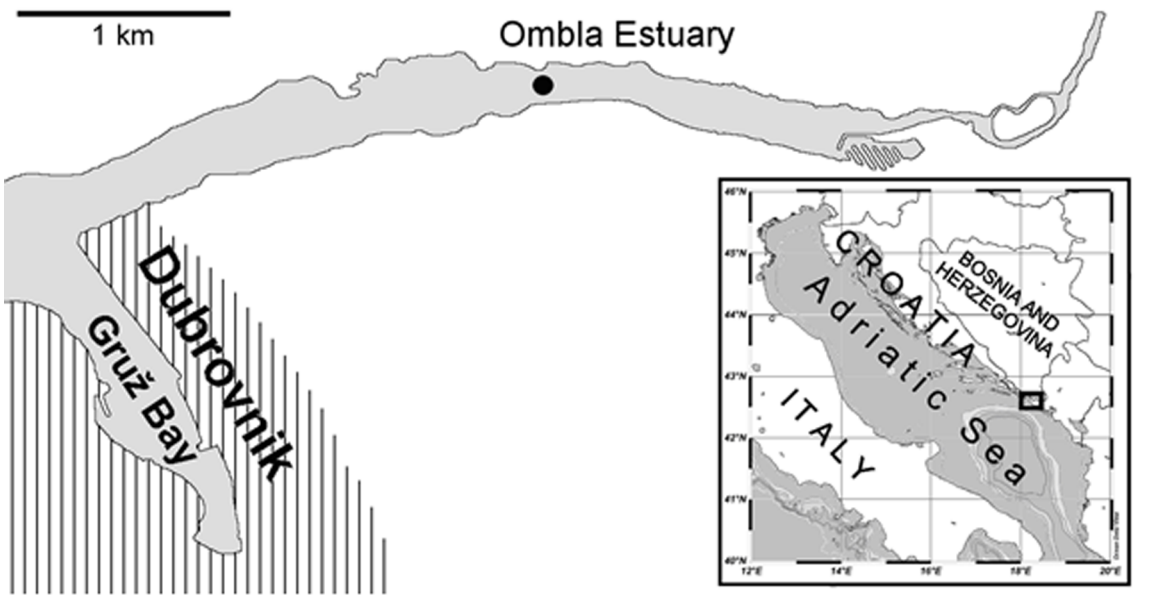

Figure 1. Geographical position of the sampling station in the middle part of the Ombla Estuary.

Table 1. Additional data for the sampling days

Date (D/M/Y)	15/2/'11	13/4/'11	17/6/'11	2/8/'11	4/10/'11	13/1/'12
(Season)	(winter)	(spring)	(spring)	(summer)	(autumn)	(winter)
Averaged daily discharge of the Ombla (m^3 s^{-1})	13.7	13.7	9.78	7.59	5.94	17.4
Daily duration of sun irradiance >250 W m^{-2} (h)	3.4	2.7	10	12.4	10.2	0.1
Surface water t (°C)	14.28	16.13	20.91	22.83	22.98	15.1

Methods

Thermohaline properties, photic zone depth, water sampling, and hydrological and meteorological data

The salinity (S), temperature (t), and density (σ) of the water column were determined *in situ* by a CTD probe SBE 19plus (Sea-Bird Electronics Inc.) with a depth resolution of 1 m. Readings of the white Secchi disc (30 cm in diameter) were multiplied by three (Berman et al., 1985) to determine the compensation depth, i.e. the depth of the photic zone. Samples for analyses of dissolved oxygen (O_2 i.e. DO), nutrients, chlorophyll-*a* (Chl-*a*), APA, and microbial community were collected by Niskin samplers (5 L) from the surface (0–1 m) and near-bottom layers (15 m). Data on daily duration

of sun irradiance >250 W m^{-2} in Dubrovnik and the average daily discharge of the Ombla were provided by the Croatian Meteorological and Hydrological Service (meteo.hr) (Table 1).

Dissolved oxygen, nutrients, and TRIX

Dissolved oxygen was precipitated in Winkler bottles and determined by iodometric titration (Grasshoff et al., 1983). The samples (50 mL) for ammonium+ammonia analysis (simplified NH_4^+) were amended with 2 mL of a phenol solution (1 mol L^{-1} in 95% ethanol), kept in dark at +4°C, and analyzed according to Ivančić and Degobbis (1984) within 72 h. Samples for other nutrients were stored in 500 mL polyethylene bottles at –22°C and analyzed within a month. Samples for nutrient analyses remained unfiltered. Nutrient analyses were performed manually by PerkinElmer λ15 (10 cm path length cuvettes). Concentrations of nitrate (NO_3^-), nitrite (NO_2^-), orthosilicate (SiO_4^{4-}), SRP, and total P (TP) were determined following Strickland and Parsons (1972). Measurements of PO_4^{3-} concentrations in natural aquatic environments have the background of an acid-labile portion of dissolved organic P (DOP) (Murphy and Riley, 1962; Tanaka et al., 2006). Therefore, those measurements represent SRP rather than only PO_4^{3-}. Total P was measured as was SRP but after the acid-persulfate digestion of the samples in Teflon bottles in an autoclave (127°C, 25 min) (Koroleff, 1983). Difference between TP and SRP includes dissolved organic P (DOP) and insoluble inorganic and organic P (Suzumura, 2008). Therefore, this difference was designated as the concentration of "other P," i.e. OP. The detection limits and reproducibility for nutrients were as follows: nitrate 0.05 and 0.025 µmol l^{-1}; nitrite 0.01 and 0.01 µmol l^{-1}; (ammonia+ammonium) 0.1 and 0.098 µmol l^{-1}; silicate 0.1 and 0.06 µmol l^{-1}; SRP 0.03 and 0.03 µmol l^{-1} (Najdek et al., 2014). DIN (NO_3^-+NO_2^-+NH_4^+) (µg L^{-1}), TP (µg L^{-1}), Chl-*a* (µg L^{-1}), and an absolute value of declination (%) from the theoretically maximal saturation of DO (O_2') at certain S, t, and depth (Weiss, 1970) configure the constituents for calculating the trophic index TRIX. This index was first developed for the Italian northwestern Adriatic coastal water, and ranges from 0 to 10, in which 0-4, 4-5, 5-6, and 6-10 indicate a high, good, moderate and poor quality of the water, respectively (Vollenweider et al., 1998).

$$TRIX=[Log_{10}(TP *DIN *Chl\text{-}a * | \text{saturation of } O_2(\%)\text{-}O_2'(\%) |)+1.5]/1.2.$$

Size, abundance, and taxonomy of microphytoplankton and nanophytoplankton

From the samples (100 mL) preserved in neutralized solution of formaldehyde (~4% vol/vol), the taxonomic composition, size and abundance of MICRO (>20 µm) and NANO (2-20 µm) (Sieburth et al., 1978) were detected by Olympus IX 71 microscope following the method by Utermöhl (1958).

Chlorophyll-*a* and biomass of phytoplankton

The filtration procedure for Chl-*a* (500 mL per sample) in NANO and PICO was identical to procedure for AP collection (only volume of the samples differed) in the same size fractions (see "APA sampling procedure"). PICO Chl-*a* result was not used to calculate biomass of PICO but only to complete the budget of Total Chl-*a* i.e. to calculate the result for the Chl-*a* of MICRO. Total Chl-*a* was determined after the filtration (500 mL per sample) by using GF/F Whatman filters (pore size 0.7 µm). All filters were stored in the dark at -22°C, and Chl-*a* detected according to Holm-Hansen et al. (1965) by using Turner TD 700 fluorometer. Biomass (B) of NANO (B_{nano}) was calculated using the factor of conversion (FC) 50 µg C:1 µg Chl-*a* (Antia et al., 1963). Biomass of MICRO (B_{micro}) was calculated indirectly via the same FC, after Chl-*a* in NANO and PICO were subtracted from the Total Chl-*a.*

Size, abundance and biomass of picoplankton

The samples (50 mL) for PICO were preserved in a neutralized formaldehyde solution (~4% vol/vol) and stored in the dark at +4°C until analyses. Abundance and size of the identified PICO groups were determined using a Zeiss Jenalumar epifluorescent microscope (magnification 1500 X). Abundance of

HBa was detected according to Hobbie et al. (1977). Heterotrophic picoflagellates (HPF), autotrophic picoflagellates (APF), and picocyanobacteria (Cyano) were determined using proflavine (Haas, 1982). The HBa biomass was calculated by FC 20 fg C $cell^{-1}$ (Lee and Fuhrman, 1987). The Cyano biomass was estimated by FC 250 fg C $cell^{-1}$ (Kana and Glibert, 1987). The volumes (μm^3) of HPF and APF cells were calculated by equations according to morphological classes: ellipsoid ($V=\pi Lw^2/6$) and sphere ($V=4\pi r^3/3$), in which L is the length (µm), w is the width (µm), and r is the radius of a cell (µm). The HPF biomass was calculated via B=NVFC, in which N is the abundance, V is the average cell volume (μm^3), and FC equals 0.22 pg C μm^{-3} (Børsheim and Bratbak, 1987). For the APF biomass, the equation $0.433(\mu m^3)^{0.863}$=pg C $cell^{-1}$ (Verity et al., 1992) was used as FC by considering average cell volumes. The sum of all the biomass of PICO groups is designated as B_{pico}.

APA sampling procedure

Hand vacuum pumps were employed at a ≤13 kPa pressure in all filtrations to determine APA (and Chl-*a*) in NANO and PICO. In total, 200 mL per sample were filtered over "Nitex" 20 µm mesh nets. The filtrates (200 mL) were filtered on "track-etched" 2 µm pore polycarbonate filters (Whatman). The filtrates under 2 µm (200 mL) were filtered at the same 0.2 µm pore quality filters. Filters for APA (and Chl-*a*) analyses in PICO and NANO were stored at −22°C (Zohary and Robarts, 1998; Rengefors et al., 2003; Hrustić et al., 2013). All APA samples (filters with biological material and seawater samples) were stored in sterile tubes. Total (original sample without filtration; APA_{tot}) and Free APA (filtrates below 0.2 µm) were measured in triplicates from 3 mL aliquots. Nanophytoplankton APA (NANO APA) and picoplankton APA (PICO APA) were detected from the material collected on filters with 2 and 0.2 µm pores by single measurements, respectively. All measurements took place within 96 h from sampling. Particulate APA, i.e. APA in >0.2 µm fractions was calculated as a difference between Total and Free APA. Microphytoplankton APA (MICRO APA), i.e. APA in fractions >20 µm=Total APA-NANO APA-PICO APA-Free APA. Picoplankton sAPA (Pico sAPA), nanophytoplankton sAPA (Nano sAPA), and microphytoplankton sAPA (Micro sAPA) denote APA in a certain size fraction divided by the associated B_{pico}, B_{nano}, B_{micro}, respectively.

APA measurements

Samples were kept at room temperature in the dark for ~0.5-1 h. The components were added in sterilized tubes (10 mL) and the reaction was started by introducing *p*-nitrophenyl phosphate (*p*-NPP) to a final concentration (S_0) of 1812 µmol L^{-1} (Hrustić et al., 2013). S_0 was much higher than expected K_m of AP for a *p*-NPP at the reaction conditions at 37°C (Orhanović and Vrančić, 2000). The method was adapted to follow the guidelines of less than 7% of S_0 (at a saturation level for V_{max} measurements) being degraded to prevent competitive inhibition by PO_4^{3-} (Orhanović and Vrančić, 2000). Samples were incubated under a dim light at 37°C with an occasional stir in a water bath. The reaction was stopped by abrupt cooling below 10°C within a few minutes after the tubes were transferred to a -22°C environment. All APA reactions were stopped after 8 hours. As much as possible, equal conditions for all incubations were ensured by adding 3 mL of autoclaved seawater from the associated Niskin samplers in tubes with filters to introduce divalent cations as activators of AP and avoid introducing any active biological material. Every tube (reaction volume of 5 mL) had 3 mL of seawater (biologically active estuarine water or sterilized oligotrophic sea), 1.9 mL of buffer solution (Tris-HCl 0.1 M, pH 8.25), and 0.1 mL of the substrate solution. Calibration plots were performed by measuring 10 concentrations (20–200 µmol L^{-1}) of *p*-nitrophenol (*p*-NP) in triplicates using VIS-spectroscopy (λ=400 nm, PerkinElmer λ15, 1 cm path length cuvettes). APA is the difference in concentration of *p*-NP between the end and start of the reaction, corrected for blank measurements of autoclaved sea with buffer and S_0 (p-NPP), divided by time. The factors of 0.025 (5 mL reaction volume/200 mL sample) for PICO APA and NANO APA and 1.67 (5 mL reaction volume/3 mL sample) for Total APA and Free APA were considered in the final APA calculations.

Statistics

"Statistica for Windows" (Statsoft Inc., USA) was used to calculate Spearman's Rank-Order

correlation coefficients (r) at a (p)<0.05 probability. As an unequal number of samples in different seasons was collected, t tests were performed to evaluate the significant differences between the layers without a seasonal aspect.

Results

Thermohaline properties of the water column

The significant shift in the t of the water column occurred from April (mean t 15.52°C) to June (mean t 18.77°C) (Figure 2A). The subsurface t_{max} (23.15°C) developed at a 2-m depth along the sharp halocline ($\Delta S_{1-2\ m}$ -2.78 m^{-1}, $S_{1-2\ m}$ 34.40–37.18 m^{-1}) in August (Figure 2B).

The subsurface t_{max} (24.35°C) developed at 3-m depth in the warmest water column in October (mean t 24.11°C) accompanied by a significant but less intense halocline ($\Delta S_{1-3\ m}$ -0.82 m^{-1}, $S_{1-3\ m}$ 36.83–38.47) than in August (Figures 2A and 2B). Temperatures failed to significantly differ between the surface and bottom water, whereas S and σ were significantly (p<0.05) higher in the bottom water (Table 2).

Table 2. Averages±sd, minima and maxima of the thermohaline properties, nutrient concentrations and index of eutrophication in the middle part of the Ombla Estuary for the studied period

Surface water	S	T (°C)	σ	DO (mg L^{-1})	NO_3 (µM)	NO_2 (µM)	NH_4 (µM)	DIN (µM)	SRP (µM)	OP (µM)	TP (µM)	DIN/ SRP	SiO_4 (µM)	SiO_4/ DIN	Total Chl-*a* (µg L^{-1})	TRIX
Average	36.36	18.71	24.98	8.61	6.16	0.05	0.48	6.70	0.09	0.12	0.21	69.16	9.15	2.16	0.67	3.46
sd	1.08	3.98	2.77	0.71	7.72	0.02	0.43	8.13	0.04	0.07	0.07	67.56	5.40	0.84	0.55	0.91
Min	34.40	14.28	21.22	7.48	0.92	0.03	0.23	1.39	0.05	0.07	0.14	19.61	3.32	0.76	0.26	2.08
Max	37.62	22.98	28.30	9.35	20.93	0.08	1.35	22.36	0.14	0.24	0.28	191.71	17.06	3.04	1.77	4.65
Bottom water	S	T (°C)	σt	DO (mg L^{-1})	NO_3 (µM)	NO_2 (µM)	NH_4 (µM)	DIN (µM)	SRP (µM)	OP (µM)	TP (µM)	DIN/ SRP	SiO_4 (µM)	SiO_4/ DIN	Total Chl-*a* (µg L^{-1})	TRIX
Average	38.48	17.35	28.06	8.04	0.71	0.07	0.60	1.38	0.04	0.13	0.17	32.00	2.02	2.86	0.40	3.03
sd	0.13	3.55	0.89	0.54	0.72	0.08	0.78	1.01	0.00	0.06	0.06	24.13	0.41	2.64	0.06	0.58
Min	38.31	14.71	26.33	7.32	0.05	0.02	0.22	0.28	0.04	0.06	0.10	6.20	1.44	0.70	0.33	2.01
Max	38.58	24.08	28.76	8.77	1.80	0.22	2.18	2.75	0.05	0.23	0.27	67.92	2.59	7.16	0.46	3.50
t test	0.002		0.021						0.013				0.012			

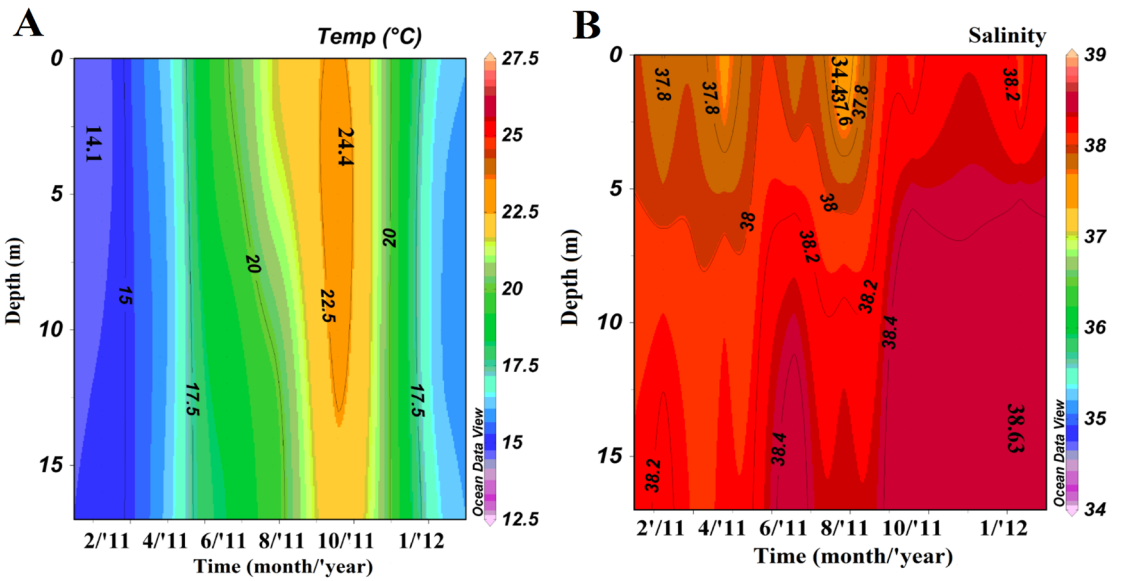

Figure 2. Temperature (A) and Salinity (B) in the the middle part of the Ombla Estuary in the studied period.

River discharge, nutrients, and TRIX

SRP concentration (0.05-0.14 µmol L^{-1}) showed no clear seasonal trend and we found the highest value in the surface water in June (Figure 3) under a significantly decreased Ombla discharge (Figure 4, Table 1). On the other hand, DIN and SiO_4^{4-} concentrations (0.28-22.35 µmol L^{-1} and 1.44-17.06 µmol L^{-1}, respectively) had marked seasonal trends with summer values of about an order of magnitude lower than winter ones (Figure 3). Values for all nutrients were higher in the surface than in bottom water (Figure 3, Table 2). Nitrate prevailed (70-93% contribution to DIN; data not shown) in surface water DIN, whereas NH_4^+ significantly contributed to DIN in summer and autumn (up to 30%). NH_4^+ was the most important inorganic N species in bottom water during summer and autumn (45 and 79%, respectively).

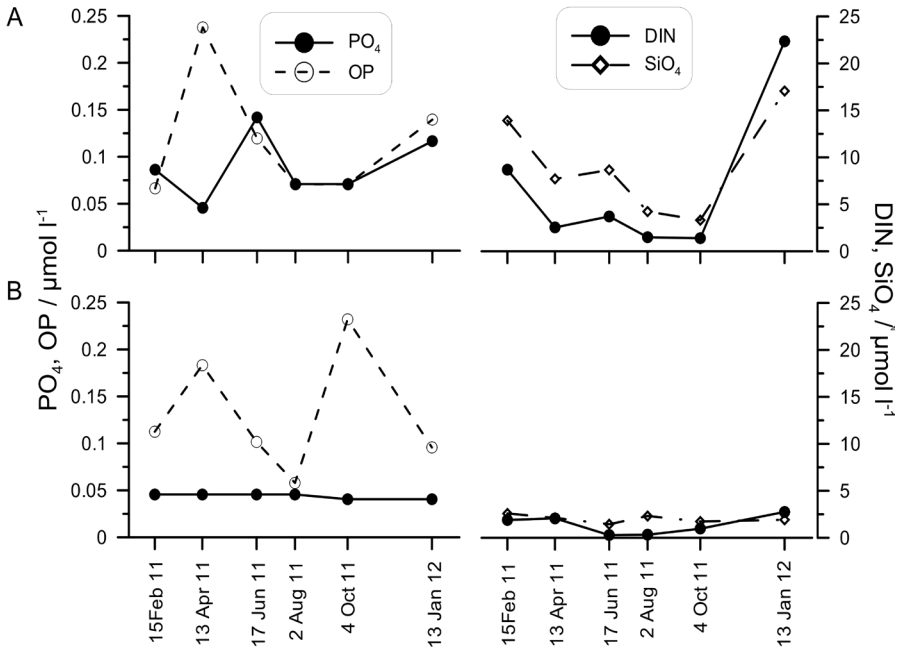

Figure 3. Concentration of nutrients in the surface (A) and bottom water (B) in the middle part of the Ombla Estuary in the studied period.

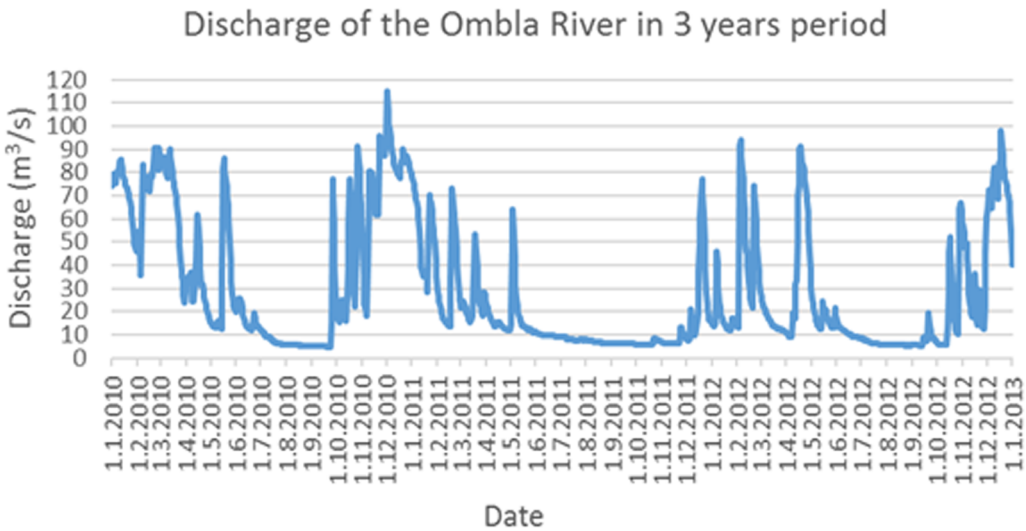

Figure 4. Average daily discharge of the Ombla River before, during and after the studied period.

Correlations of nutrients ($p<0.05$) with S (SRP, r=-0.67; NO_3^-, r=-0.69; SiO_4^{4-}, r=-0.75) indicated that the Ombla constituted a significant source of nutrients to OE. In surface waters, OP concentrations (0.07-0.14 µmol L^{-1}) generally resembled SRP ones, except for about their five times higher OP value (0.24 µmol L^{-1}) in April (Figure 3). On the other hand, bottom water OP concentrations (~0.1-0.25 µmol L^{-1}) were significantly ($p<0.05$; t test not shown) higher than those of SRP (Figure 3, Table 2). We only found a TRIX value (4.65) above four in the surface water in January (Figure 3), being an exceptional case of mesotrophic conditions in dominantly oligotrophic OE (Table 2).

Depth of the photic zone and the biomass of phytoplankton and picoplankton

Secchi disc readings (6–7 m) suggested that the whole water column belonged to the photic zone (Table 1). Microbial biomass (C_{tot}) varied from a minimum of 17.4 µg C L^{-1} in the bottom water in January to a maximum of 129.7 µg C L^{-1} in the surface water in June, when B_{micro} and B_{pico} equally dominated (43%) B_{nano} (14%) (Figure 5). B_{pico} was generally the dominant part of C_{tot}, contributing with 54±15% and 55±13% to the C_{tot} of the surface and bottom waters, respectively. We found its minimal contributions (36%) for both layers in January and its maximal contributions to surface water (80%) and bottom waters (71%) in August and April, respectively (Figure 5). B_{pico} consisted dominantly of picophytoplankton (Cyano+APF) (46-80% in the surface water and 49-73% in the bottom water, Table 3). HBa showed a maximum 9.28 µg C L^{-1} in the surface water in June along with the maximum of the Total Chl-*a* (1.77 µg L^{-1}), SRP (0.14 µmol L^{-1}), B_{micro} (56.31 µg C L^{-1}), B_{nano} (18.09 µg C L^{-1}), and B_{pico} (55.27 µg L^{-1}) and the maximal share of picophytoplankton within B_{pico} (80%) (Figure 3 and Figure 5). B_{pico} was positively correlated with SRP (r=0.66, $p<0.05$). HBa had a significantly higher biomass in the surface than in the bottom water (Table 3).

B_{micro} was the second largest contributor to C_{tot} (27±13%) in the nutrient richer surface water, whereas B_{nano} was the second largest contributor to C_{tot} (27±9%) in more oligotrophic bottom waters (Figure 5). The highest contribution of B_{micro} in C_{tot} (56%) appeared in the cold and nutrient-rich surface water (the highest TRIX) in January (Figure 3). Minimal B_{micro}, below the limit of detection, occurred in both layers in April and in the surface water in October. B_{nano} had 8% minimal contribution to C_{tot} in the surface water in January and maximum of 46% in the surface water in April.

Table 3. Averages±sd, minima and maxima of the APA, biomass and sAPA of plankton size fractions with significant differences (t test) between the surface and the near-bottom water in the middle part of the Ombla Estuary

Surface water	T APA (nM h^{-1})	M APA (nM h^{-1})	N APA (nM h^{-1})	P APA (nM h^{-1})	Free APA (nM h^{-1})	T bio-mass (µg C L^{-1})	C MICRO (µg C L^{-1})	C NANO (µg C L^{-1})	C PICO (µg C L^{-1})	C HBa (µg C L^{-1})	C Cyano (µg C L^{-1})	C APF (µg C L^{-1})	C HPF (µg C L^{-1})	M sAPA (nM/ µg h)	N sAPA (nM/ µg h)	P sAPA (nM/ µg h)
Average	2148.91	830.52	106.95	80.89	1130.55	47.52	16.86	7.44	23.22	6.01	10.27	6.04	0.90	86.15	16.20	3.71
Sd	3728.05	1537.23	99.77	72.45	2085.68	41.27	20.84	5.72	16.35	2.00	11.22	4.85	0.61	148.02	15.61	3.54
Min	144.71	0.00	13.62	11.37	99.69	20.06	0.00	2.82	10.90	4.08	3.56	0.42	0.24	0.56	3.06	0.51
Max	9707.41	3907.20	256.92	167.35	5375.93	129.67	56.31	18.10	55.27	9.28	30.89	13.11	1.99	307.77	37.70	8.65
Bottom water	**T APA (nM h^{-1})**	**M APA (nM h^{-1})**	**N APA (nM h^{-1})**	**P APA (nM h^{-1})**	**Free APA (nM h^{-1})**	**T bio-mass (µg C L^{-1})**	**C MICRO (µg C L^{-1})**	**C NANO (µg C L^{-1})**	**C PICO (µg C L^{-1})**	**C HBa (µg C L^{-1})**	**C Cyano (µg C L^{-1})**	**C APF (µg C L^{-1})**	**C HPF (µg C L^{-1})**	**M sAPA (nM/ µg h)**	**N sAPA (nM/ µg h)**	**P sAPA (nM/ µg h)**
Average	1936.97	745.25	99.06	66.00	1026.66	26.35	4.57	6.82	14.96	4.17	5.35	4.12	1.33	144.18	18.47	4.74
Sd	2067.85	806.80	62.38	48.50	1196.37	5.34	2.61	2.03	5.23	0.65	3.90	2.66	1.00	110.10	17.90	3.34
Min	262.04	0.00	31.25	14.11	119.89	17.39	0.00	3.76	6.31	3.12	0.95	0.00	0.10	0.00	3.09	0.74
Max	5387.71	1990.81	196.79	120.47	3079.65	32.60	7.03	10.11	20.27	4.94	11.88	7.20	2.88	293.20	52.34	9.92
t test										0.037						

APA unit is nmol P l^{-1} h^{-1}, sAPA unit is nmol P µg C^{-1} h^{-1}

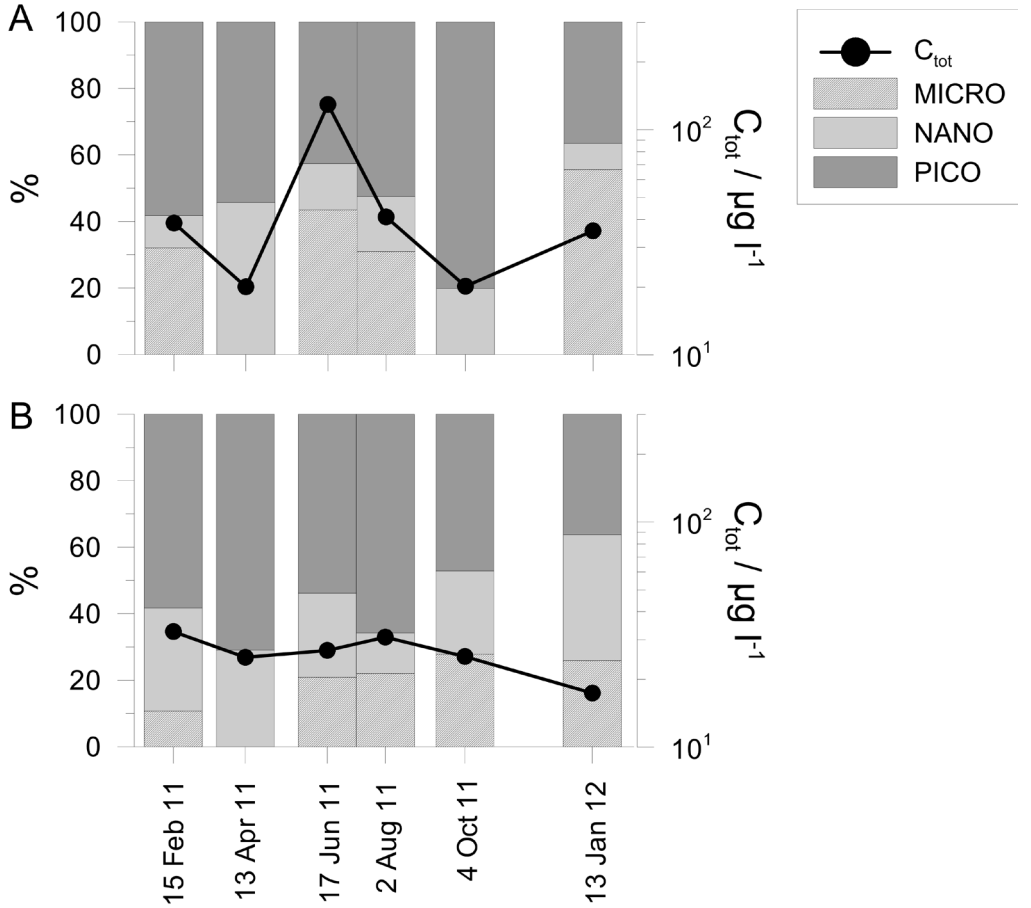

Figure 5. Biomass of MICRO, NANO and PICO in the surface (A) and the bottom water (B) in the middle part of the Ombla Estuary in the studied period

APA, sAPA, and phytoplankton

Total APA showed a seasonal trend with higher values during summer and autumn (Figure 6). We found the highest total APA in August (9708 and 5387 nmol L^{-1} h^{-1} in the surface and bottom waters, respectively; Figure 6). In particulate fraction, MICRO APA generally predominated over NANO and PICO APA (Figure 6).

We detected the highest shares of MICRO APA in total APA (58% and 61%) in the surface and bottom waters in June, followed by 46% and 45% in the surface and bottom waters in February, respectively (Figure 6). We found no MICRO APA in the bottom water in January and April and in the surface water in October (Figure 6). We only observed a significant contribution of NANO APA to total APA in bottom waters in February (44%), which remained below 10% (Figure 6). The contribution of PICO APA totaled 10%, with a somewhat higher contribution (up to 17%) in the surface water in October and January (Figure 6). Contributions of Free APA were significant (46-86%) and usually higher than those of particulate APA, with the highest values (80 and 86% in the surface and bottom waters, respectively) in April (Figure 6). The only exception we found occurred in June when the maximal share of particulate APA in total APA (81%) coincided with that of Chl-*a*, MICRO, NANO, HBa, and SRP maxima (Figure 3, Figure 5, Figure 6) along with the second highest NO_3^- concentration (3.37 µmol L^{-1}), which contributed to 91% of the DIN (Figure 3). We found free APA_{max} (5376 nmol L^{-1} h^{-1}) in the surface water in August and the second largest value (3079 nmol L^{-1} h^{-1}) at the same time in the bottom water. We also observed its considerably high value (1898 nmol L^{-1} h^{-1}) in the bottom water in October (Figure 6).

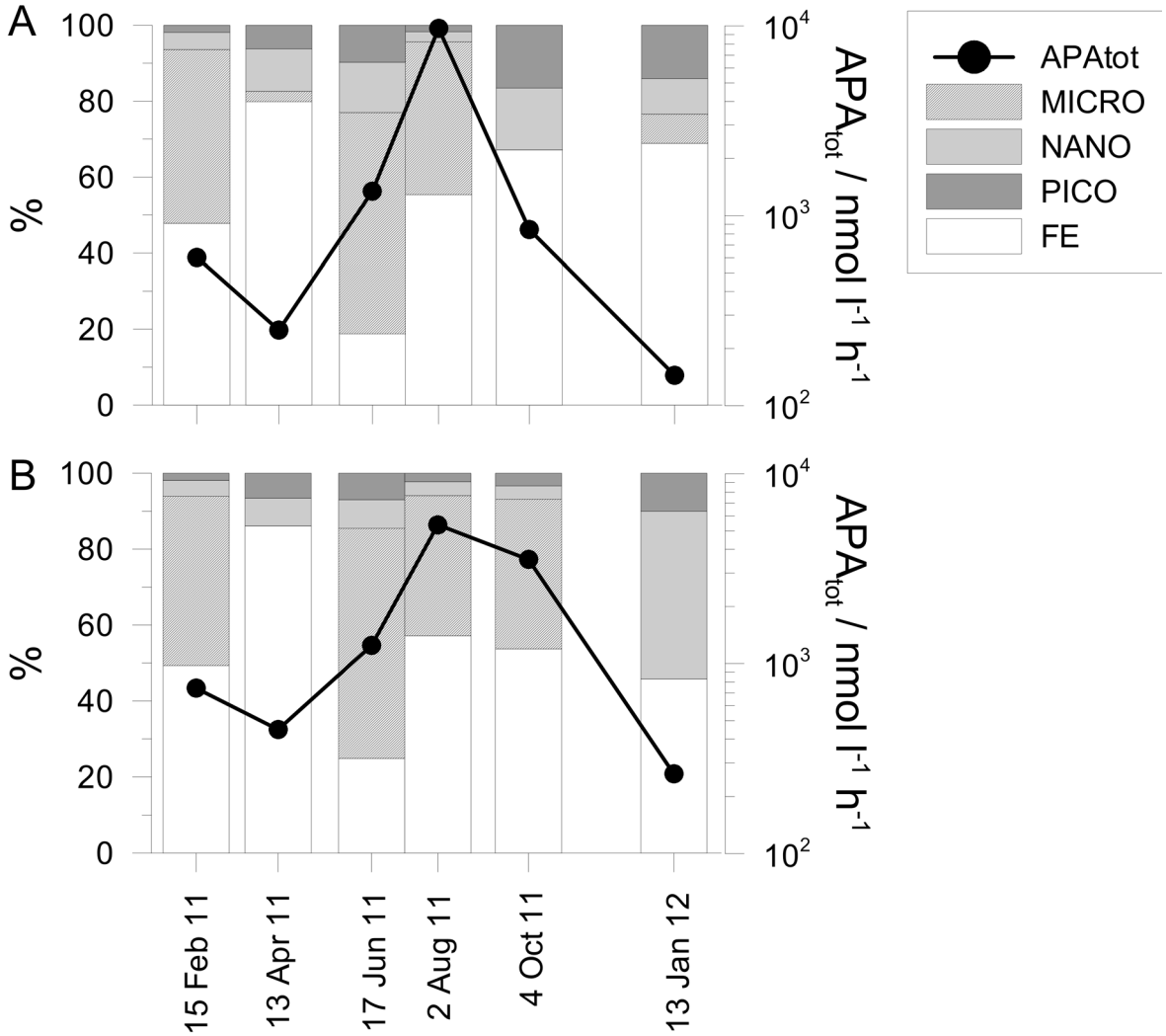

Figure 6. MICRO, NANO, PICO and FE APA with their contributions to APA_{total} in the surface (A) and the bottom water (B) of the middle part of the Ombla Estuary in the studied period.

sAPA showed a seasonal trend in all fractions (Figure 7). Higher values generally appeared in summer and autumn and lower values in winter and spring. Seasonal trends were similar in both layers, although the trends for NANO sAPA and PICO sAPA in the bottom water were smoother than those in the surface water. Values of sAPA decreased in the following order MICRO > NANO > PICO (Figure 7, Table 3). This was particularly evident during summer and autumn, in which MICRO sAPA (135-308 nmol µg C^{-1} h^{-1}) were an order of magnitude higher than NANO (9.8-37.7 nmol µg C^{-1} h^{-1}) and PICO (2.4-8.6 nmol µg C^{-1} h^{-1}) sAPA (Figure 7). In January, MICRO sAPA was very low (0.56 nmol µg C^{-1} h^{-1}) in the surface water and undetectable in the bottom water, despite the significant contributions of B_{micro} to C_{tot} (56 and 26%, respectively; Figure 5). MICRO consisted of coccolithophorids (965 cell L^{-1}) and diatoms (2617 cell L^{-1}) in the sample without MICRO APA in the bottom water in January (Figure 8). We detected no significant ($p<0.05$) controlling role of SRP on microbial APA and sAPA.

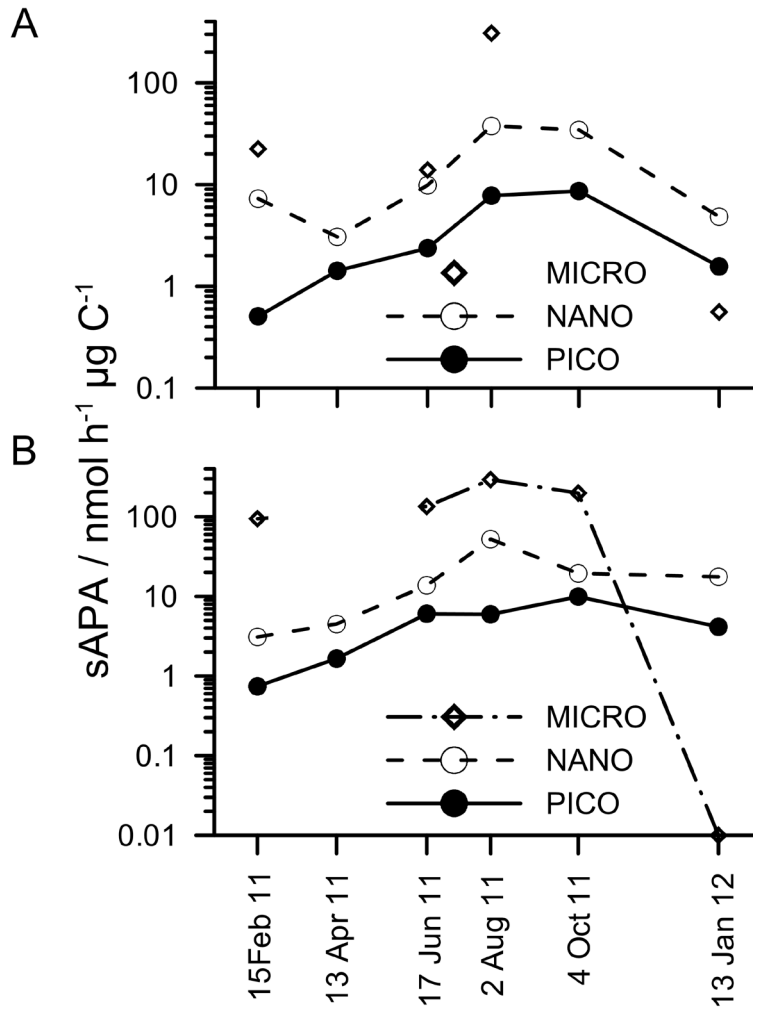

Figure 7. Specific APA of MICRO, NANO and PICO in the surface water (A) and the bottom water (B) in the middle part of the Ombla Estuary in the studied period.

We were unable to calculate MICRO sAPA for the April samples and for the surface water in October. In April, we found no B_{micro} via Chl-*a* (Figure 5), MICRO abundance was extremely low in April (Table 4), whereas MICRO APA was very low in the surface water (6.8 nmol h^{-1} L^{-1}) and undetectable in the bottom water in April (Figure 6). In the surface water in October, B_{micro} was undetectable via Chl-*a* (Figure 5) as was MICRO APA (Figure 6). Hence, we were unable to calculate MICRO sAPA despite finding 8468 MICRO cell L^{-1} in that sample (Table 4, Figure 8).

Euglenophytes (1025 cell L^{-1}), chryptophytes (568 cell L^{-1}) and dinoflagellates (120 cell L^{-1}) (Figure 8) with low APA (Figure 6) dominated MICRO in the surface water in April (Table 4). The increase in temperature of the water column, along with a sharp decline of the Ombla discharge rate from early May (Figure 2A and Figure 4), involving extended water retention time, i.e. flushing time, and consequently a greater availability of nutrients, provoked a considerable growth of *Prorocentrum* spp. ($70x10^3$ cell L^{-1}) (MICRO) and domination (>99%) of dinoflagellates (94436 cell L^{-1}) within MICRO (95024 cell L^{-1}), followed by $NANO_{max}$ ($917x10^3$ cell L^{-1}) in the surface layer in June (Figure 8). Dinoflagellates made up 84% of MICRO (Table 4) that had MICRO $sAPA_{max}$ of 308 nmol µg C^{-1} h^{-1} in the surface water in August (Figure 7). MICRO $sAPA_{max}$ for the bottom water (293 nmol µg C^{-1} h^{-1}) also appeared in August in the sample with notably more balanced MICRO

community and lower abundances of MICRO and NANO (Table 4, Figure 7, Figure 8). We found NANO $sAPA_{max}$ for the surface water (38 nmol µg C^{-1} h^{-1}) and bottom water (52 nmol µg C^{-1} h^{-1}) in August (Figure 7, Table 3). Interestingly, PICO $sAPA_{max}$ for the surface water (8.7 nmol µg C^{-1} h^{-1}) and bottom water (9.9 nmol µg C^{-1} h^{-1}) appeared in October (Figure 7, Table 3).

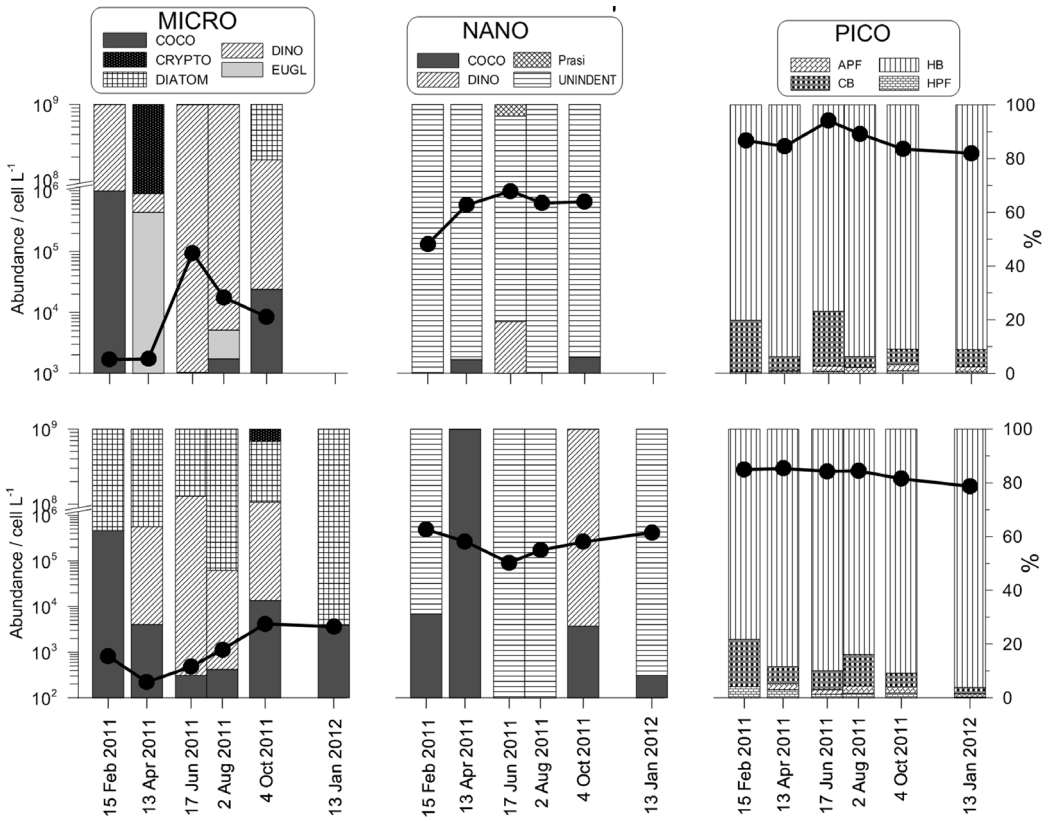

Figure 8. Abundance of microphytoplankton, nanophytoplankton and picoplankton in the surface water (A) and the bottom water (B) in the middle part of the Ombla Estuary in the studied period.

Table 4. Microphytoplankton and nanophytoplankton abundances in the surface and the bottom water in the middle part of the Ombla Estuary between February 2011 and January 2012.

Date	Depth	Tot--MICRO	COCCO-M	CRYPTO-M	DIATOM-M	DINO-M	EUGLENO-M	Tot-NANO	COCCO-N	DINO-N	Prasi-N	UNIDENT-N
15.2.2011.	0 m	1.672	1.134	0	0	0.538	0	124.163	0	0	0	124.163
13.4.2011.		1.713	0	0.568	0	0.12	1.025	538.041	27.592	0	0	510.449
17.6.2011.		95.024	0.398	0	0.2	94.426	0	916.799	0	175.761	39.417	701.621
1.8. 2011.		17.673	0.945	0	0	14.837	1.891	583.37	0	0	0	583.37
4.10.2011.		8.468	2.646	0	1.741	4.081	0	611.619	36.789	0	0	574.83
13.1.2012.		n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
15.2.2011.	15 m	0.818	0.509	0	0.309	0	0	441.469	137.959	0	0	303.51
13.4.2011.		0.22	0.06	0	0.08	0.08	0	242.808	242.808	0	0	0
17.6.2011.		0.48	0.04	0	0.12	0.32	0	82.776	0	0	0	82.776
1.8. 2011.		1.14	0.12	0	0.6	0.42	0	155.668	0	0	0	155.668
4.10.2011.		4.169	1.512	0.189	0.945	1.523	0	236.501	63.067	173.434	0	0
13.1.2012.		3.582	0.965	0	2.617	0	0	378.408	31.539	0	0	346.869

All abundances presented as 10^3 cell l^{-1}; n/a-not analyzed

DISCUSSION

Our results suggest that the Ombla River configures a significant source of nutrients for the OE. However, the Ombla Estuary experiences a substantial influence from the coastal sea throughout the year, but especially in winter-early spring after the mixing events in the South Adriatic Pit (Batistić et al., 2012; Ljubimir et al., 2017; Šilović et al., 2018). These waters have a greater flow rate along the eastern Adriatic coast in winter (Zavatarelli et al., 1998). Therefore, the structure of the plankton population of the OE changes under the influence of the nutrient and salinity contents of the two major water sources to the OE. Of course, other factors shape the plankton population structure of the OE but those factors lie outside the scope of this study. The OE is generally oligotrophic with a great transparency of its water column. The bottom water under the halocline is even more oligotrophic. However, mesotrophic conditions may prevail in winter, as in the surface water in January (TRIX 4.65), in which we found exceptionally high nutrient concentrations during the high Ombla discharge rate (Figure 3 and Figure 4). Minimal APA and sAPA in winter and early spring at a relatively higher river discharge rate and the probable great influence of the mixed and nutrient rich coastal waters inflow confirm the tendency for AP synthesis repression under the sufficient supply of P to the microbial community. The maximal APA and sAPA during summer and autumn at lower riverine nutrient supply confirms the tendency of increased AP synthesis in oligotrophic environments. Higher temperatures, extended exposure to light, and prolonged water retention in the OE in summer and autumn support phytoplankton growth, which becomes limited by the lack of nutrients, most commonly of SRP (Carić et al., 2012). We confirmed this by our APA and sAPA results. Moreover, we observed an indication of N-limitation in the surface water in October at the lowest river discharge, accompanied by the lowest DIN and total Chl-*a* concentrations (1.39 and 0.26 μg L^{-1}, respectively), and undetectable MICRO APA. Nitrogen is an essential element both for Chl-*a* and enzyme synthesis and the MICRO in the surface water was potentially more limited by N than P.

According to the contribution of each plankton size group in total APA and by their sAPA, we can conclude that MICRO was the most P-stressed group, followed by NANO and PICO. This was particularly evident during the summer, in which MICRO sAPA had an order of magnitude higher sAPA than the other size classes of the plankton community. To overcome nutrient limitation, microbes induce synthesis of extracellular enzymes (e.g. AP in the case of P-limitation) to use PME or they can adapt to use low nutrient concentrations efficiently. Larger cells require more nutrients for growth, causing higher half saturation constants of nutrients uptake (K_m) (Eppley and Thomas, 1969). This can consequently become a disadvantage in competition for nutrients uptake with smaller phytoplankton. Oh et al. (2010) reported that K_m might be an important estimator of PO_4^{3-} uptake affinity and of the threshold for APA induction. In fact, in oligotrophic conditions during the summer, the dominating plankton group (PICO), mainly composed of autotrophs, had for an order of magnitude lower sAPA compared to larger plankton group (MICRO). In short, PICO is a better competitor at low nutrient concentrations (Moutin et al., 2002), and hence the significantly lower Pico sAPA than sAPA of larger phytoplankton was most likely connected to the more efficient uptake of SRP at its lower concentrations. Higher surface to volume ratio enables picoplankton to grow with a greater success than NANO and MICRO, particularly in the bottom water (with the most severe oligotrophic conditions). A positive correlation of B_{pico} with SRP ($r=0.66$, $p<0.05$) indicates that PICO was the most successful competitor for orthophosphate in the oligotrophic waters. Therefore, PICO was probably the most active plankton fraction in the fast recycling of PO_4^{3-} in the surface water with a shorter retention time than the bottom water. We observed the lowest and undetectable MICRO APA at conditions of relatively high MICRO biomass in comparatively cold and nutrient-rich waters in January, suggesting that MICRO showed no P limitation at the time. Nutrient-rich and cold waters favor the slow growth of bigger phytoplankton species. We have ignored the concentration of polyphosphates but we estimate that, in the cases with a very low or undetectable APA in nutrient-rich and cold waters, polyphosphate levels were either stable or in the

phase of accumulation, thus failing to cause notable P deficiency responses, i.e. APA. We suspect that the major contributors to high MICRO APA during late spring and summer were dinoflagellates because of their large predominance within MICRO, particularly in the surface water. The river discharge reduction and relatively high importance of NH_4^+ (from 0.30 to 0.23 µmol L^{-1} from June to August) and the depletion of NO_3^- (from 3.37 to 1.24 µmol L^{-1} from June to August) probably supported a competitive advantage for dinoflagellates (Domingues et al., 2011) as mixotrophs over autotrophic phytoplankton in this period. Apart from the significant positive correlation with temperature in the OE (Carić et al., 2012), domination of dinoflagellates in the MICRO community during late spring-autumn could be favored by the lower DIN/SRP (~7–25, compared to 55-100 in other seasons). Heterotrophs are better adapted to lower DIN/SRP ratio than autotrophs (Goldman et al., 1987).

Unlike the rest of phytoplankton, dinoflagellates might have a significant share of constitutive AP (Dyhrman and Palenik, 1999; Rengefors et al., 2003). Large copepods, major phytoplankton grazers (Calbet et al., 2000) whose abundance rises in spring–summer in the OE (Carić et al., 2012) as they follow prey, i.e. phytoplankton (Viličić et al., 1995), possibly had a notable share in MICRO APA and an impact on Free APA generation in spring–summer, similar to findings for the NW Mediterranean in summer (Bogé et al., 2002). Some attached bacteria on the zooplankton larvae can also produce APA (Jean et al., 2003). Note that MICRO sAPA was the most prone to error introduction since some MICRO constituents have less Chl-*a* per biomass than the used FC (e.g. dinoflagellates) (overestimated sAPA). Although we have shown two important sources of MICRO sAPA overestimation, those should fail to significantly influence the general conclusion of decreasing ranking from MICRO to NANO to PICO regarding P stress in the oligotrophic OE. Some PICO members have a constitutive APA expression (primarily HBa) but its APA should be approximately linear in comparison to its biomass. Therefore, in the case of some degree of underestimation of PICO sAPA, this error should fail to change the main results of our study. Carbon to Chl-*a* ratios should not be enormously different between MICRO and NANO. Hence, the most sensitive point in our biomass estimations between MICRO and NANO, on one hand, and PICO, on the other hand, lies in the fact that we used FC via Chl-*a* concentration for MICRO and NANO and cell abundances and their volumes for the FC of PICO members. Moreover, photoacclimatization of the photosynthetic apparatus (less Chl-*a* per phytoplankton cell in the surface than in the bottom water) (Geider et al., 1997) provoked some overestimation and underestimation of the sAPA values in the surface and the bottom water, respectively.

We found no significant ($p<0.05$) controlling role of SRP on microbial APA and sAPA, an unexpected result falsifying the hypothesis based on the repeatedly found relation between APA and orthophosphate concentrations (Boekel and Veldhuis, 1990; Li et al., 1998; Li and Dittrich, 2019). This is probably due to our small number of samples (n=12) from the estuary (a complex natural environment) but even more so by the shortcomings of the used sampling procedure for nutrient analysis without filtration. Furthermore, N-limitation of the phytoplankton growth could also have occurred in the bottom water during summer and autumn according to DIN concentrations (especially <0.6 µmol L^{-1}), representing an upper limit of K_m range for NO_3^- and NH_4^+ uptake by natural phytoplankton communities in oligotrophic conditions (MacIsaac and Dugdale, 1969). Hence, we consider that the phytoplankton community in the middle part of the OE could experience growth limitation due to low concentrations of DIN and DIP in the most oligotrophic conditions during summer-autumn.

High contribution of Free APA in APA (46-86%) agrees with Duhamel et al. (2010), who found a high contribution of Free APA in APA (>65%) at station Aloha in an oligotrophic North Pacific subtropical gyre. Their results are confirmed (12-100%; 41±23%) (Duhamel et al., 2011) and explained by experiments performed in the same area. Namely, N addition provoked more cell-bound AP, i.e. added nitrogen caused P limitation and engaged in AP synthesis, whereas P addition provoked a release of cell-free AP

that can also recycle nitrogen during N limitation. Intense sun irradiance probably contributed more to the release of Free AP in the surface water of the OE in late spring, summer, and autumn (June, August and October) than in other sampling months (Table 1) due to the detrimental impact of UV-B rays on the microbial community (Garde and Gustavson, 1999).

CONCLUSIONS

Our results indicate that the phytoplankton community in the middle OE (with the clearest impact on MICRO) might be experiencing a shift from P-limitation in late spring and summer toward N-limitation in early autumn. It seems that nutrient limitation disappears in winter. This pattern, with different intensities of nutrient limitations, might be occurring in most, if not all, the highly stratified estuaries of the eastern Adriatic Sea, depending on the significance of the volatility in supply of nutrients by river discharges and the coastal waters inflow throughout the year. Our results can serve as a starting point for more specific explorations of the excess or lack of bioavailable N and P not only in the OE but along the eastern Adriatic coast and wherever the variation of the supply of nutrients can have an important impact on coastal ecosystems (Hrustić et al., 2017).

AUTHOR CONTRIBUTIONS

E.H.: Conceptualization, Methodology, Investigation, Formal Analysis, Writing – original draft, Writing – review & editing.
S.L.: Investigation, Formal Analysis, Writing – review & editing.
S.B.-Ć.: Formal Analysis, Writing – review & editing.
I.I.: Conceptualization, Writing – review & editing.

Acknowledgments

We would like to thank our colleagues from the Institute for marine and coastal research at the University of Dubrovnik and the crew of R/V “Naše More” for their help during sampling. We thank the Croatian Meteorological Service for sharing their meteorological and hydrological data and the three anonymous reviewers for their great help and rich comments on how to improve the presentation of this study.

This work has been conducted within the project 275-0000000-3186 supported by the Ministry of Science, Education and Sports 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*(2), 166–183. DOI: https://doi.org/10.4319/lo.1963.8.2.0166

Batistić, M., Jasprica, N., Carić, M., Čalić, M., Kovačević, V., Garić, R., Njire, J., Mikuš, J. & Bobanović-Ćolić, S. 2012. Biological evidence of a winter convection event in the South Adriatic: A phytoplankton maximum in the aphotic zone. *Continental Shelf Research*, *44*(1), 57–71. DOI: https://doi.org/10.1016/j.csr.2011.01.004

Berman, D. W., T., Walline, P. D., Schneller, A., Rothenberg, J. & Townsend, D. W. 1985. Secchi disk record: A claim for the eastern Mediterranean. *Limnology and Oceanography*, *30*(2), 447–448.

Boekel, W. H. M. van & Veldhuis, M. J. W. 1990. Regulation of alkaline phosphatase synthesis in Phaeocystis sp. *Marine Ecology Progress Series*, *61*(3), 281–289.

Bogé, G., Jamet, J. L., Richard, S., Jamet, D. & Jean, N. 2002. Contribution of copepods, cladocerans and cirripeds to phosphatase activity in mediterranean zooplankton. *Hydrobiologia*, *468*(1/3), 147–154. DOI: https://doi.org/10.1023/a:1015266722757

Bonacci, O. 2001. Analysis of the maximum discharge of karst springs. *Hydrogeology Journal*, *9*(4), 328–338. DOI: https://doi.org/10.1007/s100400100142

Børsheim, K. Y. & Bratbak, G. 1987. Cell volume to cell carbon conversion factors for a bacterivorous Monas sp. enriched from seawater. *Marine Ecology Progress Series*, *36*(2), 171–175.

Calbet, A., Landry, M. R. & Scheinberg, R. D. 2000. Copepod grazing in a subtropical bay: species-specific responses to a midsummerincrease in nanoplankton standing stock. *Marine Ecology Progress Series*, *193*, 75–84.

Carić, M., Jasprica, N., Kršinić, F., Vilibić, I. & Batistić, M. 2012. Hydrography, nutrients, and plankton along the longitudinal section of the Ombla Estuary (south-eastern Adriatic). *Journal of the Marine Biological Association of the United Kingdom*, *92*(6), 1227–1242. DOI: https://doi.org/10.1017/s002531541100213x

Civitarese, G., Gačić, M., Lipizer, M. & Eusebi Borzelli, G. 2010. On the impact of the Bimodal Oscillating System (BiOS) on the biogeochemistry and biology of the Adriatic and Ionian Seas (Eastern Mediterranean). *Biogeosciences*, *7*(12), 3987–3997. DOI: https://doi.org/10.5194/bg-7-3987-2010

Domingues, R. B., Barbosa, A. B., Sommer, U. & Galvão, H. M. 2011. Ammonium, nitrate and phytoplankton interactions in a freshwater tidal estuarine zone: potential effects of cultural eutrophication. *Aquatic Sciences*, *73*(3), 331–343. DOI: https://doi.org/10.1007/s00027-011-0180-0

Duhamel, S., Björkman, K. M., Wambeke, F. V., Moutin, T. & Karl, D. M. 2011. Characterization of alkaline phosphatase activity in the North and South Pacific

Subtropical Gyres: Implications for phosphorus cycling. *Limnology and Oceanography*, *56*(4), 1244–1254. DOI: https://doi.org/10.4319/lo.2011.56.4.1244

Duhamel, S., Dyhrman, S. T. & Karl, D. M. 2010. Alkaline phosphatase activity and regulation in the North Pacific Subtropical Gyre. *Limnology and Oceanography*, *55*(3), 1414–1425. DOI: https://doi.org/10.4319/lo.2010.55.3.1414

Dyhrman, S. T. & Palenik, B. 1999. Phosphate stress in cultures and field populations of the dinoflagellate *Prorocentrum minimum* detected by a single-cell alkaline phosphatase assay. *Applied and Environmental Microbiology*, *65*(7), 3205–3212. DOI: https://doi.org/10.1128/aem.65.7.3205-3212.1999

Eppley, R. W. & Thomas, W. H. 1969. Comparsion of half-saturation constants for growth and nitrate uptake of marine phytoplankton. *Journal of Phycology*, *5*(4), 375–379. DOI: https://doi.org/10.1111/j.1529-8817.1969.tb02628.x

Froelich, P. N. 1988. Kinetic control of dissolved phosphate in natural rivers and estuaries: A primer on the phosphate buffer mechanism. *Limnology and Oceanography*, *33*(4 part. 2), 649–668. DOI: https://doi.org/10.4319/lo.1988.33.4part2.0649

Garde, K. & Gustavson, K. 1999. The impact of UV-B radiation on alkaline phosphatase activity in phosphorus-depleted marine ecosystems. *Journal of Experimental Marine Biology and Ecology*, *238*(1), 93–105. DOI: https://doi.org/10.1016/s0022-0981(99)00005-2

Geider, R. J., Macintyre, H. L. & Kana, T. M. 1997. Dynamic model of phytoplankton growth and aclimation: responses of the balanced growth rate and the chlorophyll a: carbon ratio to light, nutrient-limitation and temperature. *Marine Ecology Progress Series*, *148*(1–3), 187–200.

Goldman, J. C., Caron, D. A. & Dennett, M. R. 1987. Regulation of gross growth efficiency and ammonium regeneration in bacteria by substrate C: N ratio1. *Limnology and Oceanography*, *32*(6), 1239–1252. DOI: https://doi.org/10.4319/lo.1987.32.6.1239

Grasshoff, K., Kremling, K. & Ehrhardt, M. (eds.). 1983. *Methods of Seawater Analysis* (2nd ed.). Berlim: Wiley. DOI: https://doi.org/10.1002/9783527613984

Haas, L. W. 1982. Improved epifluorescence microscopy for observing planktonic micro-organisms. *Annales de l'Institute Océanographique*, *58*(Supl S), 261–266.

Hobbie, J. E., Daley, R. J. & Jasper, S. 1977. Use of nuclepore filters for counting bacteria by fluorescence microscopy. *Applied and Environmental Microbiology*, *33*(5), 1225–1228. DOI: https://doi.org/10.1128/aem.33.5.1225-1228.1977

Holm-Hansen, O., Lorenzen, C. J., Holmes, R. W. & Strickland, J. D. H. 1965. Fluorometric Determination of Chlorophyll. *ICES Journal of Marine Science*, *30*(1), 3–15. DOI: https://doi.org/10.1093/icesjms/30.1.3

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

Hrustić, E., Carić, M., Čalić, M. & Bobanović-Ćolić, S. 2013. Alkaline phosphatase activity and relative importance of picophytoplankton in autumn and early spring (Mljet lakes, eastern Adriatic Sea). *Fresenius Environmental Bulletin*, *22*(3), 636–648.

Hrustić, E., Carić, M. & Garić, R. 2011. Alkaline Phosphatase Activity in the NE Adriatic. *In*: Özhan, E. (ed.), *Proceedings of the tenth international conference on the mediterranean coastal environment* (pp. 521–532). Dalyan: MEDCOAST.

Hrustić, E., Lignell, R., Riebesell, U. & Thingstad, T. F. 2017. Exploring the distance between nitrogen and phosphorus limitation in mesotrophic surface waters using a sensitive bioassay. *Biogeosciences*, *14*(2), 379–387. DOI: https://doi.org/10.5194/bg-14-379-2017

Ivančić, I. & Degobbis, D. 1984. An optimal manual procedure for ammonia analysis in natural waters by the indophenol blue method. *Water Research*, *18*(9), 1143–1147. DOI: https://doi.org/10.1016/0043-1354(84)90230-6

Jean, N., Bogé, G., Jamet, J.-L., Richard, S. & Jamet, D. 2003. Seasonal changes in zooplanktonic alkaline phosphatase activity in Toulon Bay (France): the role of Cypris larvae. *Marine Pollution Bulletin*, *46*(3), 346–352. DOI: https://doi.org/10.1016/s0025-326x(02)00450-2

Jonge, V. & Villerius, L. 1989. Possible role of carbonate dissolution in estuarine phosphate dynamics. *Limnology and Oceanography*, *34*(2), 332–340. DOI: https://doi.org/10.4319/lo.1989.34.2.0332

Kana, T. M. & Glibert, P. M. 1987. Effect of irradiances up to 2000 µE m-2 s-1 on marine Synechococcus WH7803—I. Growth, pigmentation, and cell composition. *Deep Sea Research Part A. Oceanographic Research Papers*, *34*(4), 479–495. DOI: https://doi.org/10.1016/0198-0149(87)90001-x

Koroleff, F. 1983. Determination of phosphorus. *In*: Grashoff, K., Ehrhardt, M., & Kremling, K. (eds.), *Methods of seawater analysis* (pp. 117–138). Weinheim: Verlag-Chimie.

Krom, M., Kress, N., Brenner, S. & Gordon, L. 1991. Phosphorus limitation of primary productivity in the eastern Mediterranean Sea. *Limnology and Oceanography*, *36*(3), 424–432. DOI: https://doi.org/10.4319/lo.1991.36.3.0424

Krom, M. D., Woodward, E. M. S., Herut, B., Kress, N., Carbo, P., Mantoura, R. F. C., Spyres, G., Thingstad, T. F., Wassmann, P., Wexels-Riser, C., Kitidis, V., Law, C. S. & Zodiatis, G. 2005. Nutrient cycling in the south east Levantine basin of the eastern Mediterranean: Results from a phosphorus starved system. *Deep-Sea Research II*, 52, 2879-2896. doi:10.1016/j.dsr2.2005.08.009

Lee, S. & Fuhrman, J. A. 1987. Relationships between Biovolume and Biomass of Naturally Derived Marine Bacterioplankton. *Applied and Environmental Microbiology*, *53*(6), 1298–1303. DOI: https://doi.org/10.1128/aem.53.6.1298-1303.1987

Li, H., Veldhuis, M. J. W. & Post, A. 1998. Alkaline phosphatase activities among planktonic communities in the northern Red Sea. *Marine Ecology Progress Series*, *173*, 107–115. DOI: https://doi.org/10.3354/meps173107

Li, J. & Dittrich, M. 2019. Dynamic polyphosphate metabolism in cyanobacteria responding to phosphorus availability. *Environmental Microbiology*, *21*(2), 572–583. DOI: https://doi.org/10.1111/1462-2920.14488

Ljubimir, S., Jasprica, N., Čalić, M., Hrustić, E., Dupčić Radić, I., Car, A. & Batistić, M. 2017. Interannual (2009–2013) variability of winter-spring phytoplankton in the open South Adriatic Sea: Effects of deep convection and lateral advection. *Continental Shelf Research*, *143*, 311–321. DOI: https://doi.org/10.1016/j.csr.2017.05.007

MacIsaac, J. J. & Dugdale, R. C. 1969. The kinetics of nitrate and ammonia uptake by natural populations of marine phytoplankton. *Deep Sea Research and Oceanographic Abstracts*, *16*(1), 45–57. DOI: https://doi.org/10.1016/0011-7471(69)90049-7

Moutin, T., Thingstad, T. F., Wambeke, F. V., Marie, D., Slawyk, G., Raimbault, P. & Claustre, H. 2002. Does competition for nanomolar phosphate supply explain the predominance of the cyanobacterium *Synechococcus* ? *Limnology and Oceanography*, *47*(5), 1562–1567. DOI: https://doi.org/10.4319/lo.2002.47.5.1562

Murphy, J. & Riley, J. P. 1962. A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta*, *27*, 31–36. DOI: https://doi.org/10.1016/s0003-2670(00)88444-5

Najdek, M., Paliaga, P., Šilović, T., Batistić, M., Garić, R., Supić, N., Ivančić, I., Ljubimir, S., Korlević, M., Jasprica, N., Hrustić, E., Dupčić-Radić, I., Blažina, M. & Orlić, S. 2014. Picoplankton community structure before, during and after convection event in the offshore waters of the Southern Adriatic Sea. *Biogeosciences*, *11*(10), 2645–2659. DOI: https://doi.org/10.5194/bg-11-2645-2014

Nausch, M. 1998. Alkaline phosphatase activities and the relationship to inorganic phosphate in the Pomeranian Bight (southern Baltic Sea). *Aquatic Microbial Ecology*, *16*(1), 87–94. DOI: https://doi.org/10.3354/ame016087

Nedoma, J., Garcia, J. C., Comerma, M., Simek, K. & Armengol, J. 2006. Extracellular phosphatases in a Mediterranean reservoir: seasonal, spatial and kinetic heterogeneity. *Freshwater Biology*, *51*(7), 1264–1276. DOI: https://doi.org/10.1111/j.1365-2427.2006.01566.x

Oh, S. J., Kwon, H. K., Noh, I. H. & Yang, H.-S. 2010. Dissolved organic phosphorus utilization and alkaline phosphatase activity of the dinoflagellate Gymnodinium impudicum isolated from the South Sea of Korea. *Ocean Science Journal*, *45*(3), 171–178. DOI: https://doi.org/10.1007/s12601-010-0015-2

Orhanović, S. & Vrančić, M. P. 2000. Alkaline Phosphatase activity in Seawater: Influence of reaction conditions on the kinetic parameters of ALP. *Croatica Chemica Acta*, *73*(3), 819–930.

Orret, K. & Karl, D. M. 1987. Dissolved organic phosphorus production in surface seawaters. *Limnology and Oceanography*, *32*(2), 383–395.

Rengefors, K., Ruttenberg, K. C., Haupert, C. L., Taylor, C., Howes, B. L. & Anderson, D. M. 2003. Experimental investigation of taxon-specific response of alkaline phosphatase activity in natural freshwater phytoplankton. *Limnology and Oceanography*, *48*(3), 1167–1175. DOI: https://doi.org/10.4319/lo.2003.48.3.1167

Sieburth, J. M., Smetacek, V. & Lenz, J. 1978. Pelagic ecosystem structure: Heterotrophic compartments of the plankton and their relationship to plankton size fractions 1. *Limnology and Oceanography*, *23*(6), 1256–1263. DOI: https://doi.org/10.4319/lo.1978.23.6.1256

Šilović, T., Mihanović, H., Batistić, M., Dupčić Radić, I., Hrustić, E. & Najdek, M. 2018. Picoplankton distribution influenced by thermohaline circulation in the southern Adriatic. *Continental Shelf Research*, *155*, 21–33. DOI: https://doi.org/10.1016/j.csr.2018.01.007

Spiteri, C., Cappellen, P. V. & Regnier, P. 2008. Surface complexation effects on phosphate adsorption to ferric iron oxyhydroxides along pH and salinity gradients in estuaries and coastal aquifers. *Geochimica et Cosmochimica Acta*, *72*(14), 3431–3445. DOI: https://doi.org/10.1016/j.gca.2008.05.003

Su, B., Song, X., Duhamel, S., Mahaffey, C., Davis, C., Ivančić, I. & Liu, J. 2023. A dataset of global ocean alkaline phosphatase activity. Scientific Data. *Scientific Data*, *10*(205). DOI: https://doi.org/10.1038/s41597-023-02081-7

Suzumura, M. 2008. Persulfate chemical wet oxidation method for the determination of particulate phosphorus in comparison with a high-temperature dry combustion method. *Limnology and Oceanography: Methods*, *6*(11), 619–629. DOI: https://doi.org/10.4319/lom.2008.6.619

Tanaka, T., Henriksen, P., Lignell, R., Olli, K., Seppälä, J., Tamminen, T. & Thingstad, T. F. 2006. Specific affinity for phosphate uptake and specific alkaline phosphatase activity as diagnostic tools for detecting phosphorus-limited phytoplankton and bacteria. *Estuaries and Coasts*, *29*(6), 1226–1241. DOI: https://doi.org/10.1007/bf02781823

Torriani-Gorini, A. 1994. The Pho regulon of Escherichia coli. Iin. *In*: Torriani-Gorini, A., Yagil, E., & Silver, S. (eds.), *Phosphate in Microorganisms: Cellular and Molecular Biology* (pp. 1–4). Washington, DC: ASM Press.

Utermöhl, H. 1958. Zur Vervollkommnung der quantitativen Phytoplankton-Methodik. *SIL Communications, 1953-1996*, *9*(1), 1–38. DOI: https://doi.org/10.1080/05384680.1958.11904091

Verity, P. G., Robertson, C. Y., Tronzo, C. R., Andrews, M. G., Nelson, J. R. & Sieracki, M. E. 1992. Relationships between cell volume and the carbon and nitrogen content of marine photosynthetic nanoplankton. *Limnology and Oceanography*, *37*(7), 1434–1446.

Viličić, D., Kršinić, F., Carić, M., Jasprica, N., Bobanović-Ćolić, S. & Mikuš, J. 1995. Plankton and hydrography in a moderately eastern Adratic bay (Gruž Bay). *Hydrobiologia*, *304*(1), 9–22. DOI: https://doi.org/10.1007/bf02530699

Vollenweider, R. A., Giovanardi, F., Montanari, G. & Rinaldi, A. 1998. Characterization of the trophic conditions of marine coastal waters with special reference to the NW Adriatic Sea: proposal for a trophic scale, turbidity and generalized water quality index. *Environmetrics*, *9*(3), 329–357.

Weiss, R. F. 1970. The solubility of nitrogen, oxygen and argon in water and seawater. *Deep Sea Research and Oceanographic Abstracts*, *17*(4), 721–735. DOI: https://doi.org/10.1016/0011-7471(70)90037-9

Zavatarelli, M., Raicich, F., Bregant, D., Russo, A. & Artegiani, A. 1998. Climatological biogeochemical characteristics of the Adriatic Sea. *Journal of Marine Systems*, *18*(1–3), 227–263. DOI: https://doi.org/10.1016/s0924-7963(98)00014-1

Zohary, T. & Robarts, R. D. 1998. Experimental study of microbial P limitation in the eastern Mediterranean. *Limnology and Oceanography*, *43*(3), 387–395. DOI: https://doi.org/10.4319/lo.1998.43.3.0387