

RESEARCH ARTICLE

## A case study on the application of the MSFD to Mediterranean coastal systems: the Po plume, as a transitional water system in the Northern Adriatic basin

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### Abstract

- 1 - In the frame of the Marine Strategy Framework Directive (MSFD) of the European Community, in order to assess the Good Environmental Status (GES) of the marine coastal and estuarine waters, the eutrophication descriptors include several aspects of the phytoplankton communities (such as composition, abundance and biomass).
- 2 - Two oceanographic campaigns were carried out in the area off the Po River plume, where a highly dynamic frontal zone separates an inshore and an offshore system. The coupling of size structure and diagnostic pigments of phytoplankton communities in relation to different environmental contexts are tested as an expedite and informative tool to assess water quality *sensu* MSFD.
- 3 - The Po plume creates a very dynamic frontal zone, resulting in strong trophic gradients within a relatively small area.
- 4 - The spatio-temporal variability of salinity in coastal waters and in transitional waters plays a pivotal role in structuring phytoplankton communities.
- 5 - The dynamics of forcing factors drive changes in cell-size structure, in the functional group composition as well as a shift in size within the same functional group.
- 6 - The combination of phytoplankton size-structure and chemotaxonomic compositions is proposed as an expedite tool for investigating, at the appropriate scales, the ecology of transitional water systems.

**Keywords:** Phytoplankton size-fractions; HPLC pigments; Chemotaxonomical composition; Photosynthetic parameters; Northern Adriatic Sea; Transitional waters

### Introduction

The implementation of guidelines aimed at the assessment of the environmental quality of aquatic systems is a challenging objective in both theoretical and applied terms. Approaches and methodologies adequate to provide

reliable managerial tools are matter of broad debate, reflected by a rich, heterogeneous and often contradictory literature (Mouillot *et al.*, 2006; Borja and Dauer, 2008; Devlin *et al.*, 2009; Ferreira *et al.*, 2011; Pachés *et al.*, 2012; Garmendia *et al.*, 2013).

Several reviews have dealt with different components of the systems, spanning from a monofactorial to an integrative approach, taking into account both environmental typologies and managerial objectives. Nevertheless, the literature appears to address the complex problem of environmental quality primarily through an observational and analytical grid that underestimates the differences in the environmental context at regional and local levels, particularly for those regions where environmental forcing responds to entirely distinct natural and socio-economic variables. Within the European marine systems, the Mediterranean subregion is among the most challenging for its highly heterogeneous geographical and environmental setting, which contrasts the identification of common quality indexes; on the opposite side, the Atlantic subregion shows a coastal system essentially represented by the estuarine typology, driven by the tidal forcing.

The need for assessing the trophic status of aquatic ecosystems is a priority due to the serious impacts caused by worldwide eutrophication phenomena (Cloern, 2001). In the frame of Marine Strategy Framework Directive (MSFD), the Descriptor 5 (Eutrophication) is regarded as a tool for minimizing the negative effects of anthropogenic eutrophication on the marine ecosystem. The MSFD considers several criteria, among which total chlorophyll concentrations and species shift in floristic composition of phytoplankton communities (e.g. diatom to flagellate ratio) (Druon *et al.*, 2004; Devlin *et al.*, 2007; Ferreira *et al.*, 2007; 2011; Garmendia *et al.*, 2013). The evaluation of water quality status should ideally incorporate suitable multimetric indices (Borja and Dauer, 2008; Diaz *et al.*, 2004; Domingues *et al.*, 2008; Spatharis and Tsirtsis 2010) taking into consideration as many of the fundamental attributes of phytoplankton as possible (i.e. biomass,

community structure, and frequency of blooms). However, the criteria for the assessment of Good Environmental Status (GES) are not yet clearly defined and agreed upon.

Phytoplankton cells span more than 4 orders of magnitude in size, ranging from cells smaller than a micrometer to large diatom chains that can reach several millimetres in length (Sheldon *et al.*, 1972; Margalef 1978; Falkowski *et al.*, 2004) and more than 6 orders as biovolume (e.g. Stanca *et al.*, 2013). As a result, the pathways and efficiencies of transfer of carbon and energy to higher trophic levels depend on the abundance and composition of phytoplankton communities (Falkowski *et al.*, 2003; Cloern and Dufford 2005; Domingues *et al.*, 2008).

Cell size not only defines the metabolic activity, growth rates and numerical abundance (Malone, 1980; Chisholm, 1992; Belgrano *et al.*, 2002), but also strongly influences biogeochemical cycles via size-dependent sinking (Falkowski *et al.*, 1998) and affects community structure and dynamics via size-dependent species interactions (Banse, 1992; Cohen *et al.*, 2003). Phytoplankton abundance is sensitive to changes of environmental factors such as light availability, micro- and macro-nutrients concentrations, temperature, mixing and turbulence, depth of euphotic zone and selective grazing and may respond by shifting in cell size and/or taxonomic composition (Margalef, 1978; Sprules and Munawar, 1986; Gaedke, 1992; Rodríguez *et al.*, 2001; Jiang *et al.*, 2005; Kudela *et al.*, 2006; Finkel *et al.*, 2010). Cell size and size spectra were also found to respond to anthropogenic pressures, being incorporated in multimetric indices of phytoplankton ecological status (Lugoli *et al.*, 2012; Vadrucchi *et al.*, 2013). Coastal phytoplankton communities undergo large variations in abundance, size structure and species diversity due to the dynamic nature of the environment such as potential

mixing of distinct water masses of estuarine, neritic and open-ocean origin (Ahel *et al.*, 1996; Pelegrí *et al.*, 2005; Mangoni *et al.*, 2008; Sabetta *et al.*, 2008). In coastal areas affected by seasonal upwelling as well as anthropogenic input, biological changes are even more pronounced, as nutrient-rich upwelled waters favour the dominance of large organisms (micro-plankton, usually diatoms) and high levels of primary productivity (Tilstone *et al.*, 2003; Cermeño *et al.*, 2005). The absence of canonical phytoplankton patterns might be expected, given the multiplicity of anthropogenic, atmospheric and terrestrial forcing that drive physical and community dynamics of near shore marine ecosystems (Cloern and Jassby, 2008). Phytoplankton biomass, mainly in terms of chlorophyll *a* concentrations, has been used as an indicator of trophic conditions since the Sixties of the last century. In recent years, the HPLC (High Performance Liquid Chromatography) analysis of phytoplankton pigments has been used to single out and estimate phytoplankton functional groups (Zapata *et al.*, 2000; Pearl *et al.*, 2003; Havskum *et al.*, 2004; Wright and Jaffrey, 2006; Henriksen *et al.*, 2011; Roy *et al.*, 2011). Several accessory pigments ('marker pigments') are unique to specific taxonomic groups (e.g. prasinoxanthin in some prasinophytes and peridinin in most dinoflagellates) and others are found mainly in one or a few groups (e.g. 19-hexanoyloxyfucoxanthin in haptophytes and some dinoflagellates, and fucoxanthin in diatoms, chrysophytes, haptophytes and raphidophytes) (e.g. Bjørnland and Liaen-Jensen, 1989; Wright and Jeffrey, 2006). Therefore, pigment analysis is a powerful mean of recognizing nano- and pico-planktonic organisms, which are normally unrecognizable by light microscopy and are often difficult to preserve in their morphological characteristics (Andersen *et al.*, 1996; Peeken, 1997; Ansotegui *et al.*,

2003; Rodriguez *et al.*, 2006; Latasa 2007; Mangoni *et al.*, 2011; Klöpper *et al.*, 2013). The approach to size structure and pigment spectra of the phytoplankton communities adopted in this article is a contribution to identify an expedite and informative tool to assess water quality *sensu* MSFD. In this study, we analyzed the distribution of phytoplankton communities in terms of size fractions and contributions of main phytoplankton functional groups (e.g. diatom to flagellate ratio) across the Po river plume in the Northern Adriatic Sea, where a highly dynamic frontal zone separates an inshore from an offshore system. We discuss the changes in phytoplankton size classes and chemotaxonomic composition in relation to salinity and macro-nutrient concentrations on different spatial scales and their eco-physiological response to the ensuing environmental conditions.

## Materials and methods

### *Study area*

The remarkable hydrodynamics observed in the Northern Adriatic Sea offer a good scenario to study mesoscale variations of phytoplankton assemblages in relation to environmental forcing. In this area, the combination of natural environmental features and anthropogenic impact results in a variety of different trophic contexts over different space and time scales (Harding *et al.*, 1999). The Adriatic Sea physical and biological oceanographic characteristics have already been extensively studied focusing mainly on factors controlling dissolved nutrient concentrations and primary production processes within the Northern basin (e.g. Malanotte Rizzoli and Bergamasco, 1983; Franco, 1984; Degobbis *et al.*, 1986; 2000; 2005; Smodlaka, 1986; Gilmartin *et al.*, 1990; Revelante and Gilmartin, 1992; Artegiani *et al.*, 1993; Zoppini *et al.*, 1995; Bergamasco *et al.*, 1996; Fonda Umani, 1996; Gačić *et al.*, 1997; Vadrucci *et al.*, 2002; Fonda Umani *et*

*al.*, 2005; 2012; Mauri *et al.*, 2007; Tedesco *et al.*, 2007; Mangoni *et al.*, 2008; Mozetič *et al.*, 2010; 2102; Bernardi Aubry *et al.*, 2012; Cozzi *et al.*, 2012).

The basin is characterised by wide inter-seasonal and inter-annual variations of physical parameters (e.g. temperature and salinity) and circulation, strongly dependent by atmospheric forcing and riverine inputs (Russo and Artegiani, 1996; Artegiani *et al.*, 1997). The Po accounts for about 70% of the freshwater discharge and nutrient load, significantly influencing large areas of the northern Adriatic Sea (Degobbis and Gilmartin, 1990; Raicich, 1996; Cozzi and Giani, 2011). The combined effects of freshwater input, wind regime and seasonal heat budgets contribute to increase complexity and drive the stratification dynamics of the water column and the extension of the river plume (Fonda Umani, 1996). Nutrient availability has been suggested as the main factor determining phytoplankton biomass concentrations related to the high variability of the river flow and nutrient input (e.g. Gilmartin *et al.*, 1990; Vollenweider *et al.*, 1992; Harding *et al.*, 1999; Mauri and Poulain 2001; Zavatarelli *et al.*, 2000; Artioli *et al.*, 2008; Cozzi *et al.*, 2012). Varying trophic conditions characterize therefore the basin and recurrent algal blooms occur in the coastal area more directly influenced by the Po while very low biomass characterises the area outside the coastal front (Fonda Umani, 1996; Mangoni *et al.*, 2008; Giordani *et al.*, 1999). However, phytoplankton biomass and primary production levels are relatively modest in the northern Adriatic compared to other coastal embayment and estuaries with similar hydrological characteristics and/or nutrient inputs - e.g. Chesapeake Bay, Baltic Sea- (Boynton *et al.*, 1995; Zoppini *et al.*, 1995). Exceptionally high surface Chla concentrations (up to 800 mg m<sup>-3</sup>) (Gilmartin and Revelante, 1983) and primary production levels (up to 400 g C m<sup>-2</sup>yr<sup>-1</sup>) (Boynton *et*

*al.*, 1982; Giordani *et al.*, 1999) have been occasionally reported in the coastal waters facing the Po delta. In addition, very low concentrations of phosphate, coupled with exceptionally high chlorophyll *a* values, suggest that the statements indicating phosphorous as the classical growth-limiting factor of phytoplankton abundance in the north western Adriatic Sea must be reconsidered (Tedesco *et al.*, 2007). A recent consideration of a century long time series of chlorophyll *a* (Chla) concentrations demonstrated a significant decline of phytoplankton biomass in the northern Adriatic (Djakovac *et al.*, 2012; Gašparović, 2012; Mozetič *et al.*, 2010; 2012). Reduced river discharges may be responsible of this new ecological scenario through variations in the input of nitrogen and silicate. In fact, the decrease of nutrient loads from 2003 to 2007 indicates that recurrent crisis in river water availability might significantly lower the trophic level in coastal zone (Cozzi *et al.*, 2012). Furthermore, Zanchettin *et al.* (2008) indicated that freshwater discharge seems to be a good hydrological tool for assessing the northern Adriatic response to background climatic changes on decadal and multi-decadal time scales, so that the observed decrease could therefore be related to larger-scale climatic forcing (Giani *et al.*, 2012).

#### *Sampling and analysis*

In the frame of the Italian national project PRISMA, two oceanographic campaigns were carried out in summer 1996 in the area off the Po River delta on board R/V “Coopernaut Franca”.

The cruises were conducted from 17 to 22 July in the first campaign and from August 31 to September 3 in the second one. In the two campaigns, 112 and 74 stations were respectively sampled. Selected stations, 18 for the first campaign and 21 for the second one, were sampled for biological parameters (Fig. 1a, 1b).

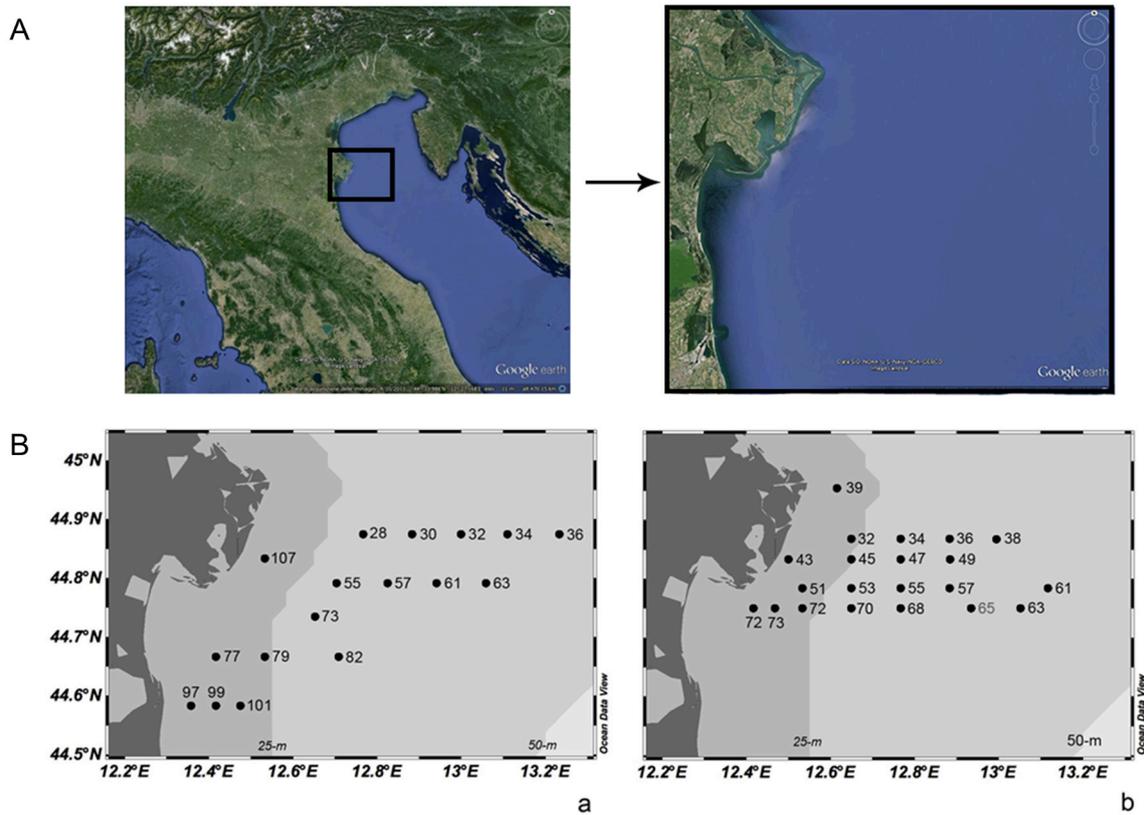


Figure 1. A- Geographic position of the studied area. B- The sampling stations: July, for the first campaign (a) and August-September, for the second (b).

Vertical profiles of temperature, salinity and oxygen from surface to bottom were performed using a SBE 911 Plus CTD probe equipped with a SBE Oxygen sensor. At biological stations, from 2 to 4 depths were sampled on the basis of temperature and salinity profiles. Water samples were collected with a SBE 32 Carousel sampler equipped with Niskin bottles (12 l). Reactive silicate, phosphate, nitrate, nitrite and ammonia concentrations were measured according to Hansen and Grasshoff (1983) with a Technicon II analyzer. Five litres of seawater were drawn from the Niskin bottle and, after careful mixing, subsamples were collected for the determination of total and size-fractionated Chla, pigment spectra and PvsE experiments.

Fractionation of phytoplankton biomass was performed by serial filtration. One subsample was collected on a Whatman GF/F filter (GF/F tot); two subsamples were passed through a Nitex net (20  $\mu\text{m}$  mesh size) and a Nuclepore membrane (2  $\mu\text{m}$  pore size) and thus collected on Whatman GF/F filters. For more details, see Mangoni *et al.* (2008). The filters for spectrofluorometric analyses of Chla and phaeopigments were stored at  $-80^{\circ}\text{C}$ , analyzed a week after sampling with a Spex Fluoromax (Jeffrey and Humphrey, 1975); the instrument was checked daily with a Chla standard solution (*Anacystis nidulans* by Sigma).

Subsamples (3 l) were filtered through 47 mm GF/F Whatman filters and stored at  $-80^{\circ}\text{C}$  for HPLC analyses (Beckman Systems

Table 1 – Abbreviations used throughout this article for photosynthetic pigments.

Pigment	Abbreviation
Chlorophyll <i>a</i>	Chl <i>a</i>
Chlorophyll <i>b</i>	Chl <i>b</i>
Fucoxanthin	Fuco
Peridinin	Perid
19'hexanoyloxyfucoxanthin	Hex
19'butanoyloxyfucoxanthin	But
Alloxanthin	Allo
Zeaxanthin	Zea
Diadinoxanthin	Dd
Diatoxanthin	Dt

Gold analyser, Mantoura and Llewellyn, 1983). Calibration of the instrument was carried out using twenty standard pigments (International Agency for <sup>14</sup>C Determination, VKI Water Quality Institute). Abbreviations of the photosynthetic pigments are reported in Table 1.

The photosynthetic performance of phytoplankton assemblages were analyzed through PvsE experiments (Babin *et al.*, 1994) in relation to the different water column dynamics as well as to the strong differences in the amplitude of upper mixed layer (UML). During the first campaign, two PvsE

experiments were conducted in coastal (St. 55) and in offshore (St. 63) stations, in water columns characterized by different physical structures. During the second campaign, the PvsE experiments were conducted only at the surface in order to assess the photosynthetic parameters along a coastal-offshore gradient (from St. 74 to St. 68). At coastal St. 74, a PvsE experiment was also conducted at 11-meter depth. For methodological details, see Mangoni *et al.*, 2008.

The photosynthetic parameters were derived from the model by Platt *et al.* (1980):  $P^B(E) = P^B_s [1 - \exp(-\alpha E / P^B_s)] \exp(-\alpha E / P^B_s)$ . Symbols and units are reported in Table 2.

The contribution of the main phytoplankton groups to total Chl*a* was estimated on the basis of the concentrations of biomarker pigments using the chemical taxonomy software CHEMTAX (Mackey *et al.*, 1996; Muylaert *et al.*, 2006). In order to assess the trophic status, we have tested the Fp ratio (Fuco+Perid)/(Fuco+Perid+But+Chl*b*+Hex+Allo+Zea) (Claustre, 1994).

In order to assess the evaluation of the photo-acclimation processes of phytoplankton communities, we have analyzed the photo-protective response through Diatoxanthin (Dt) and Diadinoxanthin (Dd), involved in the epoxidation-de-epoxidation cycle regulated by light (Mangoni *et al.*, 2009; Brunet *et al.*, 2011).

Table 2 – List of symbols and units of the photosynthetic parameters.

Symbol	Definition	Unit
$P^B_s$	The potential light-saturated chl <i>a</i> -normalized rate of photosynthesis in absence of photoinhibition	mg C (mg chl <i>a</i> ) <sup>-1</sup> h <sup>-1</sup>
$P^B_{max}$	Photosynthetic capacity (maximum Chl <i>a</i> -normalized photosynthetic rate)	mg C (mg chl <i>a</i> ) <sup>-1</sup> h <sup>-1</sup>
$\alpha$	Photosynthetic efficiency	mg C (mg chl <i>a</i> ) <sup>-1</sup> h <sup>-1</sup> (μmol photons m <sup>-2</sup> s <sup>-1</sup> ) <sup>-1</sup>
$\beta$	Photoinhibition parameter	mg C (mg chl <i>a</i> ) <sup>-1</sup> h <sup>-1</sup> (μmol photons m <sup>-2</sup> s <sup>-1</sup> ) <sup>-1</sup>
$E_k$	Light saturation parameter ( $P^B_{max} / \alpha$ )	μmol photons m <sup>-2</sup> s <sup>-1</sup>
$E_m$	Optimal irradiance for photosynthesis	μmol photons m <sup>-2</sup> s <sup>-1</sup>

Principal component analysis (PCA), based on a Pearson's correlation matrix, was performed with 112 objects (sampled stations and depths described by the 12 variables, in order to investigate the relationship between the *in situ* fresh water input (salinity and macro-nutrients) and the biological features (phytoplankton biomass, percentage contribution of size classes and main phytoplankton groups).

All analyses were performed with XLSTAT 2012.3 software and the differences were deemed statistically significant at  $p < 0.01$ .

The results of a PCA are presented using diagrams of factor loadings (projection of the variables on the factor-plane described by the 1<sup>st</sup> and 2<sup>nd</sup> principal factors) and scores (projection of the sampling cases on the factor-plane described by the 1<sup>st</sup> and 2<sup>nd</sup> principal factors). Variables with the highest loadings have the greatest influence on the samples separation.

## Results

### *Hydrological features and nutrient distribution*

During the first campaign, the surface salinity varied from 31.69 in the central area of the northern transect (St. 32) to 37.75 at St. 57. The incoming fresh water generates a strong W-E gradient in the northern area, in contrast with the southern area where the water masses flow along the western coastal areas (Fig. 2a).

During the second campaign, the river input affected the entire investigated area, with a presence of a frontal structure within transitional coastal waters (Fig. 2b). Surface salinity varied from 30.25 at inshore station St. 32 to 36.09 at offshore station St. 61.

Fig. 3a and 3b show the vertical distributions of salinity, potential temperature and potential density anomaly along two selected transects representative of the maximum extension of the frontal areas during the two campaigns.

In July, along the northern transect (St. 107

to St. 63), the boundary between coastal and open waters (saline front) was located at ~55 km from the coast (Fig. 3a), while in the southern stations the frontal area was located at ~11 km from the coast. The water column salinity along the entire transect varied from 25.60 to 38.02. During the second campaign along the transect from St. 74 to St. 63, the salinity varied from 30.25 to 38.05 in the surface and bottom layers, respectively (Fig. 3b). The easternmost station of the transect was located at ~57 km from the coast.

The temperature/salinity diagrams (TS) relative to the two campaigns are reported in Fig. 4. Differences in terms of contribution of fresh water are clear. In particular, the July campaign is characterized by fresh water occupying a large layer of the water column; in contrast, the August campaign showed a high contribution of fresh water essentially confined to the surface layer.

In July, the UML of the three stations located off the saline front was 24 m at St. 63 and St. 67 and 14 m at St. 82. Within the frontal area, the mixed layer varied from 0 to 3 m, emphasizing the role of fresh water in driving the water column stability. In August, the UML varied from 0 to 10 m with a mean value of 3.5 m over the entire investigated area.

The surface distributions of Dissolved Inorganic Nitrogen (DIN) and phosphate are reported in Fig. 2a and 2b. During the first campaign the nitrate concentrations were under the detection limits along the water column in several stations, showing a mean value of 0.13  $\mu\text{M}$ . Detectable concentrations of nitrate were measured only at southern coastal stations and in the central areas (St. 30 and St. 32) of the northern transect (Fig. 2a). In contrast, phosphate and silicate showed relatively high concentrations, with mean values of 0.24  $\mu\text{M}$  and 5.46  $\mu\text{M}$  respectively (Fig. 2a). During the second campaign, the mean concentrations of nitrate, phosphate and silicate along the water column were 1.31

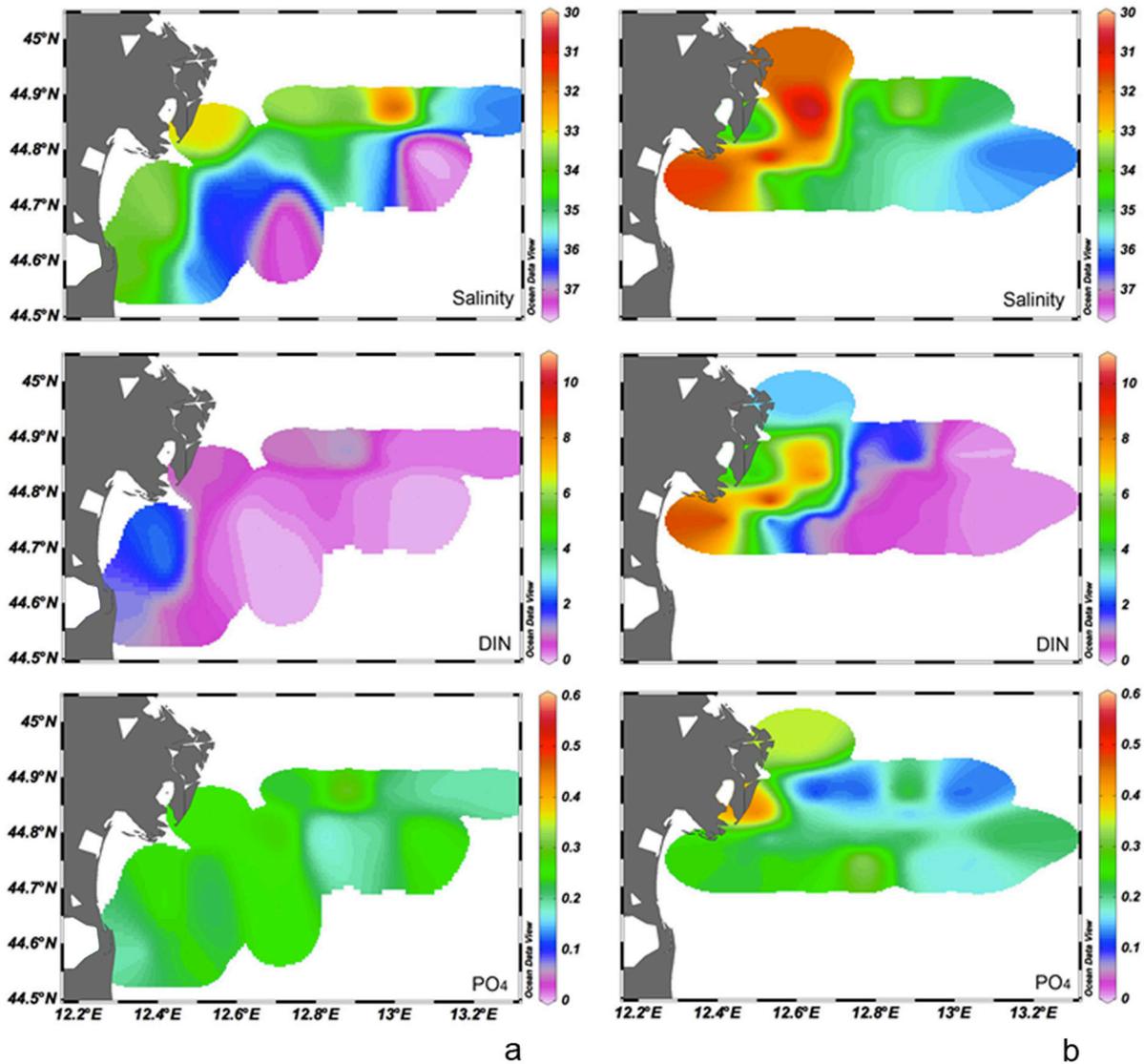


Figure 2. Spatial distribution of surface salinity, DIN ( $\mu\text{M}$ ) and phosphate ( $\text{PO}_4$ ,  $\mu\text{M}$ ) – first campaign July (a) and second, August-September (b).

$\mu\text{M}$ ,  $0.23 \mu\text{M}$ ,  $8.40 \mu\text{M}$  respectively (Fig. 2b). A clear relation among total biomass, salinity and nutrient concentration is evident only during the second campaign when the DIN was clearly related to fresh water input.

*Phytoplankton biomass and size fractions*

The surface distributions of the total biomass (Chla) and the percentage contribution of different size fractions in July are shown

in Fig. 5a. During the sampling period, Chla concentrations varied from  $0.100 \text{ mg m}^{-3}$  at St. 67, located in the eastern area characterised by the highest salinity value, to  $3.231 \text{ mg m}^{-3}$  at St 77, located in the southern coastal area. The mean value was  $0.637 \text{ mg m}^{-3}$ . The highest contribution of the micro-fraction, observed at St. 77, was associated with a maximum of biomass. The nano-fraction predominates in the whole area,

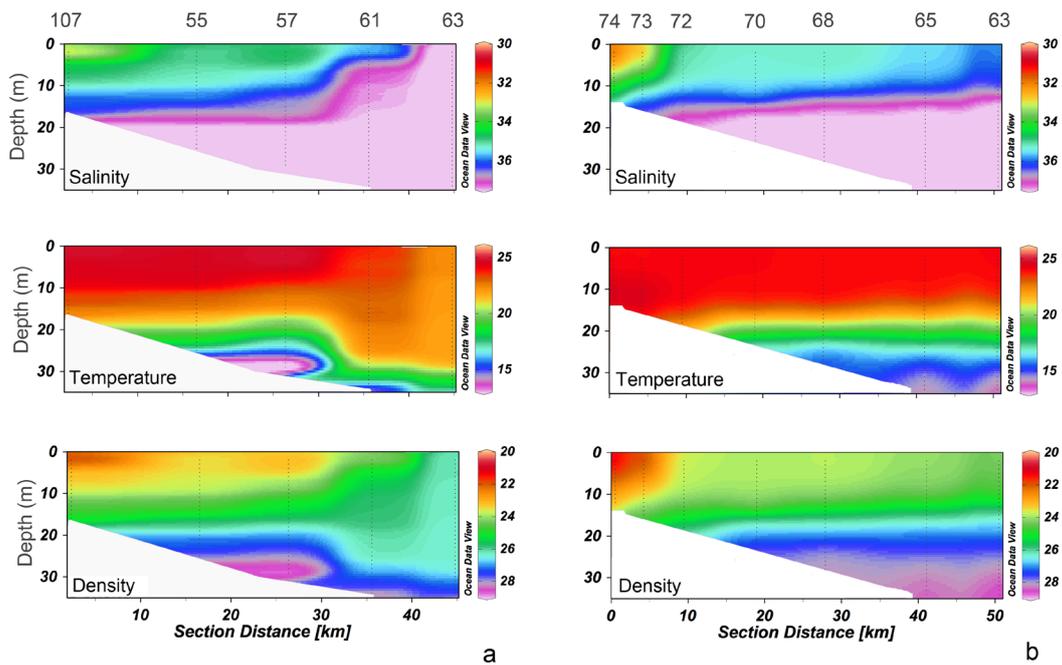


Figure 3. Vertical distribution of the salinity, temperature and density in two transect of first campaign, July (a) and second, August-September (b).

while the maximum contribution of the pico-fraction (up to 55%) was measured at St. 63, characterized by the highest salinity value.

The distribution of phytoplankton biomass and size fractions at the surface, observed during the second campaign, was completely different from that observed in July (Fig. 5b); the concentrations of Chla were twice as those reported in July. The mean value measured at surface was  $1.348 \text{ mg m}^{-3}$ ; the minimum and maximum values,  $0.336$  and  $6.417 \text{ mg m}^{-3}$  of Chla respectively, were measured at St. 61 and St 39 characterized by the highest (36.09) and the lowest (31.86) salinity values, respectively.

The distribution of the size fractions showed a NW-SE gradient with a predominance of the micro-fraction in the coastal area (80% at St. 73). The nano-fraction characterized the central area outside of the coastal front, while the pico-fraction showed the maximum contribution at St. 61, located in the south-eastern area and characterized by

the minimum concentration of biomass and maximum value of salinity (Fig. 5b and 2b). The area was characterised by a water column stability essentially driven by the fresh water input. In order to investigate the effects of such input on phytoplankton communities, the layer above (0-15 m) and below the alocline (15 m to bottom) were analysed separately. The total biomass concentration and the percentage contribution of size fractions in the two layers are reported in Table 3.

In July (first campaign), the mean contribution of size classes showed the dominance of the nano-fraction in the surface layer. In the deep layer the nano- and pico-fractions were dominant with the same relative contribution (Tab. 3a). A different picture of size classes contribution was observed during the second campaign. In the surface layer, the average contributions of micro- and nano-fractions had a quite similar percentage, while that of the pico-fraction accounted for the 23.0%.

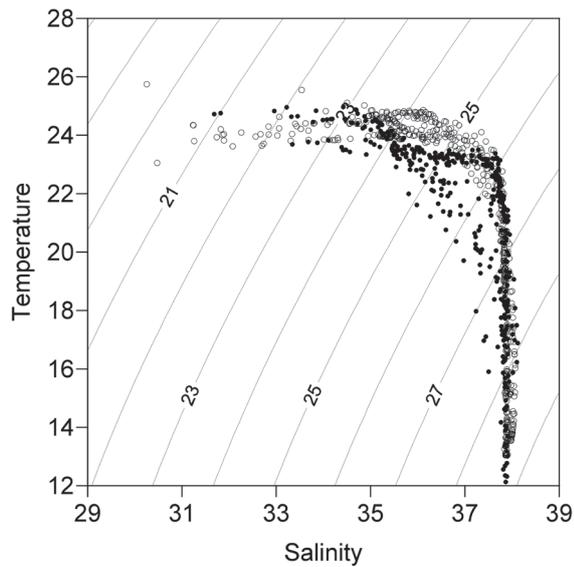


Figure 4. TS diagram of sampled stations – July, first campaign (closed circles) and August-September, second campaign (open circles).

In the deep layer, the contribution of micro- and nano-fractions changed slightly, while the pico-phytoplankton fraction remained substantially constant (Tab. 3b).

*Chemotaxonomy of the main functional groups (CHEMTAX analyses)*

During the first campaign, diatoms showed similar spatial surface distribution of total Chla as well as that of the micro- and nano-fractions. Flagellates (chlorophytes + pelagophytes + haptophytes + cryptophytes + prasinophytes), were more characteristic of the central areas and of the offshore waters characterized by low salinity (e.g. St. 32). The distribution of cyanobacteria was associated to the minimum of biomass and to the highest salinity values, in accordance to the distribution of the pico-fraction.

During the second campaign, diatoms

Table 3 - Maximum, minimum and mean values of Chla (TOT Chla mg m<sup>-3</sup>), micro- (> 20 µm), nano- (2-20 µm) and pico- (< 2 µm) fractionated as for Chla, and percentage of dinoflagellates, cyanobacteria, diatoms and flagellates in the surface and deep layers for the two campaigns: July for the first campaign (a) and August-September for the second one (b).

Surface layer								
	Tot Chla	% micro-	% nano-	% pico-	% Dino	% Cyano	% Diat	% Flag
max	3.2	64.0	77.3	53.0	31.4	10.4	87.8	43.3
min	0.2	2.1	4.2	7.2	0.3	0.0	32.0	1.98
avg	0.8 (± 0.6)	16.7 (± 15.3)	51.7 (± 16.0)	31.3 (± 13.3)	12.8 (± 8.21)	4.01 (± 3.12)	59.3 (± 14.0)	23.9 (± 12.2)

**a**

Surface layer								
	Tot Chla	% micro-	% nano-	% pico-	% Dino	% Cyano	% Diat	% Flag
max	6.4	79.5	71.9	70.2	49.8	16.9	84.0	55.0
min	0.3	0.7	14.7	4.4	3.4	0.6	22.4	7.6
avg	1.8 (± 1.7)	39.2 (± 23.0)	37.8 (± 15.1)	23.0 (± 15.3)	17.4 (± 13.0)	5.3 (± 4.3)	53.4 (± 18.0)	24.0 (± 11.2)

**b**

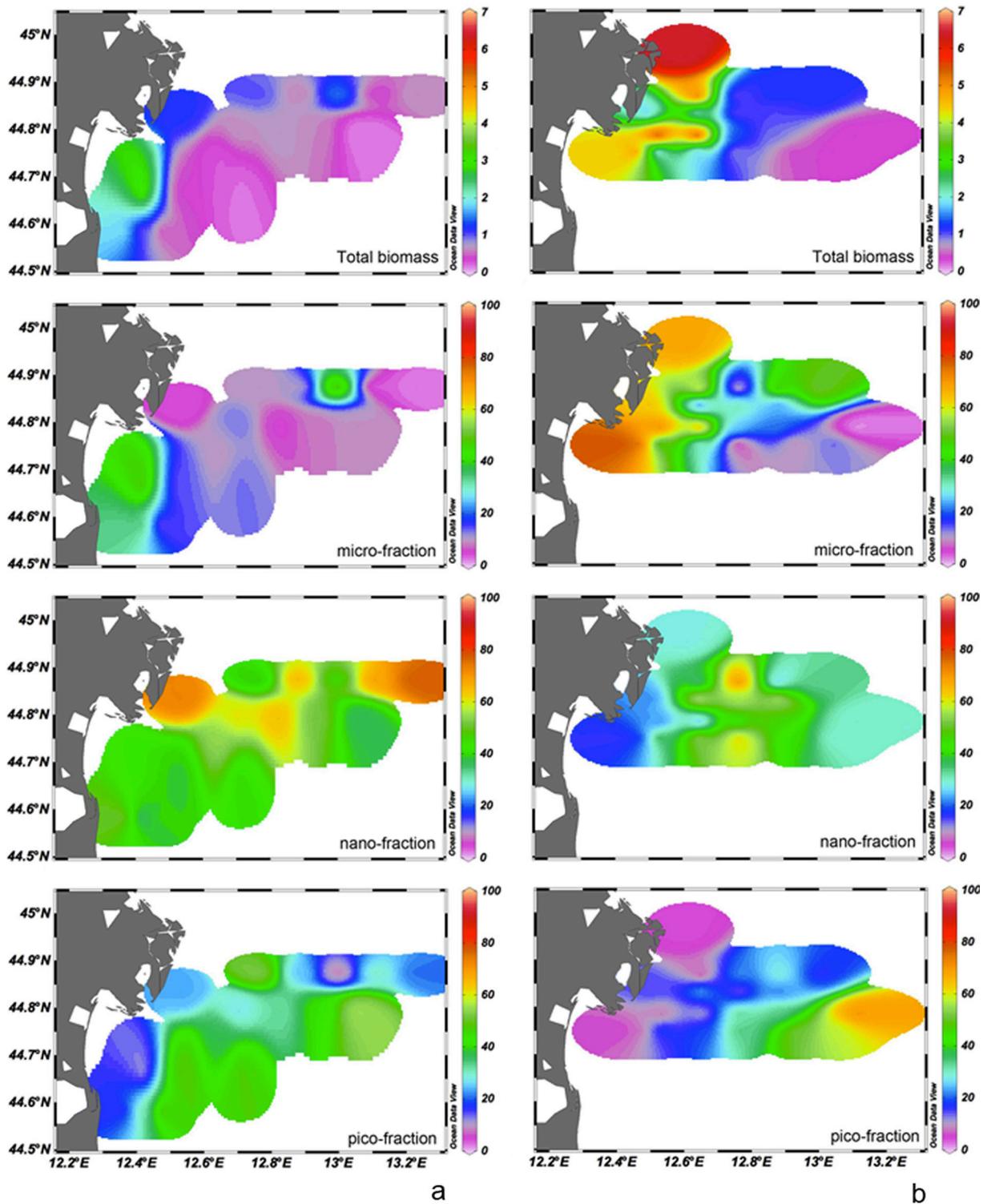


Figure 5. Spatial distribution at surface of total biomass (Tot Chl<sub>a</sub> mg m<sup>-3</sup>) and percentage contributions of the micro- (> 20µm), nano- (2-20 µm), and pico-fraction (< 2µm) - July (a) and August-September (b).

dominated at the surface over almost the whole area, except at St. 34, where their contribution was <10%. In the stations 38, 45 and 61, the relative contribution was less than 40%. The flagellates characterized the areas outside the salinity front and at St. 61 and St. 63 located in the SE area; in the coastal area, their relative contribution was <10%. Cyanobacteria were predominant in the SE sector.

During the first campaign, in the surface layer, the mean contributions of the main functional groups were 59.2% for the diatoms and 23.9% for the flagellates, 12.7% for the dinoflagellates, 4.0% for the cyanobacteria; the haptophytes contributed for more than 50% of flagellates (Tab. 3a). In the deep layer, a decrease in the percentage contribution of dinoflagellates and diatoms and an increase of cyanobacteria and flagellates were observed (Tab. 3a).

During the second campaign, in the surface layer, the mean contribution of diatoms was 53.4%, flagellates 23.9%, dinoflagellates 17.4% and cyanobacteria 5.2%. In the deep layer, a slightly increase of diatoms and flagellates and decrease of cyanobacteria and dinoflagellates was observed (Tab. 3b).

#### *Diagnostic pigments*

The strong relationship between total Chl $a$  and diagnostic pigments ( $\Sigma DP = \text{Fuco, Perid, But, Chl}b, \text{Hex, Allo, Zea}$ ) concentrations and between total Chl $a$  and the predominant diagnostic pigments (Fuco, Per and Hex) for the two campaigns are reported in Fig. 6. In particular, Fuco represents the predominant pigment in both campaigns, emphasizing the high contribution of diatoms in micro- and nano- size classes.

In order to identify the trophic status of the investigated area, we have tested the pigment index Fp ratio utilized by Claustre (1994).

In Fig. 7, the correlations between the integrated values of Chl $a$  and Fuco and Per in the entire water column, as well as between

integrated Chl $a$  and Fp ratio (Fuco+Perid)/(Fuco+Perid+But+Chl $b$ +Hex+Allo+Zea) for both campaigns are reported. The good correlations reported in Fig. 7 highlight the utility of this index also in coastal areas and in transitional waters. The index can be considered as the biomass ratio of phytoplankton involved in new production over total phytoplankton. As reported for all oceanic provinces (Claustre, 1994), the changes in Fuco and, to a lesser extent in Per, were tightly coupled to changes in Chl $a$  standing stock, while the other accessory pigments were invariable with respect to changes in Chl $a$  concentrations.

#### *PvsE experiment and photo-protection index*

The photo-physiology of phytoplankton assemblages were studied through the analyses of the photo-acclimation indexes derived from PvsE experiments as well as through the evaluation of specific carotenoids indicators of the photo-protection mechanism.

During the first campaign, the PvsE curves and the photosynthetic parameter of St. 55 (Fig. 8a) showed clear differences in the photosynthetic performance  $P^B_{max}$  of phytoplankton assemblages above and below the UML (Fig. 9a). The PvsE curves and the photosynthetic parameter of St. 63 (Fig. 8b), outside the influence of fresh water input, showed similar values of photosynthetic parameters along the well mixed water column (Fig. 9b). It should be noted that the photosynthetic efficiency of the populations of the external station was about twice as those in the coastal station (St. 55).

During the second campaign, the attention was focused on the study of the spatial variability of photosynthetic parameters along a coastal-offshore transect, in order to assess the photosynthetic parameters in areas with strong differences in fresh water input (Fig. 3b). A clear spatial gradient of photosynthetic performance was observed along the southern transect, with a decrease

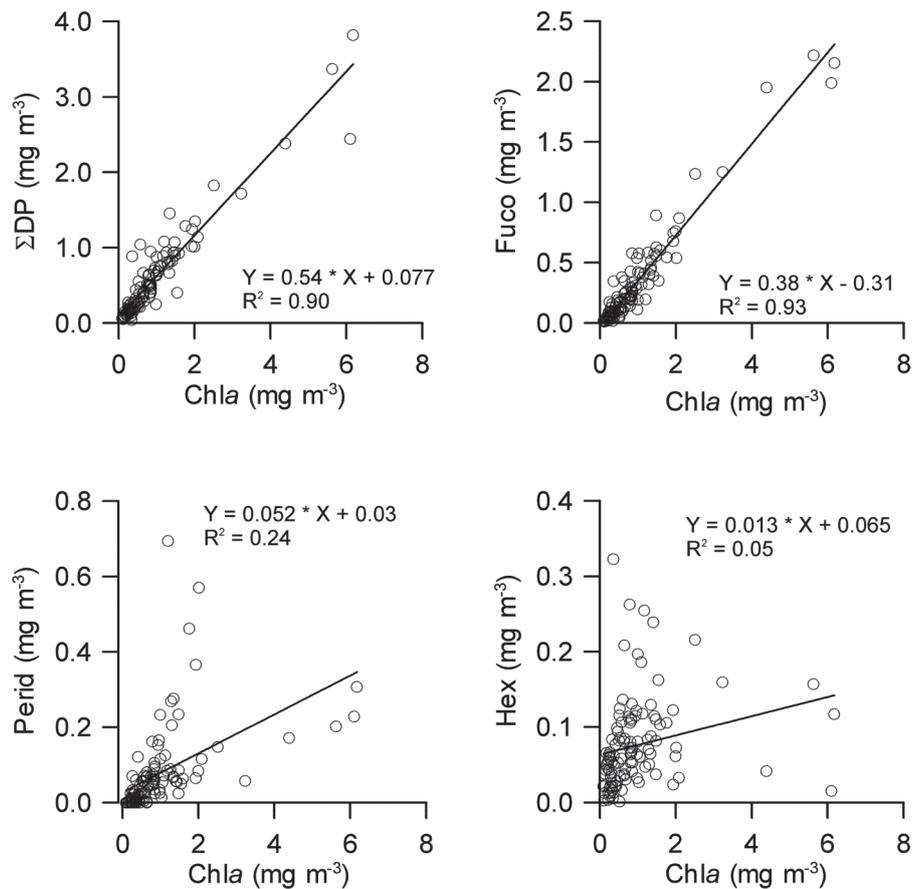


Figure 6. Correlation for all sampling depths between of total Chla and diagnostic pigments concentrations ( $\Sigma DP = \text{Fuco, Perid, But, Chlb, Hex, Allo, Zea}$ ) ( $p < 0.01$ ); Fuco ( $p < 0.01$ ); Perid ( $p < 0.01$ ); Hex ( $p < 0.05$ ).

from St. 74 to St. 68. The  $P_{\max}^B$  varied from 20.17  $\text{mgC (mgChla)}^{-1}\text{h}^{-1}$  in the coastal station St. 73 (among the highest ever recorded in the marine environment) to 3.32  $\text{mgC (mgChla)}^{-1}\text{h}^{-1}$  in the central area of the transect (St. 68) (Fig. 10). Low values of photosynthetic performance and biomass appeared anomalous in an offshore area characterised by a notable contribution of fresh water (salinity  $< 35.5$ ). In addition, at the coastal St. 74 the high values of  $P_{\max}^B$  at 11 m depth indicate that the entire surface layer (0-15 m depth) was characterized by a high photosynthetic performance.

The de-epoxidation state,  $Dt/(Dt+Dd)$  ratio, that represents a photoprotective index of

the phytoplankton assemblages in the water column, was up to 0.25 within the UML and less of 0.05 in the layer below at costal St. 55 in July; while in St. 63 was less than 0.05 for all the sampled depths. During the second campaign, the photo-protective index varied from 0.20 to 0.03.

#### Multivariate approach

The PCA analysis (Fig. 11a, 11b) revealed a strong dependence of total biomass from salinity. Low salinity samples were characterized by high Chla concentrations, high percent contribution of micro-phytoplankton and dinoflagellates and DIN concentration ( $p < 0.05$ ), as recorded at the

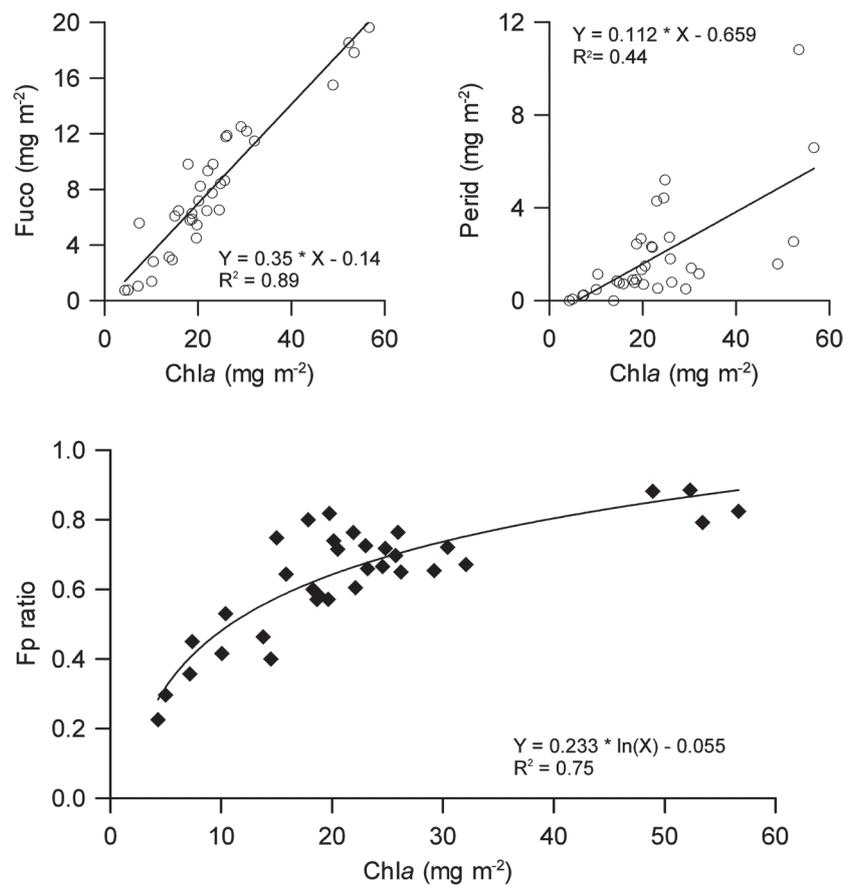


Figure 7. Correlation between integrated values of total Chla and Fuco ( $p < 0.01$ ); Perid ( $p < 0.01$ ); Fp ratio.

surface in August. The nano-fraction did not show any significant correlation to salinity but increased its contribution with phosphate concentration. The negative correlation with the percent contribution of dinoflagellates indicates that this group was essentially present in the micro-size fraction. Diatoms did not display a significant correlation with the largest fractions (micro- and nano-), but were negatively correlated with the percent contribution of pico-phytoplankton, of flagellates and of cyanobacteria ( $p < 0.05$ ); diatoms were positively correlated with silicate concentrations. The smallest size fraction was associated to high salinity values and was mainly composed by flagellates and cyanobacteria, as observed in the deep layer in July.

*Fp-index and trophic status*

All the samples were split in to three different classes based on the Fp-index (Fig. 12). The samples characterized by  $Fp < 0.40$  were grouped in the first class (A); the second class (B) was characterized by  $0.40 \leq Fp \leq 0.65$ , while the third class was composed by the samples with  $Fp > 0.65$ .

For each class, the distributions of salinity, total biomass and size structure (in terms of total biomass) were analyzed by means of box plot (Fig. 12).

A progressive decrease in salinity values and an increasing variability were observed from class A to class C, which showed the strongest variability ( $IQR = 2.71$ ) and the lowest median value (35.64). On the contrary, the total biomass showed the lowest values

(median 0.23) and variability (IQR=0.16) in the first class (A) and the highest concentrations (median 1.00) and highest variability (IQR=0.87) in class C. The similar trend of the total biomass showed the DIN concentrations with the median 0.14 in the class A (IQR=0.17), 0.33 in B (IQR=0.63) and 1.08 in class C (1.71).

As regards the size structure, class A was mainly composed by the pico-phytoplankton (median 56%); class B by the nano-fraction (median 34%) and class C by the largest phytoplankton cells (median values for nano-

43% and for micro- 31%). The percentage of the micro-fraction showed a very low variability in the class A (IQR=8) and an increase in classes B and C (IQR=33).

**Discussion**

The Po river strongly affects the thermohaline circulation in the northern Adriatic Sea (Artegiani *et al.*, 1997). The western coastal area, characterized by large riverine inputs, has a structure of the water column driven by salinity and, hence, a very stable water column. This hydrographic structure strongly affects the distributions of abiotic parameters driving primary production processes. By contrast, the offshore area had an homogeneous water column and therefore its stability was essentially driven by temperature (Degobbis and Gilmartin, 1990; Raicich, 1996).

As regards the nutrient distribution, a different picture with respect to those reported for the area was observed. Phosphorus limitation is a largely acknowledged feature of the northern Adriatic, and has become even more severe since late 80's, due to the removal of phosphates from anthropogenic discharges. The N/P ratio has always exceeded 100, but a N:P>1000 has been frequently reported, thus marking a constant, and at times severe, phosphorous limitation (Rinaldi *et al.*, 1997). In this context, the extension of coastal areas affected by the riverine input generated different subsystems, from a productive (potentially eutrophic) shallow coastal area to oligotrophic conditions in the off-shore waters, and mesotrophic ones in between.

The distribution of the surface salinity observed during the two summer campaigns emphasized the high variability in space and in time of the area, essentially driven by the discontinuous pulse of freshwater inputs from the Po, as well as by atmospheric and environmental forcings at local scales. Different subsystems can be thus identified in a restricted area off the Po delta.

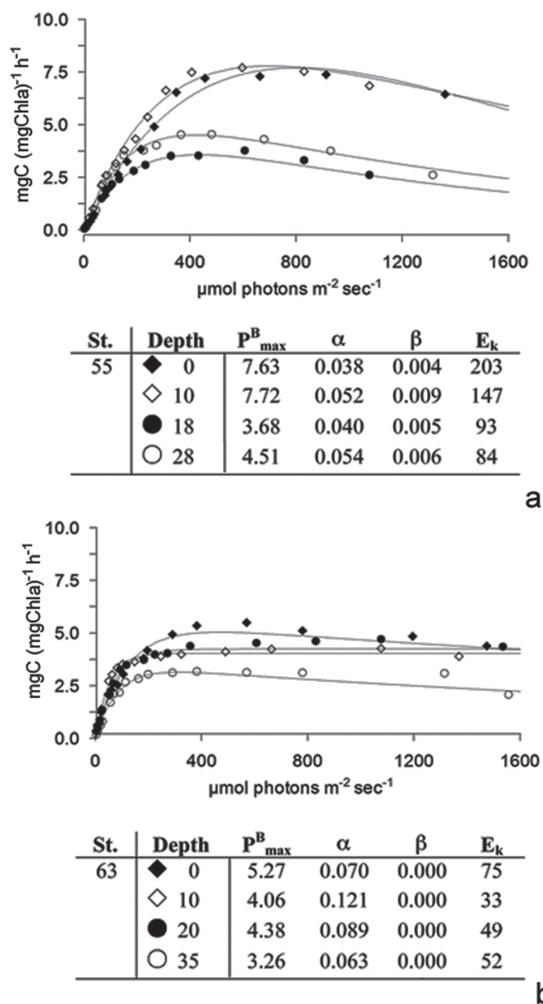


Figure 8. PvsE curves along the water column at stations 55 (a) and 63 (b) and photosynthetic parameters. July, first campaign.

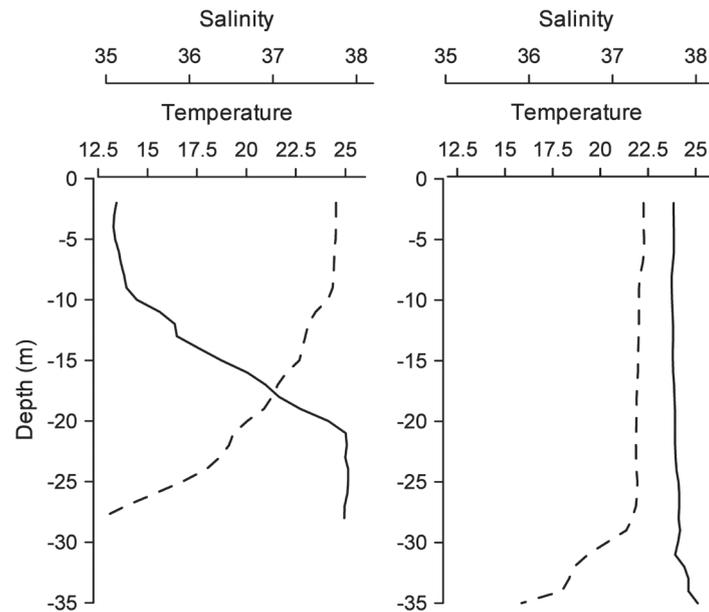


Figure 9. Vertical profiles of salinity (combined line) and temperature (dotted line) at stations 55 (a) and 63 (b). July, first campaign.

The high discontinuity along a coastal-offshore gradient observed during the first campaign giving rise to complex physical structures originating meandering patterns, probably due to different residence time, of the water masses. In addition, the northern and the southern areas showed strong differences depending on the influence of coastal waters. High salinities characterized the entire southern area at relatively low distance (~11 km) from the coast, while in the northernmost area high salinity was evident only at the offshore station (St. 36), distant more than ~55 km from the coast. This hydrological structure underlines the presence over a short distance of different and highly dynamic ecological contexts.

As regards nutrient distribution, all over the area with the exception of a coastal station (St. 77), nitrate concentration was under the detection limits; the contribution of DIN was essentially due to ammonia and the DIN/P ratio was less than 8. The area showed therefore a clear and anomalous nitrogen

limitation. The concentrations of ammonia, the relatively high presence of phosphate along the water column, might be attributed to grazing activity and to a rapid recycling of organic matter.

The completely different situation was observed during the second campaign, when the saline front was much more pronounced and the river input affected the entire studied area and generated frontal structures within the transitional coastal waters. This situation revealed differences in time in the formation of discontinuous frontal areas, probably due to meteorological forcing such as the intermittent fresh water pulses.

During this campaign, the Po river input was evident over the entire area explaining a similar distribution pattern of the macro-nutrient and salinity. More in detail, the high concentrations of nitrate were present only inshore, probably in relation with the incoming fresh water with N/P ratios higher than 20 but lower than 8 in the central and offshore waters, showing the presence of a

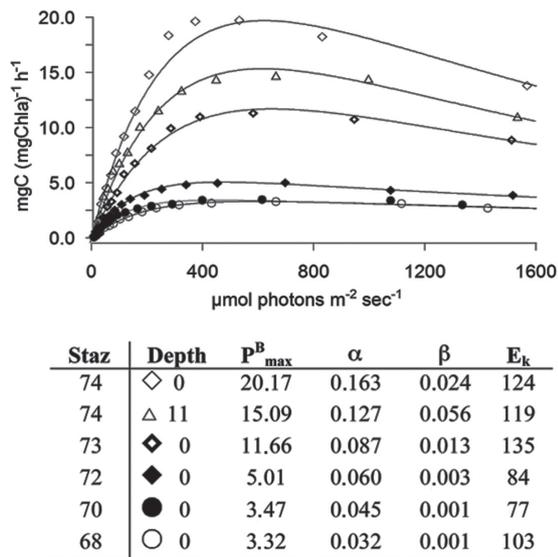


Figure 10. PvsE curves along a coastal-offshore transect (St. 74 to St. 68) and photosynthetic parameters. August-September second campaign.

nitrate limitation as reported for the first campaign. The contribution of ammonia and the relatively high concentration of phosphate indicate, once again, an efficient recycling and grazing activity.

The high hydrographic dynamics of this particular environmental context during both campaigns can be interpreted as the consequence of discontinuous inputs by the Po river, meteorological forcing and the influence of the general circulation patterns of the Adriatic basin, converging to produce different trophic characteristic at frequently-changing spatial scales. This aspect has a relevant role in determining the biological processes in terms of biomass levels, functional group composition and size structure of the planktonic communities, as well as in their photosynthetic performance. Depending on the circulation pattern, high phytoplankton biomass may accumulate inside the front and when the Po discharge is discontinuous and the water circulation slows down, nutrient limitation will occur in the plume area inducing a severe limitation

on the photosynthetic capacity of the phytoplankton community.

The PvsE experiment carried out in the coastal area during the first campaign, together with low biomass values, nitrate depletion and relatively high values of ammonia and phosphate could indicate the end of a bloom and the onset of recycled primary production processes. The lower values of photosynthetic performance below the UML may be interpreted by the light history affecting the phytoplankton community, as suggested by a lower photo-acclimation index ( $E_k$ ), induced by the strong stratification driven by salinity and temperature. In the oligotrophic external station, the relatively high photosynthetic performance of phytoplankton assemblages dominated by pico-phytoplankton, was similar along the mixed water column, confirming the ability of the smallest cells to take advantage of a broad spectrum of conditions, such as water column dynamic, and low nutrient concentrations (Agawin *et al.*, 2000).

During the second campaign, the photosynthetic parameters obtained along a coast-offshore transect showed a strong gradient. At the surface, in the coastal area directly influenced by the incoming river plume, a  $P_{max}^B$  up to 20.17 mg C (mg Chla)<sup>-1</sup> h<sup>-1</sup> was recorded. The notably high photosynthetic capacity, although not exceptional for the river plume (Mangoni *et al.*, 2008), is among the highest ever reported in marine systems and comparable to values reported for land-locked ecosystems (e.g.  $P_{max}^B = 22.5$ , Macedo *et al.*, 2001). Low values of photosynthetic performance ( $P_{max}^B < 3.32$  mgC (mgChla)<sup>-1</sup>h<sup>-1</sup>), as well as a low biomass in an offshore area characterised by a notable contribution of fresh water (salinity < 35.5), could be due to nutrient depletion in a “old” water mass characterised by a long residence time in the area. Toon *et al.* (2000) have suggested that variations in PvsE parameters may be associated with differences in the

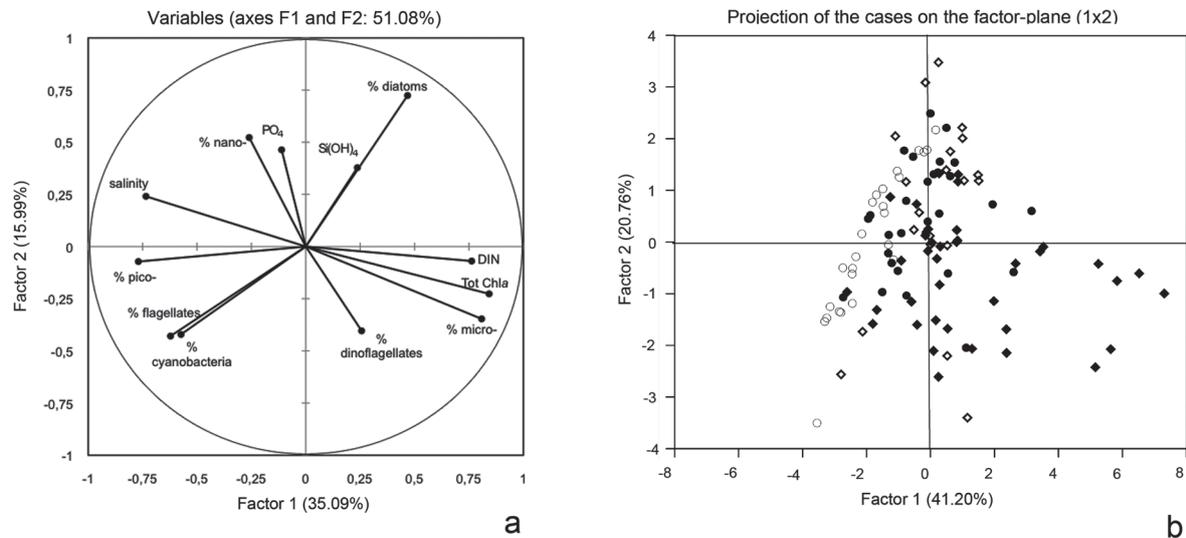


Figure 11. Results of PCA applied to salinity, nutrients and biological features (a) July (first campaign), surface layer; July (first campaign), deep layer; August-September (second campaign), surface layer; August-September (second campaign), deep layer (b).

size-structure and taxonomic composition of phytoplankton assemblages. However, our data suggest some caution, as rapidly changing environmental conditions may not allow for the replacement of phytoplankton better adapted to the instantaneous conditions. The large diatoms dominating the coastal area might have been replaced by smaller cells, including diatoms, better adapted to low nutrient conditions, if the conditions persist for a sufficient period of time.

During the first campaign, there was a clear nutrient limitation while during the second campaign in the inner part of the coastal area phytoplankton rapidly responded to new nutrients input. More in particular, during the first campaign, very low concentrations of biomass were observed and nano- and pico- fractions dominated the phytoplankton assemblages, typical community characteristics of oligo-mesotrophic conditions (Kyewalyanga *et al.*, 1998; Harrison and Platt, 1986). During the second campaign, the dimensional structure

appeared well correlated with the front, with a sharp gradient from coastal to offshore waters. Algal blooms occurred in the coastal area strongly influenced by the Po river, and low biomasses characterized the area outside the front. The mean contribution by the three dimensional classes was similar, with a slight dominance of larger forms.

In substance, during the first campaign we recorded the end of a peak of primary production, while in the second campaign the onset of another one. It is important to underline that spatial extension of eutrophication processes was linked to the distribution of fresh water rich in nutrients and/or to the ability to recycle part by the microbial loop, inducing the dominance of different size classes also within the same functional group. As regards the functional groups, the different location of the frontal zone roughly coincided with the separation between diatom- and phytoflagellate-dominated areas.

In the frame of the MSFD (Descriptor 5), the

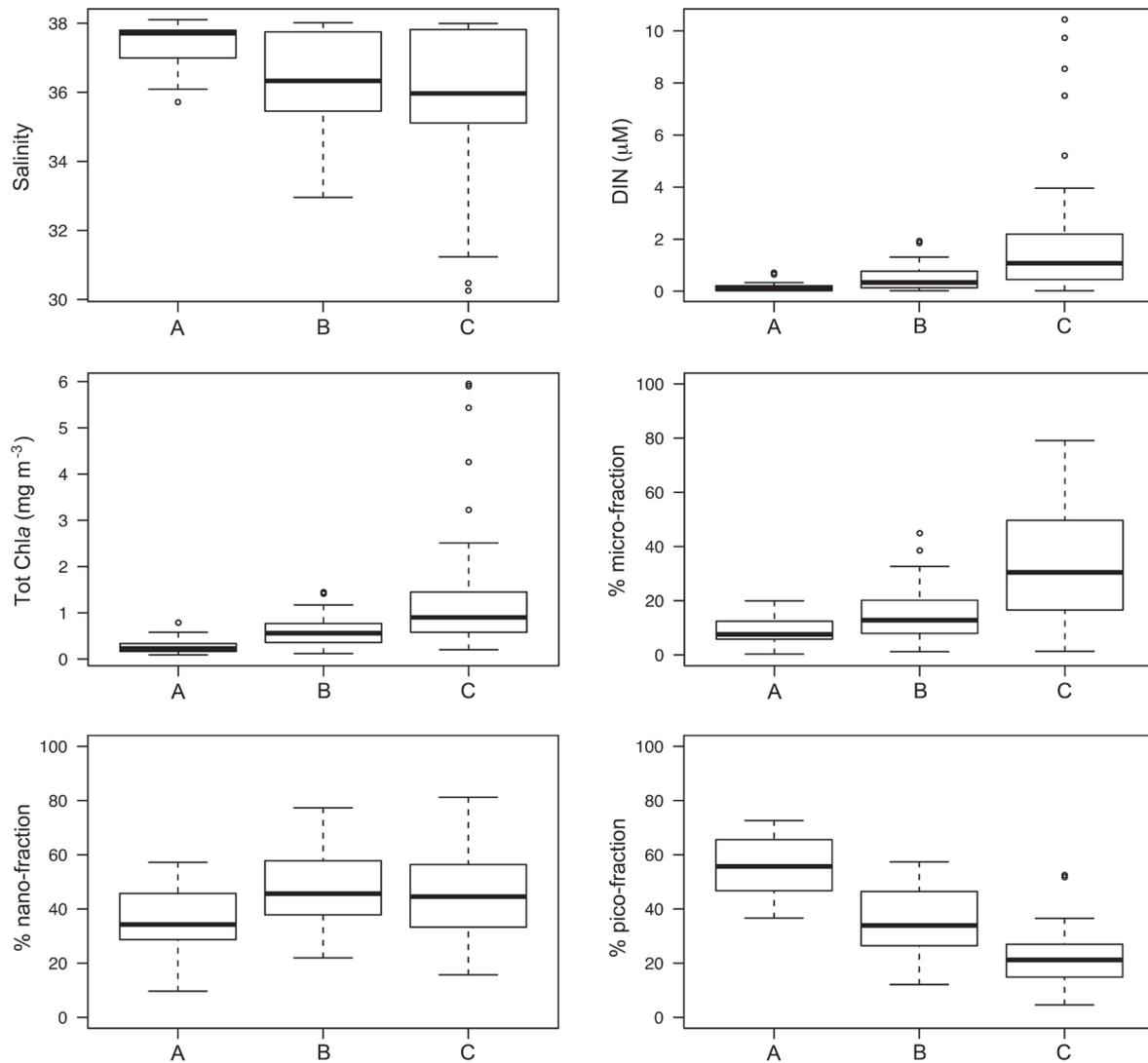


Figure 12. Distribution of salinity, DIN, total biomass (Tot Chla), percentage of micro-, nano- and pico-fractions in the three different identified classes by means of Fp-index (boxplot by classes).

changes in phytoplankton assemblages are usually addressed by light microscopy Uthermöhl technique (Uthermöhl, 1958; Edler and Elbrächter, 2010). As reported by several authors, this technique presents several drawbacks, such as loss of information on small species, damaging effects of fixatives, different biogeographic distribution of species, low reproducibility and comparability and the need of a high degree of expertise (Domingues *et al.*, 2008;

Garmendia *et al.*, 2013). More in particular, it is well known that, due to global change, the size structure of phytoplankton assemblages is shifting toward small species; some species that indicate a good ecological status in some places may not be present in others and this does not imply a different ecological status. In addition, as reported from Ferreira *et al.* (2011), HABs (Harmful Algal Blooms) should be treated only in areas where their frequency, amplitude and toxicity increase

are coupled with nutrient inputs. Finally, the low reproducibility and comparability of the information obtained by optical microscopy even in presence of a high degree of expertise, is not economically viable in large monitoring projects. The utilization of taxonomic techniques by light microscopy, as reported by several authors (Sherrard *et al.*, 2006; Garmendia *et al.*, 2013), is not advisable in the case of Descriptor 5 (Eutrophication) of MSFD, but it is fundamental in allowing a deeper knowledge on nutrition strategies within the same taxonomic group as well as in ascribing pigments to taxonomic groups in the frame of CHEMTAX program (Mackey *et al.*, 1996; Sarmiento and Descy, 2008). Obviously, traditional microscopy technique can play a key role in some other descriptors of MSFD (e.g. Biodiversity and Food Webs). Seoane *et al.* (2011) tested the chemotaxonomic technique (HPLC-CHEMTAX) to assess the ecological status of coastal and transitional waters. The results of the study showed that this technique is a good candidate for defining seasonal communities patterns of coastal and offshore waters as well as along longitudinal salinity gradient within estuaries. However, they did not find a clear relationship between phytoplankton composition and anthropogenic pressures. Claustre (1994) introduced the diagnostic pigments and proposed the Fp ratio to define the trophic status of various oceanic provinces and Ras *et al.* (2008) have used diagnostic pigments in relation to remotely sensed Chla concentrations to characterize ecosystems of extreme trophic properties such as ultra-oligotrophic regions on eutrophic upwelling zones. In addition, the pigment ratios such as Fuco/Chla and Hex/Chla have been successful utilized by Smith *et al.* (2010) as indicators of patterns and relationships among functional groups in coastal and offshore waters in Antarctic waters dominated by diatoms and haptophytes. As regards alternative methods, since the end of the Eightie, several authors

have used the size spectrum of phytoplankton as an independent tool to study community and ecosystem structure and function of aquatic environments (Chisholm, 1992; Cavender-Bares *et al.*, 2001; Rodriguez *et al.*, 2001; Sabetta *et al.*, 2005; 2008; Karp-Boss *et al.*, 2007; Garmedia *et al.*, 2013; Vadrucci *et al.*, 2013). Body size spectra, as reported by Garmedia *et al.* (2011), could be a potential descriptor of structure and function of ecosystem but they present some disadvantages in describing the shift of functional groups (e.g. diatoms:flagellates). Phytoplankton communities with similar size structure can have a completely different composition.

In order to overcome to some extent the above mentioned problems, the utilization of biochemical techniques, such as diagnostic pigment composition and size structure, could simplify the grouping of species into functional groups.

The PCA analyses (Fig. 11) synthesize the relation between environmental parameters, size structure and functional groups derived by CHEMTAX analyses and pigment ratio. Salinity was positively correlated with the pico- fraction, flagellates and cyanobacteria and negatively correlated with the micro-phytoplankton fraction, dinoflagellates and DIN. Diatoms show a good positive correlation with silicate and a negative one with cyanobacteria and flagellates. Moreover, diatoms don't show any correlation between biomass, nano- and micro- fractions and DIN. The relative dominance of this functional group suggests that the continuous change of environmental parameters could play an important role not only in the shifting on functional group, such as diatoms:flagellates but also in size fraction within the same functional group. The changes in size structure within the same functional group, could be, in our opinion, an indicator of changes in environmental conditions, such as water column dynamics, nutrient enrichment

or depletion, coastal circulation, changes in nutrient ratio, in coastal waters subject to strong and discontinuous natural and anthropogenic stress.

As regard pigment spectra, the Fp index utilized in this work well defines three different trophic conditions, from eutrophic to oligotrophic, well associate with salinity, DIN concentration, total biomass and micro-phytoplankton, as reported in the boxplot (Fig. 12). The contribution of the nano-fraction is quite similar in the Fp identified class and does not play any role in identifying the trophic characteristic of marine transitional areas. In addition this aspect suggests that this size class, associated with diatoms defines the areas subject to environmental stress by local meteorological, hydrodynamic and anthropogenic forcings.

Our data suggest that coupling of phytoplankton pigments and size spectra could represent a method allowing to overcome the disadvantages of a single method in detecting phytoplankton community composition. Pigment ratio can highlight the presence of different functional groups within an identical dimensional structure. In addition, the two methods have the advantage to be reproducible, comparable and do not require a high degree of expertise.

### Final considerations

The relatively modest phytoplankton biomass reported for the eutrophic northern Adriatic may, to some extent, be due to the high but extremely unbalanced nutrient ratio in the water discharged into the area.

The phytoplankton community efficiently responded to nutrient rich fresh water, showing a photosynthetic capacity among the highest detected in the marine environment. The phytoplankton turn-over rate was very low due to the persistent stratification of the water column and to nutrient depletion. At low Po discharge, the water circulation

slows down in the plume area and nutrient limitation will occur imposing severe limits to the photosynthetic capacity of the phytoplankton.

Changes in nutrient and biomass concentrations and a high variability in phytoplankton photosynthetic capacity occurred on small spatial and temporal scales, related to the notable time scale variations in freshwater and nutrient discharge and to the complex circulation patterns of the northern Adriatic.

It is confirmed that northern Adriatic frontal areas are an hydrographic discontinuity between an internal zone advantageous for coastal phytoplankton communities adapted to high concentrations of nutrients and an external one advantageous for oligotrophy adapted communities. In these areas, we have observed the shift in size structure within the same functional group to prevail on that among different functional groups.

On the base of the experience acquired during the present work and at the light of the existing literature specific for the northern Adriatic as well as that dealing with the general problem of environmental status assessment of the marine aquatic ecosystems, we feel confident in stating that coupling of pigment and size spectra composition may represent the best approach to assess environmental status, thus overcoming the problem affecting the traditional approach when dealing with different regional and sub-regional contexts.

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