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ORIGINAL ARTICLE

Spatial and temporal prokaryotic variability in the northern Adriatic Sea

Paola Del Negro[1], Mauro Celussi[1], Erica Crevatin[1], Alessandro Paoli[1], Fabrizio Bernardi Aubry[2] & Alessandra Pugnetti[2]

1 Dipartimento di Oceanografia Biologica, Istituto Nazionale di Oceanografia e Geofisica Sperimentale (OGS), Trieste, Italy
2 Istituto di Scienze Marine, Consiglio Nazionale delle Ricerche (CNR), Castello, Venice, Italy

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Correspondence
Paola Del Negro, Dipartimento di Oceanografia Biologica, Istituto Nazionale di Oceanografia e Geofisica Sperimentale (OGS), Via A. Piccard 54, I-34014 Trieste, Italy.
E-mail: pdelnegro@ogs.trieste.it

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Abstract

The prokaryotic community, both in terms of abundance and activity (exoenzymatic hydrolysis of proteins, polysaccharides and phosphorylated molecules and leucine uptake), was investigated seasonally for a 3-year period (2004–2006) in the Gulf of Venice (northern Adriatic Sea). By focusing on spatial and temporal variability, the prokaryote dynamics showed significant variations on a horizontal and seasonal scale, but no substantial differences were observed among years. The basin-scale variability was mainly influenced by allochthonous inputs from the Po river and the Venice Lagoon, which were the main source of nutrients, acting as a 'bottom up' control on prokaryotes. On a seasonal scale, all the microbial parameters (except the polysaccharide degradation) showed significant variations following the temperature fluctuations. The annual rate of change was very low for all the chemical, physical and biological parameters and only the abundance and phosphatase activity of the prokaryotes differed significantly among years.

Problem

The shallow northern Adriatic Sea is characterized by marked seasonal and long-term fluctuations of oceanographic and biological conditions, mainly due to atmospheric forcing, freshwater discharges, variable intrusion of high salinity waters, and a very variable and complex circulation (Franco 1982; Orlić *et al.* 1992; Poulain *et al.* 2001; Russo *et al.* 2005).

The north western Adriatic shelf area is mainly characterized by the dominant presence of two water masses. The first is a surface one characterized by reduced salinity due to the influence of the Po river flowing southwards along the Italian coast in the form of a narrow surface coastal current. The second is cold and dense seawater and formed during the winter period, resulting in a density-driven western bottom current. Along the eastern coast, a compensation occurs, carrying warmer and saltier waters from the southern Adriatic and Ionian Sea (Cushman-Roisin *et al.* 2001). This cyclonic circulation pattern is active during most of the year except during the summer period, when a semi-enclosed circulation pattern prevails in the region and thermal stratification gradually increases. In these conditions, freshened surface waters, formed along the western coast, are generally advected eastward as far as the Istrian coast, causing a significant increase in the stratification of the water column. The Po river is the major buoyancy input with an annual mean discharge rate of 1500–1700 $m^3 \cdot s^{-1}$, accounting for 51% of the total freshwater flow (Degobbis & Gilmartin 1990). In particular, freshwater discharge dynamics in spring and summer, characterized by relevant peaks of short duration (few days), may support the highly variable conditions of a pelagic ecosystem (Mangoni *et al.* 2008). The lagoon of Venice, located in its northwestern coast, is another important source of nutrients for the basin (Solidoro *et al.*

2005). In this shallow turbulent environment where increased nutrient availability is episodic, the planktonic food webs can alternatively shift from microbial to grazing pathway. In spring, large-sized phytoplankton blooms occur as a result of episodic nutrient enrichment. Conversely, the microbial food web is typical of low energy environments, mostly based on regeneration processes (Kiørboe 1996). As a consequence, the final fate of newly produced organic carbon can change greatly over time in the same environment. In view of the importance of planktonic prokaryotes in the carbon cycle, interpretation of natural patterns should constitute an important step towards an understanding of the planktonic food web dynamics.

The broad variability of the basin features leads to variations in its biological structure (Cabrini *et al.* 2002), in particular considering the riverine inputs which can enhance growth and activities of the microorganisms (Puddu *et al.* 1998; Paoli *et al.* 2006).

In the present study a field dataset covering 3 years and four sites with different water exchange and freshwater inputs was investigated. The objective was to describe the changes in the abundance and activities of the prokaryotic community both in terms of organic matter degradation (exoenzyme production) and utilization (secondary carbon production) in the northern Adriatic Sea, particularly taking into account the discharges of the Po river and the influence of the high salinity waters of southern origin. We focused on spatial and temporal variability on the basin scale in relation to hydrological conditions and availability of resources.

Methods

Sampling strategy

In the framework of the INTERREG III CBC Phare Italy Slovenia initiative, Project OBAS (Biological Oceanography of the Northern Adriatic Sea), 10 surveys were carried out on a seasonal basis (May, July, November 2004; March, May, July, November 2005; March, May, July 2006). Two stations representative of the central basin (C13, 13°00′42.04′ E, 45°14′59.28′ S and E10, 13°00′39′ E, 44°57′30.24′ S), one station strongly affected by the Po river plume (E01, 12°33′36′ E, 44°57′36′ S), and one station influenced by the exports from the Chioggia inlet (southern Venice lagoon) (C01, 12°18′46.8′ E, 45°15′00′ S) were sampled (Fig. 1). Previous studies highlighted that the C10 and E06 stations (Fig. 1) can be considered representative of the hydrological and trophic variability of the area (Pugnetti *et al.* 2004; Bernardi Aubry *et al.* 2006a,b). Station C10 is also one of the long-term research stations of the Northern Adriatic site, included in the Italian Long-Term Ecological Research Network (LTER-Italy).

Analytical determinations

At each sampling, temperature and salinity were measured using an Idronaut Ocean Seven 316 multiprobe. Water samples for the determination of the concentrations of dissolved inorganic nutrients (Grasshoff *et al.* 1983) and chlorophyll *a* (Chl *a* – Holm-Hansen *et al.* 1965) and for microbial analyses were collected at the

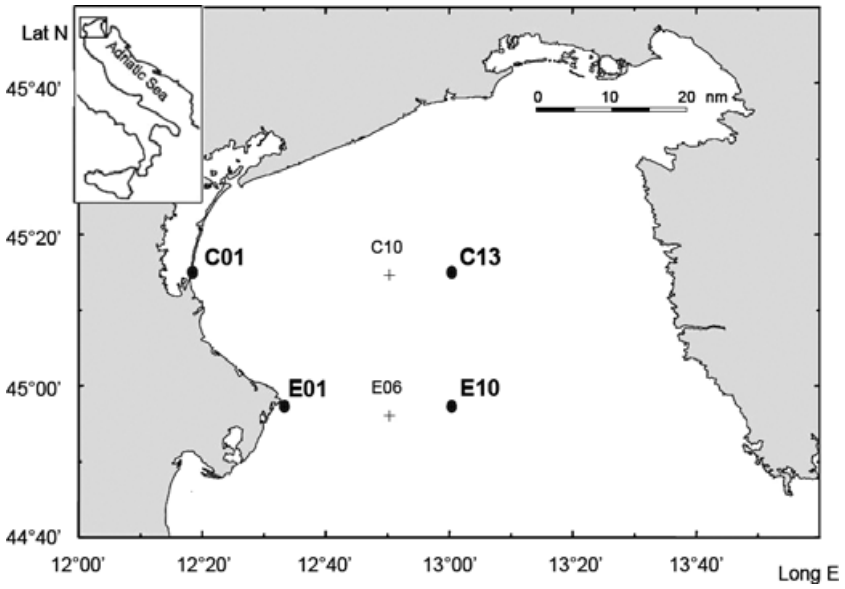

Fig. 1. Location of the sampling stations in the Gulf of Venice (northern Adriatic sea). The sampling stations considered in the study are indicated in bold.

surface (−0.5 m) with a 5-l Niskin bottle, equipped with silicon elastic and red silicon O-rings.

Samples (10 ml) taken to estimate heterotrophic picoplankton abundance (HPP) were fixed with a 2% final concentration borate-buffered formalin (pre-filtered through a 0.2-μm Acrodisc filter) and stained for 15 min with 4′6 diamidino-2-phenylindole (DAPI, Sigma) (Porter & Feig 1980) at 1 $\mu g \cdot ml^{-1}$ final concentration. Subsamples were filtered in triplicate onto 0.2-μm black-stained polycarbonate filters (Nuclepore) and preserved at −20 °C. Filters were mounted on microscope slides between layers of non-fluorescent immersion oil (Olympus), and counted under a UV filter set (BP 330–385 nm), using an Olympus BX 60 F5 epifluorescence microscope at 1000× magnification. At least 20 random fields and a minimum of 300 cells lacking photosynthetic pigments were counted for each filter. Each HPP value represents the mean of triplicate samples with a coefficient of variation lower than 5%.

Hydrolytic exoenzyme activities were measured with fluorogenic analogs of natural substrates (Hoppe 1993) derived from 7-amino-methyl-coumarin (AMC) and 4-methyl-umbelliferone (MUF). Aminopeptidase activity (AMA) was assayed as the hydrolysis rate of L-leucine-AMC. β-D-glucosidase (BGLU) and alkaline phosphatase (APA) were assayed using MUF derivatives. Enzyme activities measured using fluorogenic substrates were expressed in terms of rate of MUF or AMC production. Hydrolysis mainly ascribable to picoplanktonic cells was measured (after evaluation of saturating concentrations) by incubating 2.5-ml subsamples with 200 μM (final concentration) MUF-β-glucoside and leucine-AMC substrates and 50 μM (final concentration) MUF-phosphate for 1 h at *in situ* temperature in the dark. The fluorescence released by enzymatic cleavage of the artificial substrates was measured fluorometrically, in triplicate, at 380/365 nm excitation and 440/455 nm emission for AMC/MUF substrates using a Shimadzu RF 1501 fluorometer. Standard solutions of MUF and AMC were used to calibrate the fluorometer.

Prokaryotic carbon production (PCP) was estimated by incorporation of ^{3}H-leucine (Leu) (Kirchman *et al.* 1985). Triplicate 1.7-ml aliquots and two killed controls (90 μl 100% trichloroacetic acid – TCA) were amended with a 20-nM radiotracer and incubated at *in situ* temperature (±2 °C) in the dark. Incubations were stopped with 100% TCA after 1 h. The extraction with 5% TCA and 80% ethanol was carried out using the microcentrifugation method (Smith & Azam 1992). Activity in the samples was determined using a β-counter (Packard Tri-Carb 300) after the addition of 1-ml scintillation cocktail (Ultima Gold MV; Packard). Incorporation of ^{3}H-leucine was converted into carbon produced via bacterial protein production according to Simon & Azam (1989), assuming a twofold isotope dilution for leucine.

Statistical analysis

Differences in each dependent variable of the dataset (see Table 1 for list of abbreviations) were established using the analysis of variance (ANOVA) technique and considering the following fixed factors: F1 – stations (C1 *versus* C13 *versus* E1 *versus* E10); F2 – seasons (winter *versus* spring *versus* summer *versus* fall); F3 – years (2004 *versus* 2005 *versus* 2006). All variables were logarithmically [$\ln(x)$] transformed to comply with the assumptions of ANOVA: normal distribution and homogeneity of variance were

Table 1. Results of the ANOVA (F-test and P) on surface values of chemical, physical and biological parameters among: F1 – stations (C1 *versus* C13 *versus* E1 *versus* E10); F2 – seasons (winter *versus* spring *versus* summer *versus* fall); F3 – years (2004 *versus* 2005 *versus* 2006).

		F1		F2		F3	
Variable	abbreviation	F	P	F	P	F	P
temperature	SST	0.06	n.s.	254.65	***	0.435	n.s.
salinity	SSS	21.27	***	0.44	n.s.	1.45	n.s.
ammonium	NH_4^+	8.25	***	2.02	n.s.	0.32	n.s.
nitrite	NO_2	7.15	***	4.28	*	0.29	n.s.
nitrate	NO_3	5.71	**	1.33	n.s.	0.29	n.s.
dissolved inorganic nitrogen	DIN	6.07	**	1.43	n.s.	0.29	n.s.
silicate	SiO_4	6.39	**	1.12	n.s.	0.08	n.s.
phosphate	PO_4	7.02	***	0.86	n.s.	0.12	n.s.
particulate organic carbon	POC	3.96	*	2.46	n.s.	2.08	n.s.
chlorophyll *a*	Chl *a*	4.44	**	0.58	n.s.	0.02	n.s.
heterotrophic picoplankton	HPP	1.15	n.s.	6.34	**	4.71	*
phosphatase activity	APA	0.59	n.s.	5.51	**	6.36	**
aminopeptidase activity	AMA	3.21	*	3.02	*	2.92	n.s.
β-Glucosidase activity	BGLU	0.80	n.s.	2.70	n.s.	1.66	n.s.
prokaryotic carbon production	PCP	4.03	*	3.88	*	2.22	n.s.

*P < 0.05; **P < 0.01; ***P < 0.001; n.s. = non-significant.

estimated with Shapiro–Wilk and Bartlett tests, respectively.

Principal Component Analysis (PCA) was applied to the multivariate chemical, physical and biological dataset. This multivariate analysis rotates a cloud of data points such that the maximum variability is visible to identify the most important gradients over the study period. All the considered datasets were standardized for the analysis by subtracting the mean and dividing by the standard deviation. Varimax rotation was applied to optimize the interpretation of the PCA results.

Analyses was performed with STATISTICA software.

Results

Surface seawater temperature (SST) showed a regular seasonal fluctuation without marked differences among years or among stations, ranging from 7.5 ± 0.9 °C in winter to 26.1 ± 1.4 °C in summer. Conversely, surface salinity was more variable. The highest variability and the lowest mean value were recorded in the estuarine station E01 (average value = 27.7 ± 5.9). Freshwater inputs from the lagoon affected the station C01 (34.8 ± 1.7), whereas the stations located in the central basin showed higher average values (C13 = 37.3 ± 1.0; E10 = 36.4 ± 2.0). The broad variability of the salinity at station E01 is reflected in Dissolved Inorganic Nitrogen (DIN) and phosphate concentrations, as a consequence of the riverine outflows, as shown in Fig. 2. These macronutrients were generally low in the other sampling sites and the highest values and variability were detected in autumn (Fig. 3), with an overall general decrease from 2004 to 2006 (Fig. 4). This interannual decline was also observed for Chl *a*, which nonetheless revealed the highest phytoplankton biomass in May and generally in the coastal stations. Seasonal fluctuation in HPP and most of their activities [PCP, AMA and β-glucosidase (BGLU)] were also detected showing an increasing trend from March to July followed by a decline in November. Alkaline phosphatase was generally poorly active in autumn and winter, whereas high hydrolysis rates were registered in summer and especially in spring. All the microbial parameters expressed higher values in coastal stations rather than offshore and the general interannual decrease mentioned above was observed for all activities except HPP.

The outcome of the ANOVA carried out for testing the null hypothesis of equality among stations, seasons and years is reported in Table 1. Differences among stations were observed for all the considered variables except T, HPP, APA and BGLU. Only a few variables (T, NO_2, HPP, APA, AMA and PCP) showed significant differences among seasons and no differences occurred among years with the exception of HPP and APA.

The relationships among the chemical, physical and biological parameters for each sampling site are discussed here by using the PCA analysis (Figs 5 and 6). This multivariate analysis showed a helpful distribution of variables in new linear combinations/Principal Factors (PF). Only PF with eigenvalues > 1 are considered. The first two factors (PF1 and PF2) together explained 72.19%, 75.11%, 74.71% and 78.97% of the total variance in the C01, C13, E01 and E10 stations, respectively.

The projection of the variables on the factor plane described by PF1 and PF2 (Fig. 5) highlighted a similar grouping of stations on PF1 but slightly different grouping on PF2. In the C01 station, PF1 was found to be highly positively related to PCP, APA, AMA, BGLU, SST and Chl *a* and negatively related to SSS. The PF2 was negatively related to SSS and positively related to Chl *a*, DIN and PO_4. Both components showed a similar weak relation with HPP but were positive on PF1 and negative on PF2. In the C13 station, PF1 was highly negatively related to HPP, PCP, APA, AMA, BGLU, SST, Chl *a* and DIN and positively related to SSS, whereas the PF2 was negatively related to BGLU and SST and positively related to Chl *a*, DIN and PO_4. In the E01 station all parameters were highly related to PF1, but were positively related to HPP, PCP, APA, AMA, BGLU, SST and Chl *a* and negatively related to SSS, DIN and PO_4. Only BGLU, Chl *a* and DIN were weakly and negatively related to PF2. In the E10 station again all parameters were negatively related to PF1 except for SSS, which was highly positively related to PF1. The PF2 was positively related to APA and BGLU and negatively related to PO_4.

The diagram of the projections of the sampling cases on the PF1 *versus* PF2 factors-plane (Fig. 6) showed clearly that different distributions occurred between coastal and offshore stations. In the C13 and E10 stations all samplings were grouped together, with the exception of the ones carried out in May and July 2004, which showed an opposite position with respect to PF2. The samplings of the C01 station were generally grouped among seasons, whereas for the E01 station only the summer samplings were grouped together and the others did not show clear seasonal groupings.

Discussion

The northern Adriatic ecosystem has been described several times as a very variable shallow basin because of its chemical, physical and biological features (Franco & Michelato 1992; Fonda Umani 1996; Malej *et al.* 1999; Zaccone & Caruso 2002). Generally our results confirm the high variability, previously found for phytoplankton abundances, biodiversity and production (Bernardi Aubry

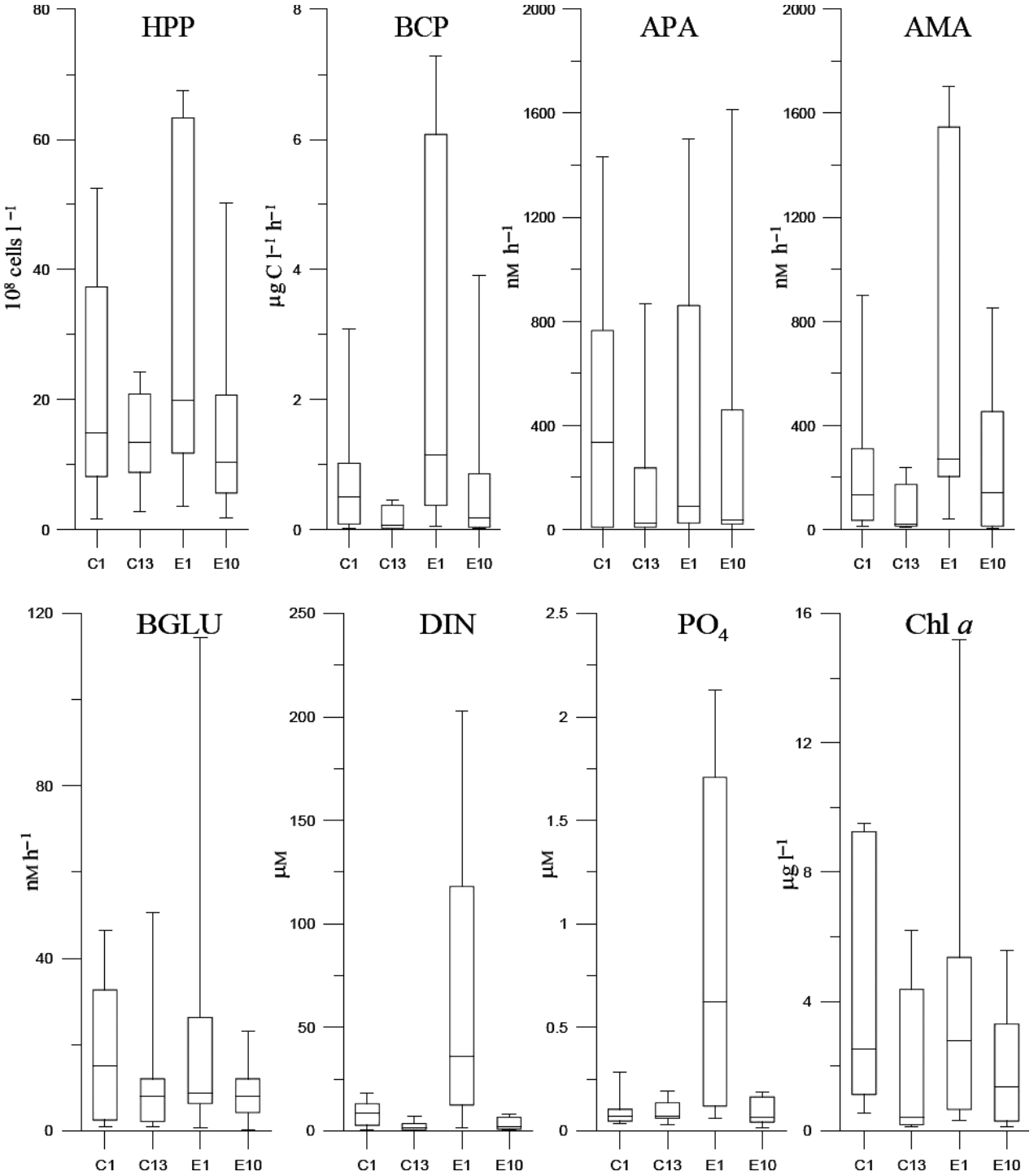

Fig. 2. Boxplots of prokaryotic abundance and activity, nutrients concentration and chlorophyll *a* in the 4 stations during the 2004–2006 sampling period. The median values (-), the first (□) and third (□) quartile, the minimum (.) and maximum (¯) values are shown. See Table 1 for list of abbreviations.

et al. 2006a,b), in heterotrophic prokaryotes dynamics on a horizontal and seasonal scale, but no substantial differences were observed among the 3 years considered (2004–2006, see Table 1).

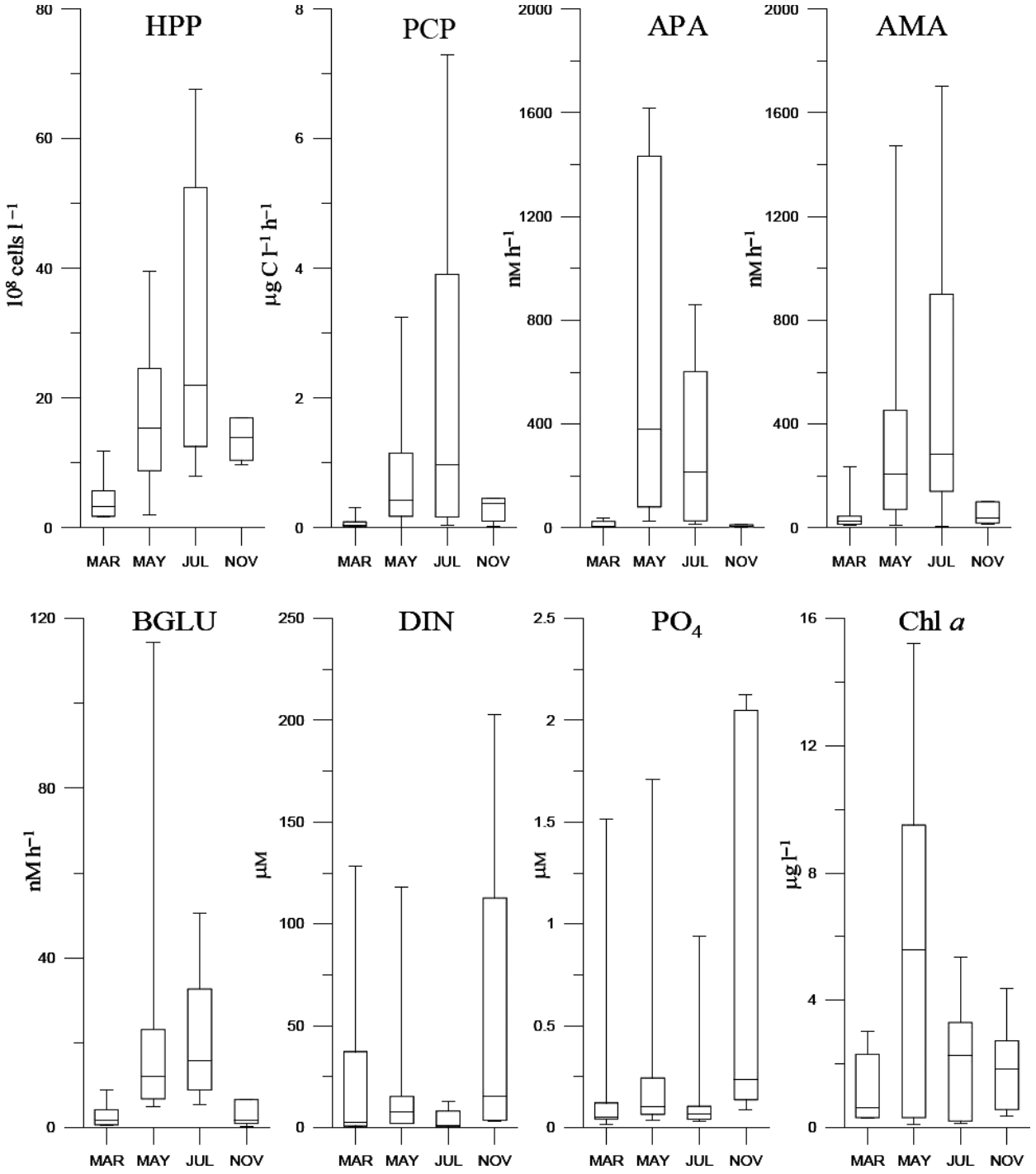

Fig. 3. Boxplots of seasonal prokaryotic abundance and activity, nutrients concentration and chlorophyll *a* considering the 4 stations during the 2004–2006 sampling period. The median values (-), the first (□) and third (□) quartile, the minimum (.) and maximum (˙) values are shown. See Table 1 for list of abbreviations.

Seasonal variability

The variability through seasons of the considered parameters were clearly less significant, as a whole, than that found among stations (Table 1). In fact, only SST, NO_2, HPP, APA, AMA and PCP varied significantly among seasons. The environmental factors were nonetheless less variable (except for temperature) than the biological ones.

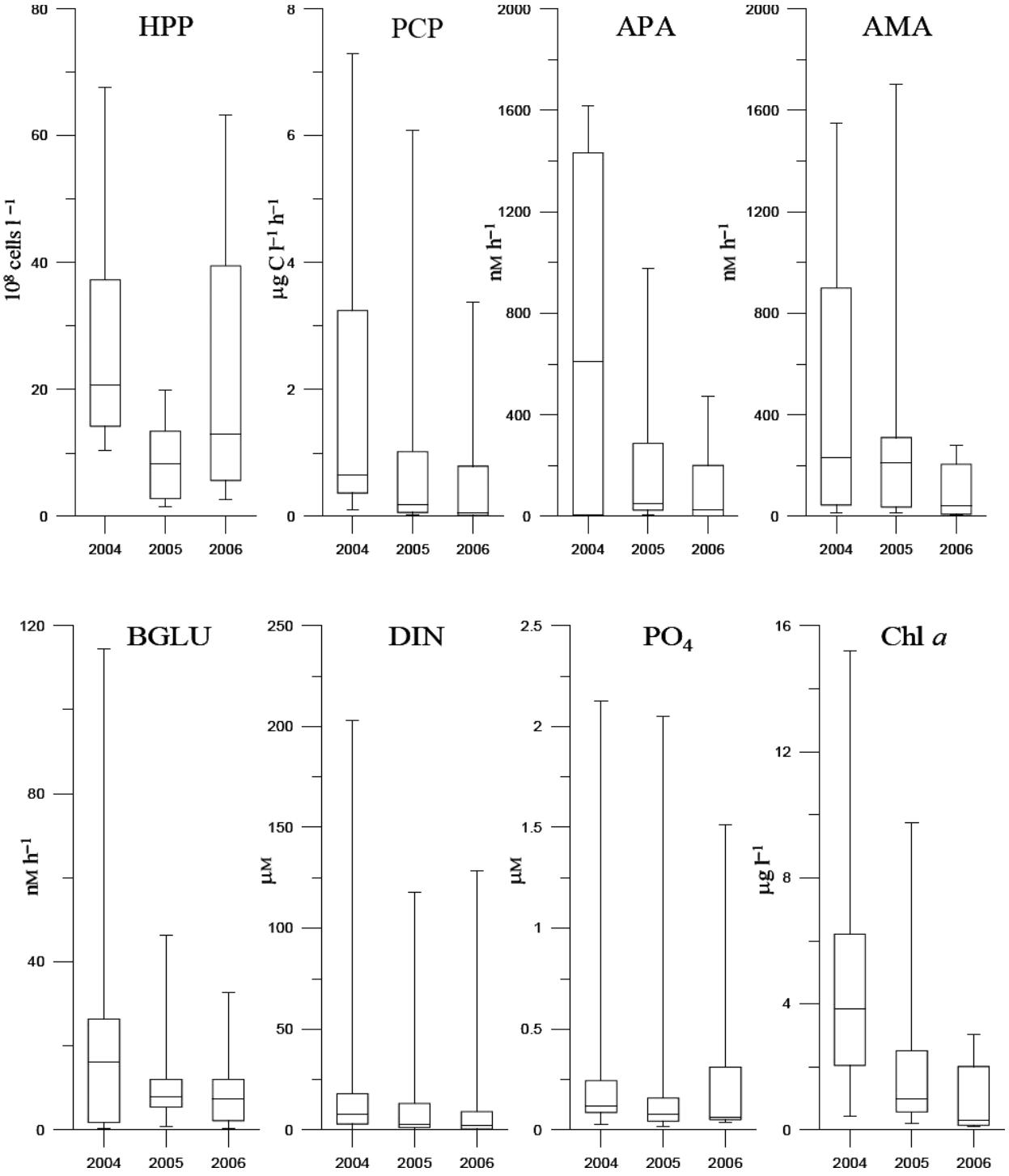

Fig. 4. Boxplots of annual (2004–2006) prokaryotic abundance and activity, nutrients concentration and chlorophyll *a*. The median values (-), the first (□) and third (□) quartile, minimum (.) and maximum (¯) values are shown. See Table 1 for list of abbreviations.

The dynamics of microbial activities in relation to chemical and physical parameters highlighted a defined seasonal pattern more marked in coastal sites (particularly in the C01 station) than in offshore waters (Fig. 6). Prokaryotic abundances followed the temperature trend and consequently also leucine uptake, protease and glucosidase activities (Fig. 3). A parallel seasonal trend was detected between Chl *a* and APA, stressing how

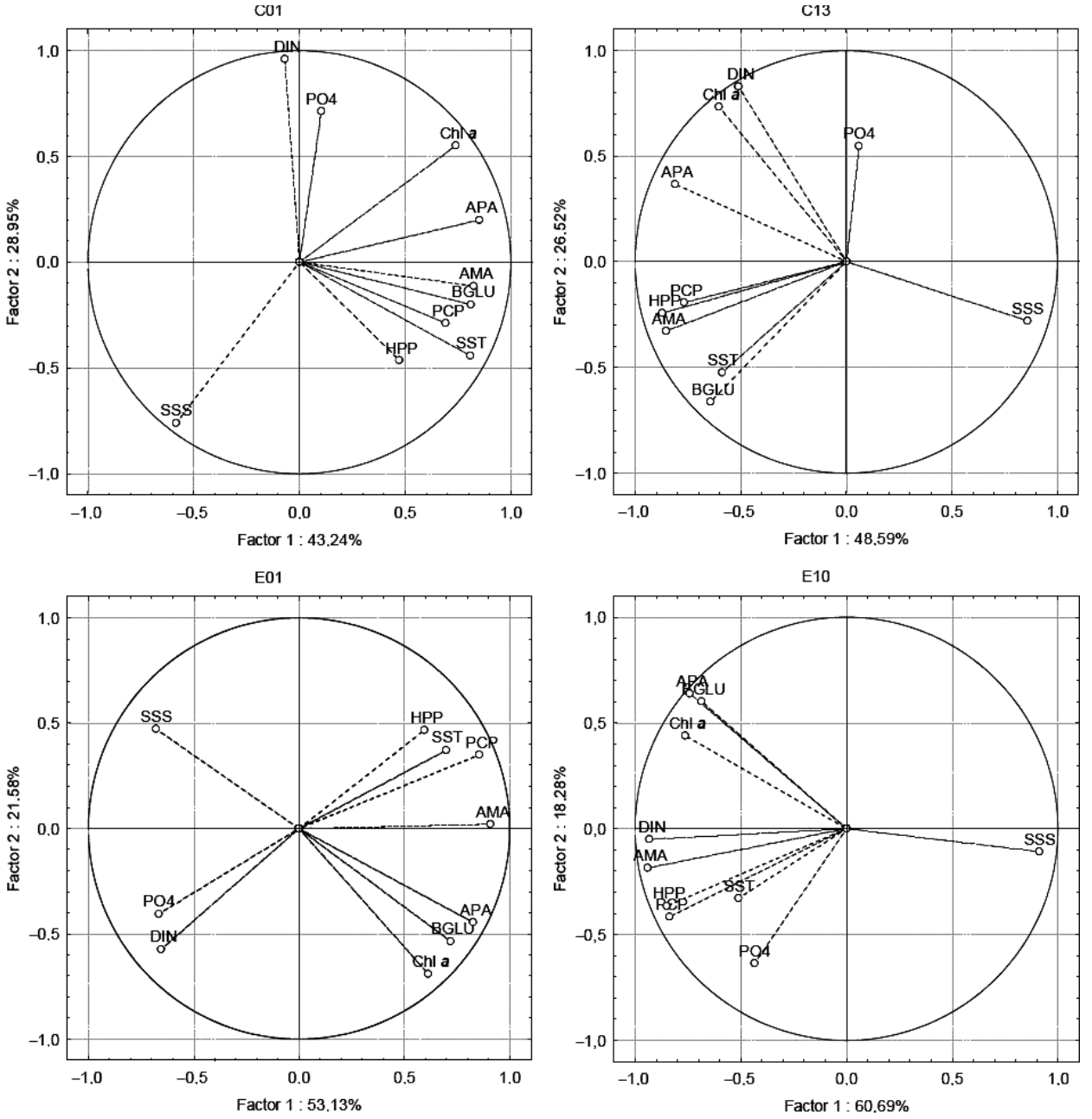

Fig. 5. Results of Principal Component Analysis applied to the chemical - physical and biological parameters dataset. The biplots of the projection of the rotated variables on the factor-plane described by the first and second principal factors and the percentage variance for each factor are indicated. See Tab 1 for list of abbreviations.

phytoplanktonic and prokaryotic release of this enzyme cannot be separated in the field (Nausch 1998). It is thus plausible to infer an important role in phosphatase production for microalgae, which reach their maximum abundances in May, when diatom blooms usually occur, especially at coastal sites (Bernardi Aubry *et al.* 2006a). The analogous trend of protease and leucine uptake suggests a quick turnover of carbon (from proteins), which would be mobilized by enzymes and immediately taken up by bacteria, driving an increase in biomass. Furthermore, the comparison of PCP with data reported by other authors for different Mediterranean areas highlights the highly productive (considered as secondary production) features of the Northern Adriatic basin, which shows, in general, regenerated production values more than one order of magnitude higher (and an extremely higher vari-

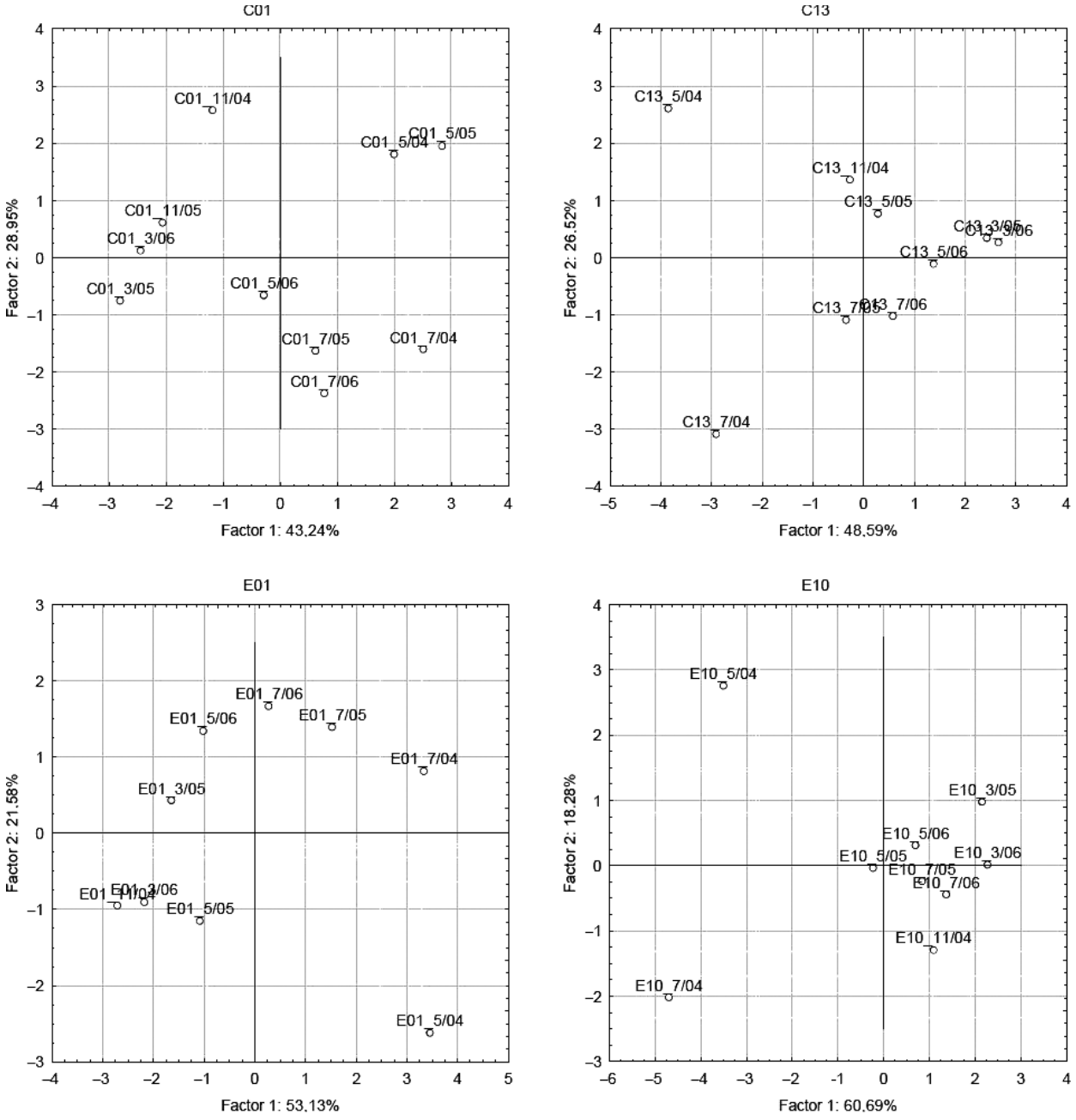

Fig. 6. Results of Principal Component Analysis applied to the chemical - physical and biological parameters dataset. The biplots of the projection of the sampling cases on the factor-plane described by the first and second principal factors and the percentage variance for each factor are indicated. See Tab 1 for list of abbreviations.

ability) than the NW Mediterranean Sea (Van Wambeke *et al.* 2001) and the Cretan Sea (Lemée *et al.* 2002).

Inter-annual variability

The long-term (interannual) variability was the least marked (see ANOVA). Only HPP and APA, in fact, presented significant variations among years. However, the year 2004 clearly showed the highest values of all the prokaryotic activities (Fig. 4). June 2004 was characterized by a massive accumulation of mucilaginous aggregates. This phenomenon is known to enhance exoenzyme production in the months immediately preceding the appearance of mucilage (Danovaro *et al.* 2005) as it is thought to be

caused by the accumulation of organic matter due to microbial loop malfunctioning (Fonda Umani *et al.* 2007). The very high hydrolytic rates recorded in May 2004 would thus have a strong influence on the dataset for the whole year (in 2004, three surveys were carried out). Moreover, the same trend detected for prokaryotic carbon production and exoenzymatic activity could also be noticed in the concentration of Chl *a*, highlighting a strong link between phytoplankton and heterotrophic picoplankton on a long-term scale. Even if the ANOVA test was not significant due to high variability, the general trend over the 3 years resulted in a decrease of all the microbial activities, expressing an uncoupling with prokaryotic abundances, which were, in contrast, significantly lower in 2005 than in the other years.

Basin scale dynamics

The surface physical and chemical parameters were extremely variable. In particular, the outflows of the Po river seemed to have a strong influence on the coastal station E01. This site, in fact, exhibited the highest degree of variability (Fig. 2) of bottom up forcing factors of the microbial food web (phosphate, DIN and Chl *a*), which is then reflected in most of the microbial parameters (HPP, PCP, AMA, BGLU). Episodic freshwater inputs, because of their organic matter and inorganic nutrients content, are known to enhance microbial activities (for the north Adriatic see Puddu *et al.* 1998; Paoli *et al.* 2006; Celussi *et al.* 2008) even on a very short-time scale (Celussi *et al.* 2008). Generally, the stations located in the centre of the basin (C13, E10) expressed the lowest variability considering nitrogen, phosphorous, chlorophyll *a*, and prokaryotes abundance and activity. The highly saline waters of southern origin seem to have a minor influence (with respect to the west-coast terrestrial inputs) on the prokaryotic community. Close to the Chioggia inlet of the Venice lagoon an extremely variable phytoplankton biomass regime (inferred through Chl *a*), not supported by variations in nutrient concentration, reflected a less variable prokaryotic activity. The lagoon of Venice is an important source of nutrients for the northern Adriatic basin (Solidoro *et al.* 2005), exporting to the sea 2590 $tN \cdot year^{-1}$, a variable portion of which passes through the Chioggia inlet and thus through the studied station. Nevertheless, the highest amount of nutrients were detected in the river-influenced site, confirming the definitely unstable feature of the coastal area and the major role of the Po river in shaping the microbial community activities.

The outcome of the PCA revealed similar groupings of variables among stations (Fig. 5) but the sampling cases clearly differed in their distributions between coastal and offshore stations (Fig. 6). Nevertheless, the May and July 2004 samplings showed extremely diverse features with respect to the other sampling, especially in the offshore sites. Moreover, these two samplings had always an opposite position in the bi-plots of the projection of the sampling cases with respect to the PF2, which generally means for DIN, PO_4, Chl *a*, APA and BGLU. In spring 2004 a particularly high Po river outflow occurred, leading to the highest monthly average of the last 15 years. Starting from the second half of June the mucilage event occurred in the central and the northern Adriatic Sea. Aggregates were found along the whole water column and their presence was registered for about a month. Mucilage disappeared at the end of July, when the monthly average of the Po river flow was fivefold lower than in May, reaching typical summer period values. This phenomenon led to strong modifications in microbial activities from the typical seasonal and basin-scale dynamics both in terms of hydrolysis rates and secondary carbon production, which were extremely high right before and after the event, respectively. In fact, exoenzyme activities were generally one order of magnitude higher than those found by Zaccone & Caruso (2002) in the same area and by Van Wambeke *et al.* (2002) in a E–W transect along the whole Mediterranean, and PCP was up to fourfold higher than reported by Fonda Umani *et al.* (2007) for the northern Adriatic Sea.

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