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Response of phytoplankton to enhanced atmospheric and riverine nutrient inputs in a coastal upwelling embayment

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ABSTRACT

Over the past decades, as a consequence of human activity, there was an increase in nutrient inputs to the ocean and they are expected to enhance even more in the future. Coastal areas, accounting for a significant proportion of marine primary productivity, are the most vulnerable zones to anthropogenic impacts. The response of phytoplankton communities to an increase in organic and inorganic nutrients levels from natural allochthonous sources was assessed in microcosm experiments conducted in a coastal system affected by intermittent upwelling events (Ría de Vigo, NW Iberia). Three nutrient addition experiments were performed in spring, summer and autumn, when surface water was supplemented with 5 and 10% of atmospheric and riverine matter. Pico-, nano- and microphytoplankton abundances, chlorophyll *a* concentration (Chl *a*) and primary production rates (PP) were measured and compared with those in the control seawater sample (without additions) after 48 h of incubation. Simultaneous experiments with controlled additions of inorganic and organic nutrients were also performed in order to describe the limiting nutrient for phytoplankton growth at each experiment. The composition of the matter inputs and the structure of the phytoplankton communities determined the type of response observed. Phytoplankton responses varied among seasons, being positively correlated with dissolved inorganic nitrogen (DIN) concentrations. As expected, the phytoplankton responses to external nutrient inputs were stronger under low nutrient levels (summer) than when phytoplankton was already growing in nutrient replete conditions (spring). Null and negative responses to the natural inputs were observed in autumn, which suggests that the oceanic phytoplankton advected to this coastal system during downwelling events could be occasionally inhibited by these nutrient inputs. In a future global change scenario, characterized by enhanced nutrient inputs from riverine and atmospheric origin, the response of phytoplankton communities will strongly depend on the concentration and chemical composition of these inputs and on the structure of phytoplankton communities able to respond to them.

1. Introduction

Phytoplankton organisms are the main primary producers in the sea being responsible for about 50% of the world primary production (Field et al., 1998). Coastal areas, despite representing only 7% of ocean's surface, account for 15–20% of global marine primary productivity (Wollast, 1993, 1998; Laruelle et al., 2009). Enhanced nutrient fluxes from continental runoff, the atmosphere and the adjacent open ocean, and the efficient nutrient recycling based on a closed coupling between pelagic production and benthic regeneration are the reasons behind the high productivity of the coastal zone (Walsh, 1991; Wollast, 1998). All these biogeochemical processes are specially intensified in coastal upwelling regions because of the enhanced entry of nutrients from the adjacent ocean in response to intense and persistent equatorward winds (Walsh, 1991; Wollast, 1998).

Due to its close proximity to human populations, coastal areas are also the most vulnerable zones to anthropogenic forcing (Jickells, 1998). Inorganic and organic nutrients and pollutants resulting from human activities reach coastal areas through atmospheric deposition, continental runoff and groundwater effluents (Jickells, 1998; Doney, 2010; Statham, 2012). Over the past decades, there was an increase in these nutrient inputs to the ocean and they are expected to expand even more in the future (Anderson et al., 2002; Galloway and Cowling, 2002; Galloway et al., 2004; Duce et al., 2008). Alterations in the magnitude

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and composition of matter inputs in coastal areas may induce significant changes in phytoplankton communities inhabiting those systems (Paerl, 1997; Peierls and Paerl, 1997; Seitzinger and Sanders, 1999; Spatharis et al., 2007). Extreme cases of nutrient enrichment may involve the development of massive phytoplankton blooms, including harmful algal species (Paerl, 1997; Anderson et al., 2002).

The Ría de Vigo is a productive embayment in the coastal upwelling system of the Northwest Iberian Peninsula (Fraga, 1981). Seasonal patterns in phytoplankton composition and productivity in this system are closely related to the hydrographic variability (Figueiras and Ríos, 1993; Figueiras et al., 2002). Upwelling events, occurring usually between March and October, introduce nutrient rich subsurface waters into the photic layer and are followed by high concentrations of phytoplankton cells and chlorophyll. On the contrary, the winter period is favorable to downwelling and it is characterized by lower biomass accounted for small-size phytoplankton cells (Figueiras et al., 2002). River discharge and atmospheric deposition may also constitute significant sources of nutrient inputs to this coastal embayment, although to a lesser extent as compared with upwelling episodes (Gago et al., 2005; Rodríguez and Macías, 2006; Alonso-Pérez and Castro, 2014; Fernández et al., in 2016). The high productivity characteristic of this system sustains an intensive mussel production and significant catches of several fish and shellfish species, which are relevant for the economy of the region (Figueiras et al., 2002; Froján et al., 2014). Thus, understanding the response of the organisms in the basis of the food web to an eventual increase in nutrients inputs in the Ría de Vigo may be determinant to anticipate its consequences to the whole ecosystem.

The outcomes of an increase in nutrients on coastal microbial plankton in this system was tested in previous experimental studies, which focused on the effect of controlled inorganic and organic nutrient additions (Martínez-García et al., 2010; Teira et al., 2011) and natural additions of rainwater (Teira et al., 2013; Martínez-García et al., 2015). In the present study, we aimed at examining the response of phytoplankton communities in the Ría de Vigo to realistic increasing amounts of dissolved matter inputs from both atmospheric and riverine origin. To calculate the current average riverine and atmospheric inputs to the Ría de Vigo we considered the average river flow of the River Oitabén-Verdugo, 17 $m^3 s^{-1}$ (Gago et al., 2005) and the average precipitation to the Ría de Vigo, 7.7 mm d^{-1}. Considering the surface area of the ría is (174 km^2), the mean surface mixing layer (2 m) and the average flushing time of this layer (5 days), the surface mixing layer of the ría must contain about 2% of river and 2% of rainwater. Therefore, the additions of 5% and 10% tested here would serve to obtain the response of the Ría de Vigo to future global change scenarios in which human activities increase the quantity without altering the quality of riverine and atmospheric inputs. Furthermore, concomitant experiments with controlled additions of inorganic and organic nutrients were also performed in order to describe the limiting nutrient for phytoplankton growth at each season.

2. Materials and methods

2.1. Sampling

Seawater for the experiments was sampled from the middle of the Ría de Vigo (42º14.09′ N, 8º47.18’ W) during the productive season in spring (May) and summer (July), and after the transition to the unproductive period in autumn (October) 2013 (Fig. 1). Temperature, salinity and *in situ* fluorescence down to 25 m depth were obtained with a SBE 9/11 CTD probe and a Seatech fluorometer attached to a rosette sampler. Sub-surface seawater (3–4 m) was collected in 12 L acid-clean Niskin bottles and filtered through a 200 µm pore size mesh to remove larger zooplankton into a large acid-clean carboy to transfer it to the coastal station (Fig. 1) to prepare the addition experiments.

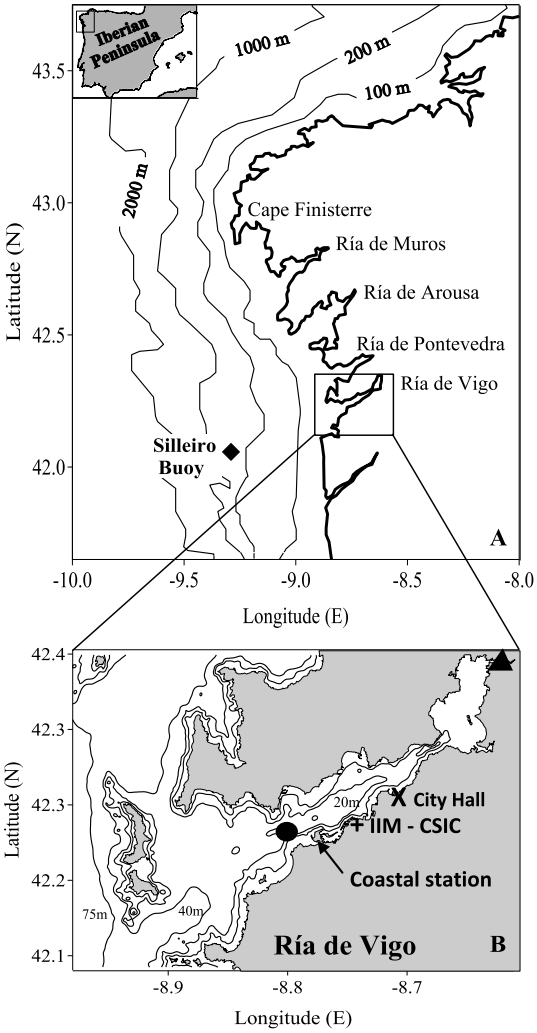

Fig. 1. (A) Map of the west Galician coast with the location of the Silleiro buoy (◆) and (B) map of the Ría de Vigo with the location of the sampling station (●), the river Oitabén-Verdugo (▲) and the meteorological stations in the IIM (+) and in the City Hall (**X**). The coastal station where addition experiments took place is indicated by an arrow.

2.2. Ekman transport and runoff

The Ekman transport (Qx; $m^2 s^{-1}$) perpendicular to the coast, a proxy for the occurrence and intensity of coastal upwelling, was calculated from wind direction and velocity, recorded at Cabo Silleiro buoy (Fig. 1) according to Bakun (1973):

$$Q_x = \frac{\rho_a * C * [V] * V_y}{f * \rho_w} \quad (1)$$

where ρ_a is the air density (1.22 kg m^{-3}), C is an empirical drag coefficient (1.3×10^{-3}, dimensionless), [V] is the wind speed (m s^{-1}) with component V_y, f is the Coriolis parameter at this latitude ($9.95 \times 10^{-5} s^{-1}$), and ρ_w is the density of seawater (~1025 kg m^{-3}). The sign of Q_x was changed to associate positive values with offshore transport (upwelling) of surface waters.

Runoff was calculated using data from River Oitabén-Verdugo (Fig. 1), according to the method described in Otero et al. (2010).

2.3. Preparation of natural waters concentrates for the addition experiments

River and rainwater samples, and < 10 µm atmospheric particles were collected and processed to obtain concentrates of the riverine and

atmospheric matter inputs to the Ría de Vigo. The objective was to reduce the volume of the original water samples 10-fold maintaining their chemical composition, i.e. without addition or loss of any component present in the natural samples. In this way, it was possible to test the effect of an increase in natural matter using small water volumes and without significant changes in salinity.

The River Oitabén-Verdugo (Fig. 1) was sampled a week before the addition experiments in April, July and October 2013. The water samples were collected upstream of the freshwater-seawater interface, to guarantee that the chemical composition of the river samples represented that of the water that mixes with the seawater of the Ría de Vigo. Five liters of each sample were gravity filtered through a prewashed (with 10 L of ultrapure water) dual-stage (0.8 and 0.2 μm) filter cartridge (Pall-Acropak supor Membrane). The filtrate was then concentrated 10-fold using rotatory evaporation with a Buchi R215 evaporator under mild conditions (bath temperature: 25 °C, vacuum: 13 mbar, condenser: acetone/CO_2) to avoid breakage of any organic compound present in the original water samples. Analysis of the concentration of inorganic (ammonium, nitrite, nitrate and phosphate) and organic (dissolved organic carbon and nitrogen) substrates confirmed that the samples were concentrated quantitatively but maintaining their original composition.

A MTX rainwater sampler (model FAS005AB) and a high volume PM10 MCV PM1025 sampler (model CAV-A/MS) installed at the roof of the Instituto de Investigaciones Marinas (IIM-CSIC) (Fig. 1) collected samples of wet and dry deposition to the Ría de Vigo. The MTX sampler was equipped with a humidity sensor opening the system only when it was raining and allowing sampling just the wet fraction of the atmospheric deposition. Rainwater was collected from four weeks to one week before the addition experiments. Samples were taken daily and frozen immediately after collection. A week before the experiment, the daily samples were thawed at ambient temperature, mixed in one volume (6 L), and quantitatively concentrated following the same procedure as for the riverine samples. Rainwater was collected only for the experiments in spring (May) and autumn (October) 2013 because wet deposition was very scarce the weeks before the summer experiment in July (36 mm accumulated from 11 June to 10 July; meteorological station of the Vigo city hall – Fig. 1). The high volume sampler was used to collect atmospheric particles (1–10 μm) on precombusted (450 °C, 4 h) 140 mm GF/F filters (48 h sampling). The particles were collected the week before each of the three addition experiments, operating during 48 h at a rate of 30 $m^3 h^{-1}$. Then, the water-soluble fraction (WSF) of one eighth of the filter was extracted in 400 mL of the corresponding rainwater concentrate by mechanical stirring during 40 min. In the summer experiment, the WSF was extracted in milli-Q water. These proportions (1/8 of the filter in 400 mL of water) were decided to obtain 10-fold the expected concentrations based on previous information about the composition of wet and dry deposition to the Ría de Vigo (Teira et al., 2013; Martínez-García et al., 2015). Final mixed extracts were filtered through precombusted (450 °C, 4 h) 47 mm diameter Whatman GF/F filters in an acid-cleaned glass filtration system, under low N_2 flow pressure, to be chemically characterized and used in the experiments as atmospheric concentrate. Similarly to riverine concentrates, quantitative concentration was maintained except for the silicate because the reduction of the rainwater volume was carried out in a glass rotary evaporator and the atmospheric particles were collected onto a glass fiber filter.

2.4. Natural and controlled addition experiments

For both natural and controlled addition experiments, 4 L UV-transparent Whirl-pak® bags were gently filled under dim light conditions until 2 L of capacity. For the natural addition experiments, riverine and atmospheric concentrates were added to subsurface seawater, collected as described in Section 2.1, in proportions of 0% (1% ultrapure water: 99% seawater), 5% (0.5% concentrate: 0.5% ultrapure water: 99% seawater) and 10% (1% concentrate: 99% seawater) of the original (previous to concentration) riverine and atmospheric materials (Fig. 2). These proportions also ensured that the final salinity of the samples was kept constant independently of the amount of extract added.

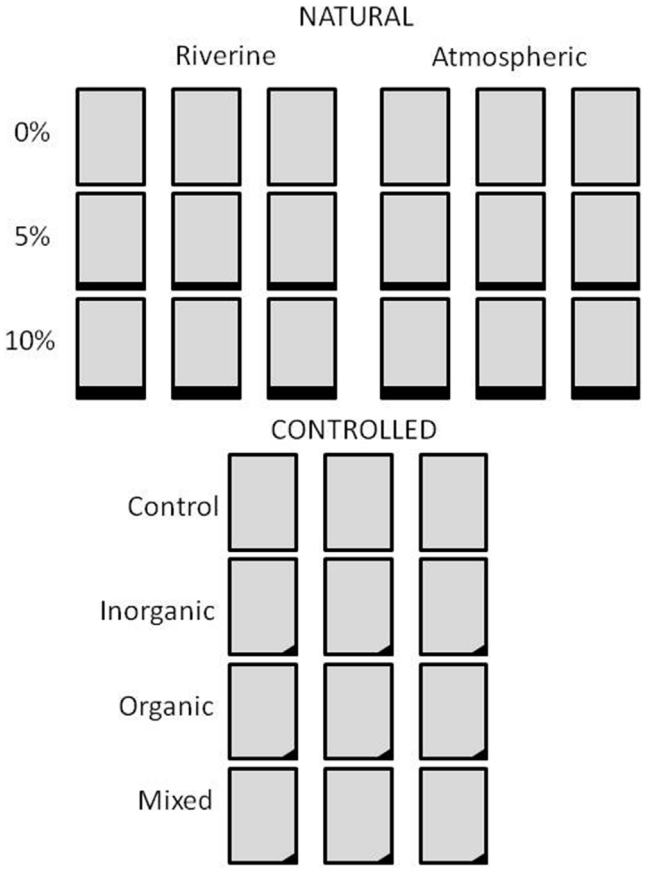

Fig. 2. Schematic representation of the natural and controlled addition treatments performed at each season (see details in section 2.4).

Controlled nutrient addition experiments were performed as in Martínez-García et al. (2010), where subsurface water collected as described in section 2.1 was spiked in the following concentrations: (Control) No addition treatment; (Inorganic) Inorganic nutrient treatment: 5 μmol L^{-1} nitrate (NO_3^-), 5 μmol L^{-1} ammonium (NH_4^+) and 1 μmol L^{-1} phosphate (HPO_4^{2-}); (Organic) Organic nutrient treatment: 5 μmol L^{-1} glucose and 5 μmol L^{-1} mix of 18 equimolar aminoacids (all the protein amino acids except cysteine and tyrosine); and (Mixed) Mixed treatment: Inorganic and organic nutrient treatments (Fig. 2).

All treatments (including natural and controlled additions) were performed in triplicate and were incubated for 48 h in a tank outside the main building of the coastal station (Fig. 1) under natural light covered with a mesh to simulate *in situ* light (~50% I_0) and with running seawater to simulate *in situ* temperature conditions. Samples for chlorophyll *a* (Chl *a*), primary production rates (PP) and phytoplankton abundance were taken at the beginning and after 48 h incubation. Inorganic and organic nutrients were sampled only at the beginning of the incubation.

Aliquots for inorganic nutrients determination (ammonium, nitrite, nitrate and phosphate) were collected in 50 mL polyethylene bottles and frozen at −20 °C until posterior analysis. Standard colorimetric methods with an Alliance Futura segmented flow analyzer (Hansen and Grasshoff, 1983) were applied for nitrate, nitrite and phosphate determinations, while ammonium was analyzed by the fluorimetric method by Kerouel & Aminot (1997). Water for the analysis of dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) was filtered

through 0.2 μm filters (Pall, Supor membrane Disc Filter) in an all-glass filtration system under positive pressure of N_2 and collected into pre-combusted (450 °C, 12 h) 10 mL glass ampoules acidified with H_3PO_4 to pH < 2. Samples were measured with a Shimadzu TOC-V total organic carbon analyzer fitted with a Shimadzu TNM-1 total nitrogen measurement unit. Dissolved organic nitrogen (DON) was obtained by subtracting ammonium, nitrite and nitrate from TDN.

Chl *a* concentrations were measured in 100 mL water samples, which were filtered through 0.2 μm polycarbonate filters. The filters were immediately frozen at −20 °C until pigment extraction in 90% acetone at 4 °C overnight in the dark. Chl *a* concentrations were determined, with a 10–AU Turner Designs fluorometer calibrated with pure Chl *a*.

Five 75 mL Corning tissue flasks (3 light and 2 dark) were filled with seawater and spiked with 185 kBq (5 μCi) $NaH^{14}CO_3$. Samples were incubated for 2 h in a temperature-controlled incubation chamber illuminated with cool white light from fluorescent tubes providing an average PAR of 240 μE m^{-2} s^{-1}. After the incubation period, samples were filtered through 0.2 μm polycarbonate filters at very low vacuum (< 50 mm Hg). Filters were exposed to HCl fumes for 24 h to remove unincorporated inorganic ^{14}C and radioactivity measured with a liquid scintillation counter using the external standard and the channel ratio methods to correct for quenching.

Abundances of autotrophic prokaryotes and picoeukaryotes were determined in fresh samples with a BD FACSCalibur flow cytometer equipped with a laser emitting at 488 nm. Pico- and nanophytoplankton were also determined in subsamples of 10 mL fixed with buffered 0.2 μm filtered formaldehyde (2% final concentration) and filtered through 0.2 μm black Millipore-Isopore filters. The filters were then immersed in low fluorescence immersion oil and examined at x1000 magnification using an epifluorescence microscope. Autotrophic organisms were recognized and enumerated under blue light excitation. Microphytoplankton was determined in subsamples of 100 mL preserved in Lugol's iodine. Depending on Chl *a* concentration, a variable volume of 5–50 mL was sedimented in composite sedimentation chambers and observed through an inverted microscope. The organisms were counted and identified to the species level when possible. Phototrophic and heterotrophic species of dinoflagellates were differentiated following Lessard and Swift (1986) and also using epifluorescence microscopy. Dimensions were measured to calculate cell biovolumes of the main groups (pico- and nanophytoplankton) or species (microphytoplankton) after approximation to the nearest geometrical shape (Hillebrand et al., 1999) and cell carbon was calculated following Menden-Deuer and Lessard (2000). In the summer and autumn experiments, only one bag of each treatment was analyzed for nano- and microphytoplankton at 48 h.

3. Results

3.1. Initial conditions

Addition experiments were performed under three contrasting hydrographic situations (Table 1; Fig. S1 supplementary material). While the first experiment in spring (May) was performed just after an intense upwelling event, the second experiment in summer (July) coincided with a relaxation of upwelling favorable winds. The third experiment in autumn (October) occurred under high runoff and after strong downwelling conditions. The highest inorganic nutrient concentrations were found in autumn. The opposite situation was observed in summer, when dissolved inorganic nutrients (except phosphate) achieved the lowest concentrations. Dissolved organic compounds showed an increasing trend from spring to autumn (Table 1).

The highest Chl *a* concentration (13.5 mg m^{-3}), phytoplankton abundance (6.9 × 10^4 cells mL^{-1}) and phytoplankton carbon biomass (167.7 mg C m^{-3}) were found in spring (Table 1; Fig. 3). While autotrophic picoflagellates (APF) showed the highest cell abundances (Fig. 3a), diatoms comprised half of the carbon biomass, followed by APF, pigmented nanoflagellates (ANF) and pigmented dinoflagellates (Fig. 3b). Among diatoms, the chain forming *Chaetoceros* spp. and *Skeletonema* cf. *costatum* were the dominant species. On the contrary, the lowest Chl *a* values (0.6 mg m^{-3}), phytoplankton abundance (9.3 × 10^3 cells mL^{-1}) and phytoplankton carbon biomass (29.74 mg C m^{-3}) at surface were found in summer (Table 1; Fig. 3). Again, APF were the most abundant group (Fig. 3a), while carbon biomass was dominated by diatoms, followed by dinoflagellates, ANF and APF (Fig. 3b). Among diatoms, small *Chaetoceros* spp., *Leptocylindrus* spp. and *Guinardia delicatula* were the dominant species. In autumn, Chl *a* values reaching 1.4 mg m^{-3} (Table 1) corresponded to the dominance of the cyanophyceae *Synechococcus* sp. achieving about 60% of total abundance, followed by APF and ANF (Fig. 3a). Carbon biomass was dominated by nanoflagellates, with smaller contributions of the other groups (Fig. 3b).

The chemical composition of the natural inputs from atmospheric and riverine origin is shown in Table 2. Riverine inputs showed higher content in DOC, DON, NO_3 and NO_2 than atmospheric inputs, while NH_4 concentration was higher in the atmospheric inputs. There was not a clear trend for phosphate concentrations. Highest concentrations (except for DON and NH_4) were found in riverine inputs in autumn (Table 2).

Table 1
Initial conditions for each experiment before natural and controlled additions of inorganic and organic nutrients. DOC: dissolved organic carbon; DON: dissolved organic nitrogen; Chla: Chlorophyll *a*; PP: primary production; Phyto: Phytoplankton.

	Spring	Summer	Autumn
Temperature (ºC)	14.3	16.6	18.5
Salinity	35.4	35.6	33.4
Nitrate (μM)	2.8	0.22	3.48
Nitrite (μM)	0.11	0.05	0.31
Ammonia (μM)	1.05	0.96	2.02
Phosphate (μM)	0.13	0.16	0.33
Silicate (μM)	5.5	3.9	12.4
DOC (μM)	65.2	76.2	84.9
DON (μM)	4.73	6.30	7.55
Chla (mg,m^{-3})	13.5	0.6	1.4
PP (mg C m^{-3} h^{-1})	17.4	1.2	3.6
Phyto abundance (cells.ml^{-1})	68814	9278	33040
Phyto biomass (mgC.m^{-3})	167.7	29.7	88.4

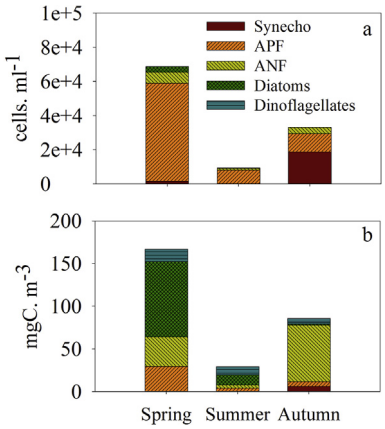

Fig. 3. *In situ* phytoplankton communities at the beginning of each experiment: a) abundance and b) biomass of major phytoplankton groups. Synecho: *Synechococcus*-type cyanobacteria; APF: autotrophic picoflagellates; ANF: autotrophic nanoflagellates.

Table 2
Chemical composition of the natural inputs added to the incubation bags at each experiment. All concentrations in μM. DOC: dissolved organic carbon; DON: dissolved organic nitrogen.

		DOC	DON	NO_3	NO_2	NH_4	PO_4
Spring	Riverine	79.5	6.4	15.1	0.04	0.15	0.01
	Atmospheric	36.9	4.1	10.5	0.004	7.5	0.07
Summer	Riverine	78.8	5.0	18.6	0.05	0.46	0.05
	Atmospheric	55.2	1.8	2.8	0.003	2.2	0.07
Autumn	Riverine	149.2	3.9	33.0	0.13	0.89	0.25
	Atmospheric	81.7	2.2	7.1	0.06	4.9	0.15

3.2. Biological responses with natural and controlled inputs

In spring, there was a significant positive response in Chl *a* and primary production (PP) rates to the addition of 10% of atmospheric and riverine inputs and to the inorganic and mixed treatments in the controlled additions (Fig. 4a and b).

In summer, Chl *a* and PP rates responded positively in all treatments, including natural and controlled additions (Fig. 4c and d). There were only two exceptions: PP rates did not respond to organic inputs and the response of Chl *a* to the 10% riverine input was marginally significant (P = 0.07). In general, the response ratio in this experiment was higher than in spring, with the response ratios to the natural inputs being higher than 1.5 and to the controlled inorganic inputs reaching 4.5 for Chl *a* and 7 for PP (Fig. 4a–d). In autumn, there was a negative response in Chl *a* to the natural inputs (~0.85) and to the organic controlled additions (~0.40) (Fig. 4e). On the contrary, inorganic and mixed controlled additions led to a slight increase in Chl *a* concentrations. PP rates increased in response to the 10% riverine inputs and to the mixed treatment, and decreased in response to the organic additions (Fig. 4f).

Statistical analyses (Table 3) demonstrated that both experiment and treatment had a significant effect in the response ratios of Chl *a* and PP. The interaction between these two factors was also significant for PP response ratios. Thus, Chl a and PP response ratios were significantly different among the three experiments ($p < 0.01$), except for the PP response ratio between spring and autumn (Table 4). Among treatments, significant differences were found for Chl a and PP response ratios between natural inputs and the inorganic treatment in the controlled additions. The response to the inorganic treatment was also significantly different from the organic treatment for Chl a and from the mixed and organic treatments for PP. No significant differences were found in Chl *a* and PP response ratios among the samples treated with both types and levels of natural inputs: riverine and atmospheric.

Table 3
Output of multivariate General Linear Model analysis for Chl a, PP and the several phytoplankton groups' response ratios as dependent variables and Experiment and Treatment as fixed factors. Synecho: *Synechococcus*-type cyanobacteria, APF: autotrophic picoflagellates, ANF: autotrophic nanoflagellates.

Tests of Between-Subjects Effects

		F	Sig.
Experiment	Chl a	23.860	0.001
	PP	34.076	0.000
	Synecho	9.405	0.010
	APF	20.515	0.001
	ANF	24.452	0.001
	Diatoms	1.333	0.323
Treatment	Chl a	15.742	0.001
	PP	24.922	0.000
	Synecho	3.788	0.052
	APF	57.782	0.000
	ANF	10.840	0.003
	Diatoms	0.946	0.519
Experiment x Treatment	Chl a	3.385	0.057
	PP	10.331	0.002
	Synecho	1.638	0.262
	APF	11.154	0.002
	ANF	3.579	0.050
	Diatoms	1.073	0.484

Experiment x Treatment refers to the interaction between both factors.

The different phytoplankton groups also responded differently to the experiments and treatments (Fig. 5, Tables 3 and 4). While *Synechococcus* showed a significant increase in the organic and mixed treatment in spring (Fig. 5a), a significant decrease was observed in autumn in response to atmospheric and organic additions (Fig. 5g). In summer, the low abundances or even absence of *Synechococcus* in some samples did not allow inferring reliable responses to the treatments. Among APF, positive responses were observed for 10% atmospheric,

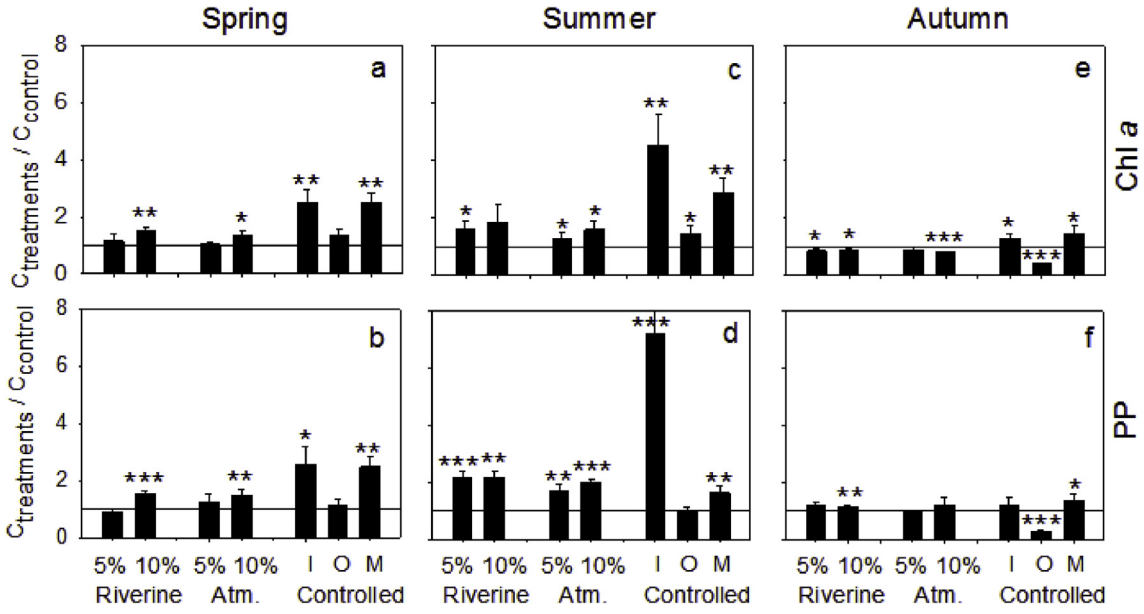

Fig. 4. Response ratios (treatment/control) for Chl *a* concentrations and primary production rates in spring (a,b), summer (c,d) and autumn (e,f). Horizontal line denotes the response ratio = 1. Atm.: Atmospheric inputs; I: controlled inorganic additions; O: controlled organic additions; M: controlled mixed additions. The levels of significance of t-tests, performed between each treatment and the control, are: *p < 0.05, **p < 0.01, ***p < 0.001.

Table 4
Bonferroni post hoc multiple comparisons from General Linear Models analysis with the response ratios of Chl a, PP and the several phytoplankton groups as dependent variables and the experiment and treatment as fixed factors. Synecho: *Synechococcus*-type cyanobacteria, APF: autotrophic picoflagellates, ANF: autotrophic nanoflagellates. For treatment, only significant results are shown.

			Sig.
Experiment			
Chl a	Spring	Summer	0.024
		Autumn	0.011
	Summer	Autumn	0.001
PP	Spring	Summer	0.001
		Autumn	0.052
	Summer	Autumn	0.000
Synecho	Spring	Summer	0.012
		Autumn	0.136
	Summer	Autumn	0.510
APF	Spring	Summer	0.545
		Autumn	0.001
	Summer	Autumn	0.012
ANF	Spring	Summer	0.182
		Autumn	0.001
	Summer	Autumn	0.013
Diatoms	Spring	Summer	1.000
		Autumn	0.682
	Summer	Autumn	0.499
Treatment			
Chl a	Inorganic	Natural	< 0.01
		Organic	0.003
PP	Inorganic	All	< 0.01
Synecho	Atm 10%	Mixed	0.037
APF	Mixed	All	< 0.01
	organic	All	< 0.01
ANF	Inorganic	Natural	< 0.05
		Organic	0.002
	Mixed	Organic	0.017

inorganic and mixed additions in spring and summer, and negative in all experiments for organic additions (Fig. 5b, e, h). ANF showed positive responses in the controlled additions and in the 5% addition of riverine inputs in spring (Fig. 5c), and in the inorganic and mixed treatments in summer (Fig. 5f). No response was observed in the autumn experiment for ANF, except a decrease in the response to the organic additions (Fig. 5i). Diatoms showed a high positive response to the inorganic treatment in the summer experiment (Fig. 5d) while a slight positive response was also observed for the mixed addition. In spring and autumn, no responses or negative effects were observed on diatoms, especially for organic and mixed additions (not shown).

It is noticeable that APF abundances were better stimulated by the combination of inorganic and organic compounds (the mixed treatment) when compared with the addition of only inorganic or only organic nutrients (Fig. 5b, e, h). In fact, the organic treatment seems to be in some way detrimental for APF, showing lower abundances than in the control. By contrast, ANF and diatoms typically grew more with the addition of only inorganic nutrients (Fig. 5c, d, f) than in the mixed treatment. *Synechococcus* showed a similar pattern to APF organisms, with highest growth under the combination of organic and inorganic compounds (Fig. 5a, g). However, in this case, the organic treatment seemed to be harmful for these organisms only in autumn (Fig. 5g).

Also for the different phytoplankton groups' response ratios, the experiment and the treatment were statistically significant factors (Table 3). For *Synechococcus*, significant differences were found between the spring and summer experiments, while for the small flagellates (APF and ANF) the significant differences occur between the autumn experiment and the other two experiments (Table 4). No differences were found between the responses to the different natural addition treatments. Most significant differences in response ratios between treatments were found between natural and controlled addition treatments.

In order to explain the different phytoplankton responses at each experiment, we analyzed them separately searching for correlations within the several variables. Chl *a* concentration was positively correlated with DIN in the three experiments (Table 5). Similarly, also several phytoplankton groups showed positive correlations with DIN, but not for all experiments. DIN was positively correlated with APF and ANF in spring, with ANF and diatoms in summer and with *Synechococcus* in autumn. No significant correlations were found between the several phytoplankton groups and organic compounds (both DOC and DON) in any of the three experiments, except for ANF and diatoms in autumn which showed a negative significant correlation with organic compounds.

Some differences in phytoplankton community composition were observed in response to nutrient amendments (Fig. 6). In spring, the proportion of diatoms increased with riverine and atmospheric inputs whereas with controlled additions the relative diatoms abundance was depressed and ANF favored (Fig. 6a). In autumn an increase in the proportion of *Synechococcus* abundance was observed in response to the organic treatment (Fig. 6c). In this same treatment, it was observed a decrease in the proportion of APF abundance (Fig. 6c).

4. Discussion

Performing the experiments in spring (May), summer (July) and autumn (October) allowed investigating the different hydrographic conditions and biological communities typical of this coastal embayment (Figueiras and Ríos, 1993; Figueiras et al., 2002; Arbones et al., 2008). Just after the first intense upwelling period of the year in spring, high Chl *a* and nutrient concentrations were observed in surface waters, with a typical community dominated in biomass by chain forming diatoms (*Chaetoceros* spp.). On the contrary, in summer, despite the previous upwelling events, we found that the surface layer was poor in nutrients and dominated by small plankton organisms. This fact was due to the sinking of the surface phytoplankton communities (dominated by large diatoms developed in the previous weeks - data not shown), resulting from the relaxation of upwelling favorable winds. In autumn, typical oceanic communities were found at the surface as a result of a previous strong downwelling event which pushed continental shelf waters into the Ría. High abundance of *Synechococcus* denotes the presence of this offshore water inside the Ría de Vigo (Rodríguez et al., 2006).

The statistically different response ratios of phytoplankton among the experiments performed in the three different seasons indicate that the initial conditions at each experiment were important to determine the magnitude and the type of response of phytoplankton communities to nutrients inputs. Differences in initial conditions among the experiments which can influence these phytoplankton responses may include dissimilarities in environmental variables (i.e. nutrients availability), in the composition of the natural matter inputs or in the structure of phytoplankton communities (Martínez-García et al., 2015). The high positive correlations found between Chl *a* (and several phytoplankton groups) and DIN concentrations in the incubation bags suggest that DIN availability is an important factor determining the response of phytoplankton communities in this investigation. In addition, the highest phytoplankton responses to the several treatments were observed in

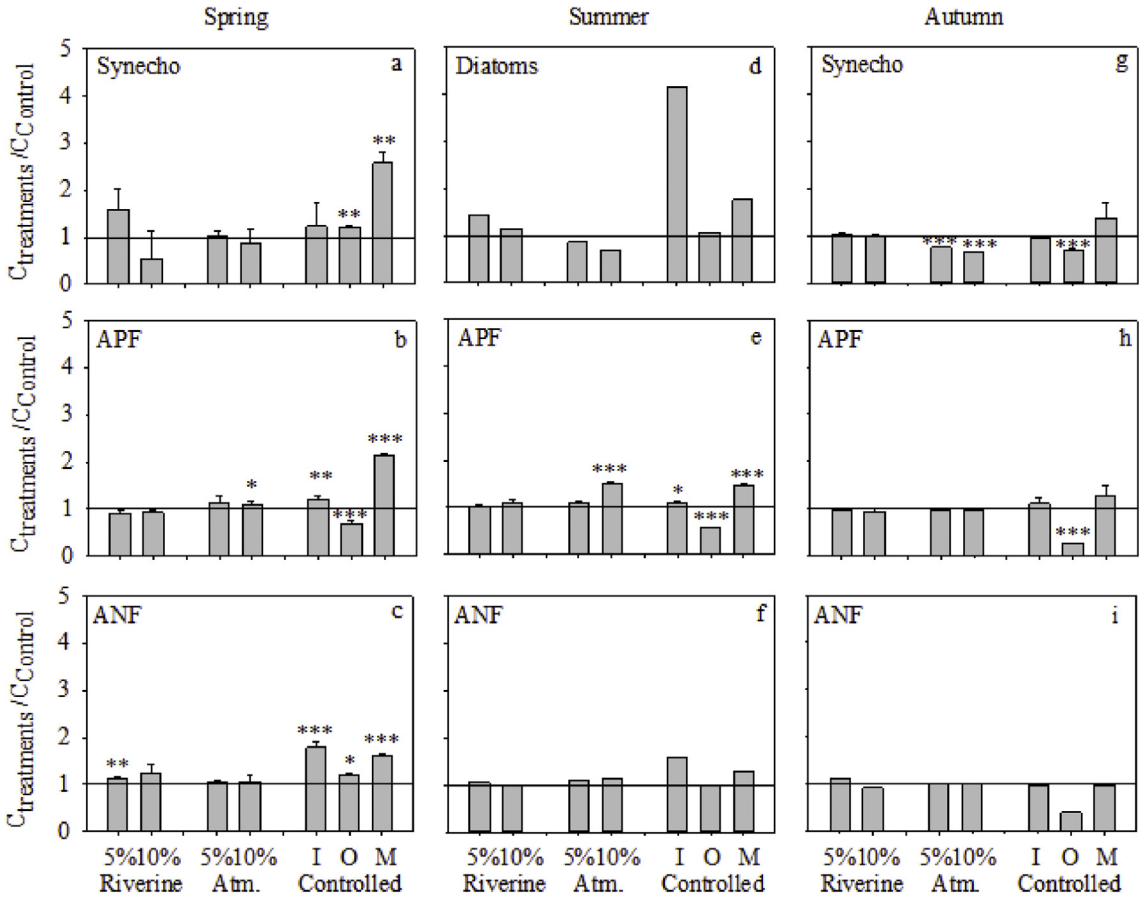

Fig. 5. Response ratios (treatment/control) of phytoplankton groups showing significant responses to treatments in spring (a–c), summer (d–f) and autumn (g–i). Synecho: *Synechococcus*-type cyanobacteria, APF: autotrophic picoflagellates, ANF: autotrophic nanoflagellates. Horizontal line denotes the response ratio = 1. Atm.: Atmospheric inputs; I: controlled inorganic additions; O: controlled organic additions; M: controlled mixed additions. The levels of significance of t-tests, performed between each treatment and the control, are: *p < 0.05, **p < 0.01, ***p < 0.001. In the experiments of July and October, only one bag of each treatment was analyzed for nano and microphytoplankton at 48h, so we don't have statistical significance levels for ANF and diatoms at these experiments.

Table 5
Pearson correlation coefficients for analyses performed between Chl a concentrations, PP rates and abundances of the several phytoplankton groups at the end of the incubation, and initial inorganic or organic nutrients levels (including *in situ* and added) at each experiment. Synecho: *Synechococcus*-type cyanobacteria, APF: autotrophic picoflagellates, ANF: autotrophic nanoflagellates.

		Chla	PP	Synecho	APF	ANF	Diatoms
Spring	DIN	**0.975*****	**0.930*****	0.563	**0.729***	**0.920*****	−0.229
	DOC	0.422	0.236	0.565	0.341	0.216	−0.497
	DON	0.421	0.232	0.573	0.349	0.210	−0.520
Summer	DIN	**0.902*****	**0.687***	0.506	0.271	**0.922*****	**0.710***
	DOC	0.117	−0.221	0.038	−0.216	0.099	−0.118
	DON	0.120	−0.214	0.088	−0.254	0.117	−0.093
Autumn	DIN	**0.657***	0,413	**0.642***	0.070	0.104	0.120
	DOC	−0.208	−0.290	0.281	−0.563	**−0.686***	**−0.775****
	DON	−0.151	−0.289	0.340	−0.621	**−0.697***	**−0.738***

Significant results are highlighted in bold. Significance levels are: * for p < 0.05; ** for p < 0.01; *** for p < 0.001.

summer, when *in situ* DIN levels were the lowest. In this case, phytoplankton growth seemed to be severely limited by nutrients and both types of inputs (natural and controlled) promoted enhanced phytoplankton growth. A similar situation, although with a lower magnitude, occurred in spring. In autumn, however, when *in situ* inorganic nutrient levels were the highest, phytoplankton did not respond or even decreased in abundance. In this case, interactions between several factors besides DIN must have occurred as discussed later on. Nitrogen has been widely recognized as the main limiting nutrient in marine systems when light intensity is sufficient (Wollast, 1998). In fact, several works have also observed stimulation in phytoplankton communities due to increases in inorganic nitrogen from atmospheric deposition (Paerl et al., 1990; Cui et al., 2016) or in controlled additions (Piehler et al., 2004; Xu et al., 2014).

In addition to inorganic nutrients, it has been observed that organic compounds may also stimulate phytoplankton growth in coastal areas (Peierls and Paerl, 1997; Seitzinger and Sanders, 1999). In fact, prior results in the Ría de Vigo showed that organic nitrogen concentration also contributed to the variability in the response of primary production and Chl *a* to rainwater additions (Martínez-García et al., 2015).

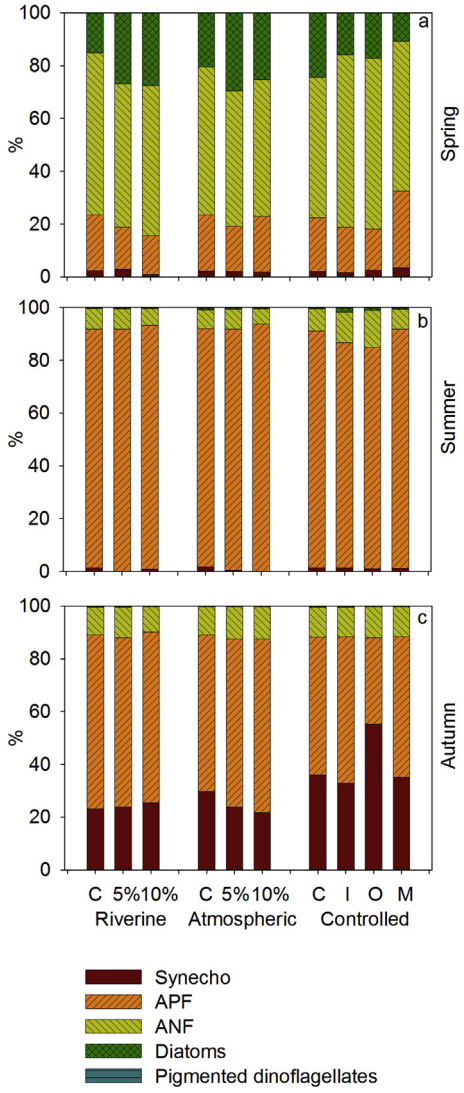

Fig. 6. Relative abundance of major phytoplankton groups at the end of the experiment in spring (a), summer (b) and autumn (c). Synecho: *Synechococcus*-type cyanobacteria, APF: autotrophic picoflagellates, ANF: autotrophic nanoflagellates. C: Control (no additions); I: controlled inorganic additions; O: controlled organic additions; M: controlled mixed additions.

However, in our work, no significant correlations were found between phytoplankton abundance and organic compounds, except a negative correlation with ANF and diatoms in autumn. The few significant positive (although low) increases in phytoplankton abundance in response to the organic treatment (*Synechococcus* and ANF in spring and Chl *a* in summer) may be related to the occurrence of mixotrophy (including direct consumption of organic compounds) in both seasons (Flynn and Butler, 1986; Eiler, 2006) or to an increase in nutrients remineralization mediated by enhanced bacterial activity in the organic treatment in summer (Teira et al., 2016). Nutrient remineralization in spring is unlikely to be important, because there were high *in situ* nutrient levels and bacteria did not respond to the organic inputs in this experiment (Teira et al., 2016). As the chemical composition of the rainwater additions here was very different from that in Martínez-García et al. (2015), it should be expected that different phytoplankton responses may occur depending on the composition of natural matter inputs in the Ría de Vigo.

Moreover, some phytoplankton groups appeared to be favored by the combination of inorganic and organic forms. This is the case of APF in spring and summer, which presented a higher response to the mixed treatment than to the inorganic or organic additions alone (Fig. 5b, e). This response can be related to the concomitant use of organic forms, namely amino acids, by these small phytoplankton cells (Flynn and Butler, 1986; Hernández-Ruiz et al., 2018). The same trend was observed for *Synechococcus* in spring (Fig. 5a) likely related to their mixotrophic capabilities (Eiler, 2006). Other explanations may also include the need of external secondary metabolites for phytoplankton growth (eg. B_{12} vitamin) produced by heterotrophic bacteria (Croft et al., 2005; Prieto et al., 2016) or the occurrence of bacterivory by the small eukaryotes (Sanders and Gast, 2012; Hartmann et al., 2013). In both cases, phytoplankton would be benefiting from the stimulation of bacterial growth by the combination of the organic and inorganic compounds in the mixed treatment (Teira et al., 2016). On the other hand, the lower response of ANF and diatoms to the mixed compared to the inorganic treatment could be explained by competition for inorganic nutrients with the organisms stimulated by the mixed treatment, including APF and bacteria (Joint et al., 2002; Martínez-García et al., 2010).

The different phytoplankton responses to the natural amendments found in autumn - null and negative responses to natural matter inputs and to the organic treatment, respectively - are likely to derive from the interaction among several processes. High *in situ* nutrient concentrations coupled to oceanic populations acclimated to low nutrient availability may explain the low reactivity of this phytoplankton community. Alternatively, the presence of organic compounds in the treatments which could have stimulated bacterial activity (Teira et al., 2016), may consequently have enhanced competition with phytoplankton by inorganic nutrients, leading to low or negative responses of the primary producers (Joint et al., 2002). Other explanations may also include increased grazing impact stimulated by enhanced bacterial biomass (Agawin et al., 2000; Joint et al., 2002).

The differential response of phytoplankton groups to the several inputs may shape the structure of phytoplankton communities, as observed in other studies (Seitzinger and Sanders, 1999; Spatharis et al., 2007; Cui et al., 2016). Some changes were also observed in our work, mainly related to large increases in nutrients (controlled additions) and the presence of organic forms, which promoted interactions among several components of the microbial community. Pronounced changes should be expected under a scenario of high nutrient inputs including both inorganic and organic nutrients, which may stimulate the smallest components of the microbial food web.

Although not considered in this work, under a climate change scenario, high nutrients inputs can be associated to high precipitation and high runoff which may induce other alterations in the environment also affecting the phytoplankton community. These include changes in the physical structure of the water column or in the residence time of the system and a decrease in salinity. Thus, these factors must also be taken in account in future studies to provide an accurate prevision of the response of the phytoplankton communities to those changes.

5. Conclusion

We observed that a realistic increase in nutrients through natural matter inputs from atmospheric and riverine sources reaching the Ría de Vigo induces variable responses in the phytoplankton communities. The magnitude and the type of these responses was closely related to DIN concentrations, but the composition of the natural matter inputs and the structure of the phytoplankton communities also modulated the kind of response observed. Responses to external nutrient inputs were stronger in coastal phytoplankton living under low nutrient levels (summer) than in phytoplankton already residing in replete nutrient conditions (spring). Oceanic phytoplankton advected to the coastal bay during downwelling events (autumn) could occasionally be inhibited by these external nutrient releases. In future global change scenarios, when predictions point to a decrease in upwelling events in this marine system and the consequent decrease in nutrient inputs through these

episodes (Álvarez-Salgado et al., 2008; Pérez et al., 2010), natural matter contributions from riverine and atmospheric origin must play a more important role fertilizing this coastal region. The response of phytoplankton communities and then, its consequences to the whole ecosystem will depend strongly on the species present and on the composition of the natural matter inputs from the different sources. This makes very difficult to predict the evolution of phytoplankton communities in the NW Iberian upwelling system to future human pressures affecting climate and fertilization.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ecss.2018.06.005.

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