Impact of atmospheric deposition on the metabolism of coastal microbial communities

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ABSTRACT

The impact of rain water collected at marine, urban and rural sites on coastal phytoplankton biomass, primary production and community composition as well as the effect on microbial plankton metabolism were studied in 3 microcosm experiments conducted under contrasting spring, autumn and winter conditions. The measured responses were highly variable. Rainwater additions increased chlorophyll *a* (Chl*a*) concentration (5 to 68% difference between rainwater treatments relative to the control) in all experiments and reduced or stimulated primary production (PP) depending on the treatment and the experiment(from –10 to +169% relative to the control). Autotrophic stimulation was highest in spring, likely related to the low initial natural nutrient concentrations. Under winter nutrient replete conditions rainwater inputs changed the phytoplankton community although this change did not promote increases in primary production. Enhancement of net autotrophy (increase of net oxygen production up to 227%) after rainwater inputs were only found during the period of low nutrient availability. Inputs of dissolved organic nitrogen (DON) explained a large fraction of the variability in the response of PP, Chl*a*, community respiration (CR)and net community production. Our results suggest that differences in the initial environmental conditions (i.e. nutrient availability), rainwater composition and the ability of the present autotrophic communities to utilize the new nutrients result in substantial changes in the microbial responses and associated biologically-mediated carbon fluxes. Atmospheric nutrient inputs into the world coastal oceans are increasing rapidly; the results presented here may help understand the effects of different inputs on the metabolism of distinct microbial communities.

1. Introduction

The terrestrial biosphere has experienced major anthropogenic transformations (Ellis, 2011), which are known to significantly alter the biogeochemical cycling of nitrogen at a planetary scale (Gruber and Galloway, 2008), also leading to changes in the fluxes of materials entering into the ocean (Duce et al., 2008). Among these fluxes, atmospheric nutrient deposition has shown a significant increase over the last decades as a result of human activities (e.g. urban, industrial and agricultural expansion), particularly in populated coastal areas (Paerl et al., 2002). The relative contribution of organic nitrogen to the total nitrogen deposited at the global scale currently ranges from 10 to 40% (Cornell et al., 1995; Prospero et al., 1996; Spokes and Jickells, 2005; Duce et al., 2008; Cornell, 2011; Cape et al., 2012; Kanakidou et al., 2012) and it is known that northern-hemisphere continental-land influenced rainwater samples may include ca. 10–30 μmol L^{-1} of organic nitrogen (Cornell et al., 2011). Furthermore, these atmospheric inputs are expected to increase massively in the next 50 years (Galloway et al., 2004; Duce et al., 2008).

Most studies propose nitrogen (N) as the nutrient controlling the rate of primary production in coastal waters (Nixon, 1983;Vitousek and Howarth, 1991;Oviatt et al., 1995;Lignell et al., 2003)and atmospheric deposition has been pointed as a potentially significant source of biologically available nitrogen (20 to 30% and up to a 70% in some areas)in estuarine and coastal systems (Paerl et al., 1990;Duce, 1991;Spokes and Jickells, 2005;Baker et al., 2007).Atmospheric nutrient inputs are known to alter the structure and metabolism of coastal microbial planktonic communities (Paerl, 1997;Peierls and Paerl, 1997;Seitzinger and Sanders, 1999).Nevertheless, the magnitude and nature of these changes are uncertain given the complex interactions and feedback mechanisms governing the dynamics of planktonic microbial communities. Several

studies have reported an increase in autotrophic community biomass and production after rainwater inputs (Paerl et al., 1990;Klein et al., 1997;Zou et al., 2000). However, less attention has been paid to the effect of organic substrates in rainwater on both auto- and heterotrophic compartments (Peierls and Paerl, 1997;Seitzinger and Sanders, 1999). Few works investigating on the effect of dust inputs on community respiration in open ocean oligotrophic areas have shown important increases in community and bacterial respiration (Pulido-Villena et al., 2008; Lekunberriet al., 2010;Marañón et al., 2010). Inorganic nutrients (Marañón et al., 2010) and/or dissolved organic carbon (Pulido-Villena et al., 2008;Lekunberriet al., 2010) in the dust have been argued as the reason behind these heterotrophic responses. Lekunberriet al., (2010) investigated on the metabolic balance response of Mediterranean microbial communities to dust inputs and reported that the composition of the phytoplankton community determined the direction of the response. Teira et al. (2013) reported increases in bacterial growth efficiency as well as changes in the composition of bacterial assemblages in response to experimental rainwater additions in the Ríade Vigo (NW Spain).However, there are not published studies investigating on microbial plankton community respiration and metabolic balance responses to rainwater inputs in coastal areas.

Previous studies have shown that microbial communities from shelf waters of the NW Iberian Peninsula may respond to the input of both inorganic and organic nutrients (Martínez-García et al., 2010; Teira et al., 2011). The microbial responses described in those studies were dependent on the magnitude and nature of the inputs and also on the structure and metabolism of the microbial communities. The aim of the present work is to assess the short-term response of coastal microbial planktonic communities collected during different oceanographic conditions to distinct natural

rainwater additions collected in contrasting areas during different seasons, with special emphasis on inorganic and organic nitrogen inputs.

2. Materials and methods

2.1. Survey area

The NW Iberian margin is characterized by the intermittent upwelling of cold and inorganic nutrient-rich Eastern North Atlantic Central Water (Fraga, 1981; Fiúza,1982; McClain et al., 1986). Conversely, warm and inorganic nutrient-poor surface ocean waters are present during downwelling episodes (Álvarez-Salgado et al., 2003). Although upwelling-favourable northerly winds prevail from March to September and downwelling-favourable southerly winds the rest of the year, out-of-season upwelling or downwelling events have been frequently recorded (Álvarez-Salgado et al., 2000; 2003).

The Ría de Vigo is a highly productive and very dynamic coastal embayment in the NW Iberian margin (Fig. 1), where different microbial communities can be found at short spatial and temporal scales (Moncoiffé et al., 2000; Cermeño et al., 2006; Arbones et al., 2008; Espinoza-González et al., 2012). Water exchange between this embayment and the adjacent shelf is determined by the balance between river discharge and on-shelf wind stress (Álvarez-Salgado et al., 2000). Riverine discharge in this area has been reported to be in the order of 1500 mg N $m^{-2}yr^{-1}$, with a contribution of DON to the inputs of about40% (Gago et al., 2005).

Several works have reported atmospheric deposition of nitrogen in this area (Vázquez et al., 2003; Rodríguez and Macías, 2006) and it has been calculated that 100–250mg N $m^{-2}yr^{-1}$is introduced by wet atmospheric deposition in the Ría de Vigo (Rodríguez and Macías, 2006). This is in the range of global estimations (300 to >1000 mg N $m^{-2}yr^{-1}$) that report that atmospheric (as wet- and dryfall) depositions are of considerable and growing importance in coastal areas and (Paerl 1997), DON contributing between 6–90% of total N (Peierls and Paerl 1997, Seitzinger and Sanders

1999). Industrial, agriculture and cattle activities are the principal sources of N emissions to the atmosphere in the NW Iberian margin. Atmospheric N deposition in thisarea has been already proved to exceed critical loads of eutrophication in local forests (Rodríguez and Macías, 2006) and the European Environmental Agency (EEA), (2001) described the NW Iberian peninsula as one of the main areas in Europe where an important hazard of water and soil eutrophication exists, being this directly related with emissions to the atmosphere and therefore, depositions. In this context, we investigated the effect of rainwater on coastal microbial planktonic communities of the NW Iberian Peninsula.

2.2. Experimental design

Three microcosm experiments were performed in (1) spring 2009, (2) autumn 2009, and (3) winter 2010 to cover a wide range of initial hydrographic and ecological conditions.

Natural seawater for the experiments was taken in the middle sector of the Ría (Fig.1).Vertical profiles of water column temperature, salinity and *in situ* fluorescence down to 25 m depth were obtained in the sampling site with a SBE 9/11 CTD probe and a Seatech fluorometer attached to a rosette sampler. Then, sub-surface seawater (4–7 m) was collected in 12–litre acid-clean Niskin bottles and filtered through a 200 μm pore size mesh to remove larger zooplankton. Subsequently, 12–litre acid-washed polycarbonate bottles were gently filled under dim light conditions.

Rainwater was collected within the previous 10 days to the microcosm experiments at Bouzas (urban), O Viso (rural) and Cíes islands (marine) sites (Fig.1). The three sites for rainwater collection were chosen on the basis of their environmental characteristics, which may determine the chemical composition of the collected

rainwater. The Bouzas station is located in the middle of a heavily populated and polluted area close to the port of Vigo (e.g. emissions from fossil fuel combustion). The Cies Islands are part of a pristine National Park located at the off-shore edge of the embayment (e.g. emissions marine aerosols). The O Viso station is located in a rural area characterized by the presence of forests, crops and farms. Total deposition collectors made with a high-density polyethylene (HDPE) bottle (10 L) connected to a HDPE funnel were used. The funnels cross-sectional area was 725 cm^2. Rainwater samples from each site were collected daily, filtered through polycarbonate filters of 0.45 μm, and frozen to –20 ºC. The day before each microcosm experiment, they were thawed and then mixed to prepare the different treatments tested in the microcosm experiments. A volume (250 mL) was kept for chemical analyses.

The experimental design included duplicate 12-litre bottles for a series of five treatment levels: 1)control; 2)urban–2.5%,addition of rainwater collected at Bouzas to a final concentration of 2.5% v/v; 3)urban–5%,addition of rainwater collected at Bouzas to a final concentration of 5% v/v;4)marine–2.5%,addition of rainwater collected at Cíes Islands to a final concentration of 2.5% v/v; and 5) rural–2.5%,addition of rainwater collected at O Viso to a final concentration of 2.5% v/v.The dilutions used correspond to the estimated rainwater concentrations for the upper 2 m of the water column after normal (2.5% v/v) and stormy (5% v/v) rain events, and are within the range used in previous studies (e.g. Paerl et al., 1990, 1999; Willey and Pearl, 1993; Klein et al., 1997).

The experimental bottles were maintained in a temperature-controlled room at in situ temperature ± 0.1 ºC (Table 1). Bottles were illuminated with cool white light from fluorescent tubes providing an average PAR of 240 μE m^{-2} s^{-1}. The photoperiod varied depending on the season and ranged from 10L:14D (winter experiment) to 12L:12D

(spring and autumn experiments).Bottles were placed on rollers rotating at ca 6 r.p.m.to prevent cell sedimentation. They were conditioned overnight and the rainwater additions were performed before the sunrise. Experiments lasted 3 days and samples to monitor changes in microbial community structure and metabolism were taken every 24 h.

2.3. Inorganic and organic nutrients

Aliquots for inorganic nutrients determination (ammonium, nitrite, nitrate, phosphate and silicate) were collected in 50 mL polyethylene bottles and frozen at –20 ºC until analysis by standard colorimetric methods with an Alpkem segmented flow analyzer (Hansen and Grasshoff, 1983). The limits of detection of these methods were 0.1 μmol $L^{-1}$for nitrate, 0.02 μmol $L^{-1}$for nitrite and phosphate and 0.05 μmol $L^{-1}$for ammonium and silicate. 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 (450ºC, 12 h) 10 mL glass ampoules acidified with H_3PO_4 to pH < 2. They were measured with a nitrogen-specific Antek 7020 nitric oxide chemiluminescence detector coupled in series with the carbon-specific Infra-red Gas Analyzer of a Shimadzu TOC-CVS analyzer (Pt-catalyst) following Alvarez-Salgado and Miller (1998). The limits of detection were 1 μmol L^{-1} for DOC and 0.3 μmol L^{-1} for TDN. Dissolved organic nitrogen (DON) was obtained by subtracting ammonium + nitrite + nitrate from TDN.

2.4. Size-fractionated chlorophyll a

Size-fractionated chlorophyll *a* (Chl*a*) concentrations were measured in 250 mL water samples which were filtered sequentially through 20, 2 and 0.2 μm polycarbonate

filters. The filters were immediately frozen at –20ºC until pigment extraction in 90% acetone at 4ºC overnight in the dark. Chl*a* concentrations were determined, with a 10–AU Turner Designs fluorometer calibrated with pure chlorophyll *a*.

2.5. Microbial plankton

Pico- and nanoplankton were determined in subsamples of 10 mL fixed with buffered 0.2 µm filtered formaldehyde (2% final concentration) and stained with DAPI at 0.1 µg mL^{-1} final concentration (Porter and Feig, 1980). After 10 minutes in the dark, samples were 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 enumerated under blue light excitation and heterotrophic organisms were counted under excitation with UV light. *Prochlorococcus*, which are not accurately counted with this technique, are not present in this coastal system (Rodríguez et al., 2003). Bacterial biomass was estimated according to Lee and Furhmann (1987). Dimensions of several individuals of the different microbial taxonomic groups were measured and cell volumes calculated assuming spherical shape. Cell carbon was estimated following Verity et al. (1992) for pico- and nanoflagellates and Bratbak and Dundas (1984) for *Synechococcus*-type cyanobacteria.

Microplankton was determined in subsamples of 250–500 mL preserved in Lugol's iodine. Depending on the Chl*a* concentration, a variable volume of 5–25 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 after approximation to the nearest geometrical shape (Hillebrand et al., 1999) and cell carbon was calculated following Menden-Deuer and Lessard (2000).

2.6. Primary Production

Five 75 mL Corning tissue flasks (3 light and 2 dark) were filled with water from each experimental bottle and spiked with 185 kBq (5 μCi) $NaH^{14}CO_3$. Samples were incubated for 2 h in the same incubation chamber as the experimental bottles. 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. Radioactivity was measured with a liquid scintillation counter using the external standard and the channel ratio methods to correct for quenching.

2.7. In vivo INT reduction rates

The reduction of the tetrazolium salt 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) to INT-formazan (INT-F) by Electron Transport System (ETS) dehydrogenase enzymes was used as estimator of community respiration (CR). Size-fractionated *r*espiration rates were estimated using the *in vivo* INT method (Martínez-García et al., 2009). The rate of reduction of INT is provided as an estimator of the respiration rate. Four 100 mL dark bottles were filled from each microcosm bottle. One bottle was immediately fixed by adding formaldehyde (2 % w/v final concentration) and used as killed-control. Samples were incubated during 1 h in the dark at the same temperature as the experimental bottles. After incubation, samples were filtered through 0.2–μm pore size polycarbonate filters.

2.8. Net community production rates

Net community production (NCP) rates were measured by changes in oxygen concentrations after light/dark bottle incubations. Dissolved oxygen concentrations were measured by automated precision Winkler titration performed with a Metrohm 721 DMS Titrino, utilising a potentiometric end point as described in Serret et al. (1999). Three gravimetrically calibrated 50 mL dark borosilicate glass bottles and three light ones were carefully filled with water from each treatment. Water was allowed to overflow during the filling, and special care was taken to prevent air bubble formation in the silicone tube. For each treatment, three replicate dark bottles were fixed immediately for the measurement of initial oxygen concentrations (t_{zero}). The three light bottles were incubated for 24 hours in incubators situated inside the temperature-controlled room under the same experimental light. NCP rates were calculated from the difference between the means of the replicate light incubated and zero time.

2.9. Statistical analysis

Repeated measure ANOVA (RMANOVA) was conducted to assess time (within subject factor), treatment (between subject factor, rainwater radditions), and experiment (between subject factor, sampling location) effects. All data fitted a normal distribution (Kolmogorov-Smirnov test); however, the homogeneity of covariance matrices failed for some datasets/variables, even after log or arcsine data transformation. For the latter case we applied the Huynh-Feldt adjustment to correct P-values (Scheiner and Gurevitch, 1993). A Bonferronni post-hoc test was conducted to assess the direction (stimulation or inhibition) of the effect of the addition treatments on the microbial parameters. In order to compare the effect of different rainwater additions on the

biomasses and rates, we calculated the percentage difference relative to the control (%Difference) as:

$$\%Difference = [(AT - C) / C]*100,$$

where AT and C are the time integrated value of the variable in the Addition Treatment and the Control over the 72 hour incubations, respectively. In the case of biomasses, time-averaged values were used. It is worth mentioning that no differences were found in the RMANOVA and in the Bonferroni post-hoc test results using data from 0 to 24, 48 or 72 hours. Stepwise regression analysis was conducted to explore the relationship among the different responses (%Difference, see above) and the concentration of inorganic and organic nutrients of rainwater.

3. Results

3.1. Initial Conditions

Initial conditions for each experiment are summarised in Table 1 and Figure 2. Different hydrographic conditions were found during each survey. In spring (April 2009), low inorganic nutrient and Chl*a* concentrations occurred while in autumn (October 2009) higher temperature, nutrient and Chl*a* concentrations were recorded. In the winter survey (February 2010), temperature was the lowest and inorganic nutrient and Chl*a* concentrations the highest.

Initial nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+)and phosphate (HPO_4^{2-}) concentrations varied over one order of magnitude between seasons: from 0.1 to 3.8 µmol L^{-1}, 0.03 to 0.40 µmol L^{-1}, 0.13 to 1.72 µmol $L^{-1}$and 0.04 to 0.43 µmol L^{-1}, respectively (Table 1). The N/P ratio of inorganic nutrients was above the Redfield ratio only in February 2010 (N/P = 18.3). Initial silicate (SiO_4H_4) concentrations ranged from

0.68 to 8.14 µmol L^{-1} (Table 1). Initial DOC and DON concentrations ranged from 60 to 83 µmol L^{-1} and from 6.8 to 8.13 µmol L^{-1}, respectively (Table 1).

Chl*a* concentrations ranged from 1.92±0.07µg L^{-1}in April 2009 to 5.10±0.17 µg L^{-1}in February 2010. Cells >2µm largely dominated the phytoplankton community in all experiments (Table 1).Primary production (PP) rates were the highest in October 2009 (7.8±1.0µg C $L^{-1}h^{-1}$) and lower values were measured in April 2009 and February 2010 (2.2±0.2and 2.3±0.2 µg C $L^{-1}h^{-1}$, respectively) (Table 1). The highest PP/Chl*a* ratio was recorded in October2009 and the lowest in February 2010 (PP/Chl*a* = 1.73and 0.45µgCµgChla^{-1} h^{-1}, respectively).The highest CR rates were measured in October 2009 (0.039±0.003µmolINT-F$L^{-1}h^{-1}$) and the lowest in February 2010 (0.012±0.002µmol INT-F $L^{-1}h^{-1}$) while intermediate values were found in April 2009 (ca. 0.025±0.001µmol INT-F $L^{-1}h^{-1}$) (Table 1). The lowest values of net community production (NCP) were registered in April 2009 and the highest in October 2009 (1.1±0.2 and 34.8±0.2µmol O_2L^{-1} d^{-1}, respectively) but in the three cases the microbial community was net autotrophic (Table 1).

Total nano- and microphytoplankton biomass was 72, 133 and 23 µg C L^{-1} in spring, autumn and winter, respectively, and represented more than 87% of total phytoplankton biomass in the three experiments (Fig. 2A).Diatoms represented 41–55% of the biomass at the beginning of the three experiments. While *Pseudonitzschia* cf. *seriata* dominated the diatom community in April 2009 (58%), unidentified small centric diatoms dominated in October 2009 (85%) and February 2010(66%) (data not shown). Dinoflagellates accounted for 44% of the total nano- and microphytoplankton biomass in the spring experiment (Fig. 2A) and were dominated by *Gymnodinium*sp., *Gyrodinium* sp. and several thecate dinoflagellates (data not shown). Other flagellates accounted for 44%and 32% of the total nano- and microphytoplankton biomass in the

autumn and winter experiments, respectively(Fig. 2A). Picophytoplankton represented a small fraction of total phytoplankton and was dominated by picoeukaryotes in spring (95%) while *Synechococcus* dominated in autumn (88%).The contribution of both groups to total picophytoplankton biomass was similar in winter (Fig. 2B).

Total heterotrophic nano- and microplankton biomass was 14µg C L^{-1} in spring, 65 µg C L^{-1}in autumn and 16 µg C L^{-1}in winter (Fig. 2C) representing 30, 58 and 35% of total heterotrophic plankton biomass in the three experiments respectively. Nanoflagellates dominated the heterotrophic nano- and microplankton biomass, particularly in the winter experiment (98%). Dinoflagellates were abundant only in the spring experiment (35%). Heterotrophic picoplankton biomass (30–46 µg C L^{-1})was dominated by heterotrophic bacteria (>80% in the three experiments) (Fig.2D).

3.2. Nutrients in rainwater

The chemical composition of rainwater collected at the three sites during the three studied seasons showed a high spatial and temporal variability (Table 2).These seasonal and geographic changes in the chemical composition of the collected material are in accordance with previous studies describing that the composition of rainwater widely change on temporal and spatial scales depending on the provenance of the air masses scavenged by precipitation (e.g. Loÿe-Pilot et al., 1990). HYSPLIT backward-trajectory model provided by NOAA (http://www.ready.noaa.gov/HYSPLIT_traj.php) showed that the air masses over the three sampling points had similar origins during each of the three periods studied, and therefore the differences observed in the chemical composition of rainwater were probably due to different local emissions.

In general, rainwater collected in April 2009 contained higher DIN, DON and DOC concentrations in the three sites studied. Higher NH_4^+concentrations were

generally found in urban as compared to marine or rural rainwater (Table 2).DON accounted from16% (October 2009, marine rainwater) to 62% (October2009, rural rainwater) of total dissolved nitrogen. Higher DON concentrations were measured in the urban and marine rainwater in April 2009,in the rural rainwater in October 2009 and in the marine rainwater in February 2010 (Table 2). DIN/DIP and DOC/DON molar ratios of the rainwater were relatively high (N/P = 26–380, C/N = 6.2–30) compared to the Redfield values (Redfield 1958) (Table 2).

3.3. Autotrophic responses to rainwater additions

Different phytoplankton responses were recorded in the 3 experiments (Figs. 3A–C, 4A–Cand 5A–B).A general decrease in Chl*a* was found in the experiment performed in April 2009although this decline was less severe, retarded or even absent (urban–5% treatment) when rainwater was added (Fig. 3A). A significant (post-hoc test, $p<0.05$)positive overall (including all time points) response to the urban–5% addition (68% difference relative to the control) was recorded (Fig.4A). A decrease in the proportion of Chl*a*> 2µm (% Chl*a*> 2µm) was observed, which was more evident in the control treatment (Fig.3B). In this experiment, rainwater additions promoted increases in PP compared to the control treatment during the first 24 h of incubation (Fig. 3C). These overall (including all time points) responses were significant (post-hoc test, $p<0.05$) in the urban–5% and marine–2.5%treatments (169% and149% difference relative to the control respectively) (Fig.4C). In spring, nano-, micro- and picophytoplankton slightly increased after the additions compared to the control (Figs.5A,B).Diatoms were the only microphytoplankton group that increased in the rainwater treatments compared to the control(Fig.5A) due to the increase in *Chaetoceros* spp. The biomass of *Pseudo-nitzschia* cf. *Seriata* remained constant in all

treatments including the control (Fig. 5A). The contribution of pigmented dinoflagellates and flagellates to total autotrophic nano- and microphytoplankton biomass decreased in the rainwater treatments compared to the control and no changes were recorded in taxonomic composition of these groups (Fig. 5A).

Statistical analysis showed that incubation time had a significant effect on Chl*a*, %Chl*a*> 2µm and PP in this experiment (RMANOVA, $p<0.05$).An overall statistically significant effect (including all time points and addition treatments) of rainwater on Chl*a*, % Chl*a*> 2µm and PP was found in April 2009 (RMANOVA, $p<0.05$).Stepwise regression analysis showed that DON inputs explained the variability in the response of Chl*a* and PP in April 2009 ($p<0.05$).

In the October 2009 experiment increases in Chl*a* and primary production during the first 24 h incubation were recorded in all treatments including the control (Figs.3AandC). These increases corresponded with increases in the % Chl*a*> 2µm (Fig.3B). All the rainwater additions promoted positive responses in Chl*a* and primary production, when compared with the control treatment, except for primary production in the marine–2.5% treatment,(up to 15 and 38% difference relative to the control for Chl*a* and PP, respectively) (Figs. 4A and C). However, these differences were not statistically significant. A general decrease in nano-, micro- and picophytoplankton biomass was found in all rainwater treatments compared to the control in the autumn experiment (Figs.5AandB).Autotrophic community composition did not change in this experiment. A small unidentified centric diatom dominated the nano- and microphytoplankton biomass in all treatments during the incubation time (Fig. 5A).

Statistical analysis showed that the incubation time had a significant effect on Chl*a*, %Chl*a*> 2µm and PP in the October 2009 experiment (RMANOVA, $p<0.05$). An overall statistically significant effect (including all addition treatments and time points)

of rainwater on PP was found in this experiment (RMANOVA, $p<0.05$).Stepwise regression analysis showed that HPO_4^{-2} and DON inputs explained the variability in the response of Chl*a* and PP respectively in October 2009 ($p<0.05$).

Increases of Chl*a*, %Chl*a*> 2 µm and PP were recorded in all treatments (including the control) in the February 2010 experiment (Figs. 3A, B and C). The slight differences between the addition treatments and the control treatment were not statistically significant (Figs. 4A, B and C).Diatoms dominated the nano- and microphytoplankton biomass which increased compared to the control only in the urban–5% and rural–2.5% treatments (Fig.5A). The relative contribution of *Skeletonema* cf. *Costatum* to total diatom biomass increased in all treatments including the control compared to the initial values and became dominant in all treatments except for the marine–2.5% in which an unidentified small centric diatom dominated the community (Fig. 5A).In the winter experiment, picophytoplankton biomass, particularly picoeukaryotes, increased in all treatments compared to the control (Fig. 5B).

Statistical analysis showed that the incubation time had a significant effect on Chl*a*, %Chl*a*> 2µm, and PP in the February 2010 experiment (RMANOVA, $p<0.05$). In this experiment the effect of rainwater (including all addition treatments and time points) was not statistically significant for any of the variables (RMANOVA F-test, $p>0.05$). Stepwise regression analysis showed that none of the studied nutrient inputs explained the variability in the response of Chl*a* and PP in October 2009 ($p>0.05$).

3.4. Heterotrophic and metabolic balance responses to rainwater additions

The effects of rainwater inputs on community respiration (CR) and the metabolic balance of the microbial community estimated as the net community production (NCP) greatly differed between the experiments (Figs.3D,E; 4D,E).

In the experiment performed in April 2009,CR increased during the first 24h in all treatments and in the control up to a maximum value of 0.056 μmol INT-F L^{-1} h^{-1}(Fig.3D).Rainwater additions resulted in increases of CR relative to the control in all rainwater treatments(up to31% increase)(Fig.4D). Increasing NCP compared to the control were recorded in all treatments in this experiment (Fig. 3E):%Difference was 28–228% relative to the control (Fig.4E). However, none of these differences were statistically significant. Heterotrophic nanoflagellates and, to a lesser extent, heterotrophic bacteria increased their biomass in the rainwater treatments compared to the control in this experiment (Figs. 5C,D).

In the experiment performed in October 2009, CR steadily increased until the end of incubation in all treatments including the control up to a maximum value of 0.14 μmol INT-F L^{-1} h^{-1}(Fig.3D). The highest increases after rainwater additions compared to the control were recorded in therural–2.5% and urban–5% treatments (up to 28%difference relative to the control) (Fig.4D).Increases in NCP were recorded in all treatments (including the control treatment) only during the first 24h of incubation (Fig.3E). However, these changes in CR or NCP relative to control were not significant for any of the addition treatments (Figs. 4D,E). Minor changes in heterotrophic nano-, micro or picoplankton biomass were registered in rainwater treatments compared to the control in the autumn experiment (Figs. 5C and D) and an increase in the relative contribution of ciliates to total heterotrophic nano-, microplankton biomass was registered in all treatments (Figs.2C and 5C).

In the experiment performed in February 2010,CR rates steadily increased during the first 48 h in all treatments and the control up to a maximum value of 0.032 μmol INT-F L^{-1} h^{-1}. Even higher values, although not significantly different, were recorded in the addition treatments compared to the control (Figs. 3D and4D). A not

statistically significant decrease of NCP in the addition treatments compared to the control was observed at 24h and 72h (Figs.3E and 4E).Due to technical problems, data for NCP rates at 48 h are not available except for the urban–5% treatment. In the winter experiment heterotrophic nanoflagellates increased in the urban treatments and heterotrophic bacteria increases were registered in all the treatments compared to the control (Figs. 5C,D).

The effect of incubation time on community respiration (CR) and net community production (NCP) was significant in all experiments (RMANOVA, $p<0.001$) but the effect of rainwater inputs (including all treatments and time points) on CR and NCP was only significant in the experiment performed in April2009 (RMANOVA F-test, $p<0.05$). Stepwise regression analysis showed that DON inputs explained the variability in the response of CR and NCP in April 2009 and CR in October 2009 ($p<0.05$). NH_4^+ inputs explained the variability in the response of NCP in February 2010 ($p<0.05$) and none of the studied nutrient inputs explained the variability in the response of NCP in October 2009 and CR in February 2010 ($p>0.05$).

4. Discussion

4.1. Autotrophic responses to rainwater additions

The significant effect that rainwater inputs had on autotrophic production and biomass and the magnitude of the changes observed were to some extent controlled by the composition and magnitude of these inputs, which is in accordance with previous field (Paerl, 1985; Mallin et al., 1993) and experimental studies (Paerl et al., 1990, 1999; Klein et al., 1997; Seitzinger and Sanders, 1999; Seitzinger et al., 2002; Zou et al., 2000) on the effect of rainwater inputs on autotrophic communities. However, phytoplankton responses found in our experiments were also dependent on the initial

natural concentration of nutrients in seawater and the ability of present microbial plankton communities to utilize these new nutrient inputs.

Rainwater inputs had the strongest effects on autotrophic communities during the period of low natural nutrient concentration (spring). In April 2009 the increase in primary production was primarily due to the contribution of *Chaetoceros* sp. to the total phytoplankton community. This might be explained by different processes: a) a higher ability for the utilization of new nutrients by *Chaetoceros* sp.; b) lower silica requirements of *Chaetoceros* sp. since the concentration of silica was low in the rainwater additions and also because *Chaetoceros* sp. presented weaken valves in this experiment (data not shown); and c) the presence in the rainwater additions of any specific organic compound that may favour *Chaetoceros* sp. against *Pseudo-nitzschia* cf. *Seriata* sp. Although diatoms auxotrophy (i.e. inability to synthesize a particular organic compound required for growth) has been previously described (Croft, 2005), little is known about specific requirements for different genera. A strong grazing pressure over the phytoplankton community in spring is suggested by higher increases in primary production than in Chl*a* concentration (Figs. 4 A and C) and the time-decreases recorded in Chl*a* concentration (Figs. 3A and B).

High initial background DIN concentration and high autotrophic biomass in both October 2009 and February 2010 experiments suggest no initial nutrient limitation, which would explain the low response of autotrophic communities to nutrient additions in these experiments. However, differences in the two responses occurred. While rainwater additions had no effect on autotrophic production in winter, a slight positive (significant) response was recorded in autumn (Figs. 3C and 4C), probably related to higher nutrient inputs in October 2009 (Table 2). The metabolic responses of the autotrophic community in autumn were not associated with changes in the

phytoplankton community composition, indicating that the phytoplankton community developed during autumn in this area (i.e. an unidentified centric diatom) is able to maintain its dominance after new nutrients inputs. Also, it is important to note that increases in Chl*a* in October 2009 were not coupled with increases in phytoplankton biomass, suggesting increases in the Chl*a* content per cell with time in this experiment. In contrast, rainwater inputs changed the phytoplankton community under winter nutrient replete conditions, without increases in primary production (Figs. 3C and 4C). These changes in the composition of phytoplankton community in the addition treatments may be related to a higher efficiency on the utilization of new nutrients by *Skeletonema* cf. *Costatum* than that of the initial dominant centric diatom. Also, it is plausible that *Skeletonema* cf. *costatum* benefited from any organic substrate present in the added rainwater.

Positive autotrophic responses (increases in Chl*a* and PP) observed in the control treatment in the experiments performed in October 2009 and February 2010 may be related to: a) the separation of the phytoplankton community from their grazers caused by the pre-incubation filtration procedure used and/or b) an increase in the light received by autotrophic cells due to the absence of natural vertical mixing during the experimental incubations. It must be noted that phytoplankton> 20 µm was responsible for most of the increases in PP and Chl*a* (data not shown). Possibly, zooplankton grazing on these large phytoplankton cells had been excluded by the pre-incubation filtration through 200 µm.

As showed by stepwise regression analysis (Table 3) DON inputs explained the variability in the response of Chl*a* and PP in most cases. This suggests a possible control of the response of autotrophic communities to rainwater additions not only by inorganic but also organic nitrogen in this area. These results are in accordance with

previous works that claimed that the input oforganic N through atmospheric deposition can be crucial for autotrophic microbial responses (Seitzinger and Sanders,1999; Seitzinger et al., 2002). Several major components of DON in atmospheric deposition such as urea and aminoacids (Cornell et al., 2011) are known to be utilized by coastal phytoplankton (Bronk et al., 2007). The relationships between stimulation of autotrophic communities and the amount of DON in rainwater additions could be also attributable to the extra inorganic nutrients available through remineralization processes due to the enhancement of heterotrophic bacterial activity after organic nitrogen inputs (Joint et al., 2002) or to the exudation by heterotrophs of secondary metabolites needed by the autotrophic community. The utilization of the organic substrates by heterotrophic communities in April 2009 cannot be disregarded since increases in CR have been found in the present work (Figs. 3D and 4D) and Teira et al. (2013) reported increases of bacterial abundance, production and respiration after rainwater treatments in this experiment. However, bacterial abundance, production and respiration did not increase in the experiment performed in October 2010 (Teira et al., 2013) and our data do not show increases in heterotrophic pico-, nano- and microplankton biomass in this experiment. Nevertheless, the positive relationships between DON and CR and PP suggest that DON (Table 3) could enhance metabolism of heterotrophic pico-, nano- and microplankton which in turn could increase PP. It can also be argued that the positive response to rainwater inputs in this experiment could be due to phosphate inputs. However, as the initial N/P ratio was below the Redfield ratio and the N/P ratios of the rainwater additions were well above Redfield, we can dismiss this explanation.

In summary, autotrophic communities composition and function may be affected by the rainwater inputs in this coastal area. Phytoplankton responses were highest when low initial natural concentration of nutrients in seawater was found (spring), being

dependent on the ability of present microbial plankton communities to utilize the atmospheric nutrient inputs. Low autotrophic responses were related to high initial nutrient concentrations (autumn and winter).However, even under inorganic nutrient-replete conditions the input of new nutrients promoted minor positive, although not significant, responses of the autotrophic community in some of the treatments in autumn and changes in the phytoplankton community composition in winter.

4.2. Responses of the autotrophic-heterotrophic balance to the rainwater additions

In the present study biases towards net autotrophy in response to rainwater inputs were only found during the period of low nutrient availability (i.e. April 2009; Fig. 4E) in which strong autotrophic responses outcompeted heterotrophic responses to nutrient inputs. These changes matched those of primary production and were probably driven by changes in autotrophic community composition from a *Pseudonitzia* cf. *seriata*-dominated community (control) to a *Chaetoceros* sp.–dominated community (rainwater treatments). Higher enhancement of autotrophy was registered in the urban treatments, coinciding with the strongest blooms of *Chaetoceros* sp. and the highest nutrient inputs.

By contrast, in autumn and winter, rainwater inputs did not affect the metabolic balance of the system (Fig. 4E). These results suggest that under the initial nutrient-replete conditions of these experiments the metabolic balance is a fairly constant characteristic of this system when receiving nutrient inputs even if autotrophic production (autumn) or community composition (winter) change as a result of the input.

Under low nutrient availability conditions (April 2009), DON in atmospheric deposition seemed to modulate the magnitude of production and respiration responses, and therefore net community production changes of microbial communities in this

coastal area (Table 3). Previous studies have shown that combined inorganic and organic additions stimulate large phytoplankton cells in this area (Martínez-García et al., 2010), which would partially compensate the enhanced community respiration after the inputs (Martínez-García et al., 2012).

Therefore, the effect of rainwater inputs on the metabolic balance of microbial communities was only important under low natural availability of nutrients. In these situations rainwater inputs have the capacity to stimulate phytoplankton communities and change their composition so primary production overcomes the increased respiration.

A weakening of the Iberian coastal upwelling has been observed in long-term analysis of climatological variables in this area (Álvarez-Salgado et al., 2008; Pérez et al., 2010). As a consequence of the increased stability of the water column, nutrient levels in surface waters have considerably decreased and the productivity of the system has declined (Pérez et al., 2010). In this scenario, atmospheric inputs of organic and inorganic nitrogen may play an important role in controlling the production and metabolic balance of microbial communities in this area.

Nitrogen limitation characterizes large coastal and estuarine areas over the world (Nixon, 1983; Oviatt et al., 1995; Lignell et al., 2003), in which the input of nutrients controls microbial activity. Furthermore, many coastal systems are subjected to eutrophication due to external nutrient loading, sometimes promoting nuisance phytoplankton blooms (Richardson, 1997). In a changing world in which atmospheric nutrient inputs (specially N) into the coastal oceans is rapidly increasing (Duce et al., 2008) it is important to understand the mechanisms through which microbial communities are affected: the community members that are benefited/excluded and the effects of the inputs in the net metabolic balance of the system.

We have shown that organic nutrients (i.e. DON) in atmospheric deposition may select and stimulate certain phytoplankton species, so primary production overcomes the increased respiration. Our results suggest that differences in the initial environmental conditions (i.e. nutrient availability), rainwater composition and the ability of the present autotrophic communities to utilize the new nutrients will result in substantial changes in the microbial responses and associated biologically-mediated carbon fluxes in coastal areas subjected to atmospheric deposition.

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Table 1.Summary of initial conditions for each experiment.Chlorophyll *a* (Chl*a*); contribution of the size-fraction larger than 2 µm to total Chl*a* (%Chl*a*); primary production (PP); community respiration (CR); net community production (NCP); dissolved inorganic nitrogen(DIN); dissolved organic carbon(DOC); dissolved organic nitrogen(DON).

	Experiment		
	April 2009	October 2009	February 2010
Temperature (ºC)	14.1	16.3	13.2
Salinity	34.71	35.38	35.42
Chl *a* (µg L^{-1})	1.92±0.07	4.47±0.02	5.10±0.17
%Chl *a*>2µm	96.8±0.4%	85.5±0.1 %	92.7±0.7%
PP (µg C L^{-1} h^{-1})	2.2±0.2	7.8±1.0	2.3±0.2
CR (µmol INT-F L^{-1} h^{-1})	0.025±0.001	0.039±0.003	0.012±0.002
NCP (µmol O_2 L^{-1} d^{-1})	1.1±0.2	34.8±0.2	12.8±0.1
NO_3^- (µmol L^{-1})	0.1	3.6	3.8
NO_2^- (µmol L^{-1})	0.03	0.46	0.33
NH_4^+ (µmol L^{-1})	0.13	0.75	1.72
HPO_4^{-2} (µmol L^{-1})	0.04	0.43	0.32
SiO_4H_4 (µmol L^{-1})	0.68	8.14	6.45
DIN (µmol L^{-1})	0.17	4.74	5.86
DOC (µmol L^{-1})	83	72	60
DON (µmol L^{-1})	8.13	6.80	6.90

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Table 2.Chemical composition of rainwater collected in urban, marine and rural sites of the Ría de Vigo (NW Iberian Peninsula) in April 2009, October 2009and February 2010. DIN, dissolved inorganic nitrogen (including nitrate, nitrite, and ammonium); N/P, inorganic nitrogen to phosphorous ratio; DON, dissolved organic nitrogen;DOC, dissolved organic carbon; C/N, organic carbon to organic nitrogen ratio.ND: no data.

	Experiment								
	April 2009			October 2009			February 2010		
	Urban	Marine	Rural	Urban	Marine	Rural	Urban	Marine	Rural
NO_3^- (µmol L^{-1})	14.8	14.6	13.9	9.1	17.5	10.1	4.9	5.6	6.5
NO_2^- (µmol L^{-1})	0.02	0.21	0.02	0.00	0.09	0.00	0.02	0.25	0.02
NH_4^+ (µmol L^{-1})	15.5	8.9	9.8	4.9	1.7	5.2	10.9	2.0	2.9
HPO_4^{-2} (µmol L^{-1})	0.08	0.70	0.17	0.33	0.23	0.51	0.16	0.29	0.05
SiO_4H_4 (µmol L^{-1})	ND	ND	ND	ND	ND	ND	0.27	0.10	0.09
DIN (µmol L^{-1})	30.4	23.7	23.7	14.0	19.3	15.3	15.8	7.8	9.4
N/P	380	34	139	42	83	30	99	26	188
DON (µmol L^{-1})	24.1	24.9	19.1	11.8	3.6	25.4	4.4	5.9	4.5
DOC (µmol L^{-1})	287	195	175	140	120	162	83	108	64
C/N	11.9	7.8	9.0	11.6	30.0	6.2	20.7	18.0	12.8

Table 3.Results of the significant stepwise regressionanalysis between the magnitude of response (%difference relative to control) of: chlorophyll *a* concentration (Chl a_{diff}); primary production (PP_{diff});community respiration(CR_{diff}); net community production (NCP_{diff})and inorganic and organic nutrients in rainwater additions.DON, dissolved organic nitrogen.NO_3^- and DOC (dissolved organic carbon) were also included in the analysis although were not significant in any of the stepwise regressions. The beta (ß) standardized coefficients are provided for each model. Only one significant model (Model 1) was found for each variable. NS: not significant.

	April 2009				October 2009				February 2010			
	Variable	R^2	F sig.	ß	Variable	R^2	F sig.	ß	Variable	R^2	F sig.	ß
Chl a_{diff}												
Model 1	**DON**	*0.874*	*0.001*	*0.935*	**$H_2PO_4^-$**	*0.554*	*0.034*	*0.744*	NS	NS	NS	NS
PP_{diff}												
Model 1	**DON**	*0.523*	*0.043*	*0.723*	**DON**	*0.712*	*0.008*	*0.844*	NS	NS	NS	NS
CR_{diff}												
Model 1	**DON**	*0.638*	*0.017*	*0.799*	**DON**	*0.520*	*0.043*	*0.721*	NS	NS	NS	NS
NCP_{diff}												
Model 1	**DON**	*0.619*	*0.021*	*0.787*	NS	NS	NS	NS	**NH_4^+**	*0.786*	*0.021*	*-0.786*

Figure 1. Map showing the sampling area. Black circles represent rainwater collection sites: Cíes Islands (marine rainwater), O Viso (rural rainwater) and Bouzas (urban rainwater); and black cross represents the seawater sampling site.

Figure2.Initial autotrophic and heterotrophic pico- and nano-/microplankton community composition of the three experiments in the control treatments.

Figure 3. Time course of mean (A) chlorophyll *a* concentration (Chl*a*); (B) contribution of the size-fraction larger than 2µm to total Chl*a* (%Chl*a*>2 µm); (C) primary production (PP); (D) community respiration (CR); and (E) net community production (NCP) in the April 2009, October 2009 and February 2010 experiments. Control: no addition, Urban–2.5%: 2.5% addition of urban rainwater; Urban–5%: 5% addition of urban rainwater; Marine–2.5%: 2.5% addition of marine rainwater; Rural–2.5%: 2.5% addition of rural rainwater. Note that different scales were used. Error bars represent the standard error from triplicates; where error bars are not visible, they are smaller than the symbol size.

Figure 4.Relative change (%) of (A) chlorophyll *a* concentration (Chl*a*) ; (B) contribution of the size-fraction larger than 2µm to total Chl*a* (%Chl*a*>2 µm); (C) primary production (PP); (D) community respiration (CR); and (E) net community production (NCP) in the April 2009, October 2009 and February 2010 experiments expressed as the difference between the time-averaged/integrated value in the rainwater treatment and the control relative to the time-averaged/integrated value in the control microcosms: time-averaged values for Chl*a* and % Chl*a* and time-integrated values for PP, CR and NCP . Control: no addition, Urban–2.5%: Urban 2.5% rainwater addition, Urban–5%: 5% addition of urban rainwater; Marine–2.5%: 2.5% addition of marine rainwater; Rural–2.5%: 2.5% addition of rural rainwater. Note that different scales were used. Error bars represent the standard error from

triplicates; where error bars are not visible, they are smaller than the symbol size. The horizontal line in each graph represents 0(no change). Asterisks represent significant overall (including all time points) changes for each addition treatment from Bonferroni post-hoc test ($p<0.05$).

Figure 5.Time-averagedautotrophic and heterotrophic pico- and nano- and microplankton community composition of the three experiments in the different treatments. Control: no addition, Urban–2.5%: Urban 2.5% rainwater addition, Urban–5%: 5% addition of urban rainwater; Marine–2.5%: 2.5% addition of marine rainwater; Rural–2.5%: 2.5% addition of rural rainwater. Note that different scales were used. The horizontal line in each graph represents the value in the control. Diatoms taxonomic composition has been specified.

Figure 1.

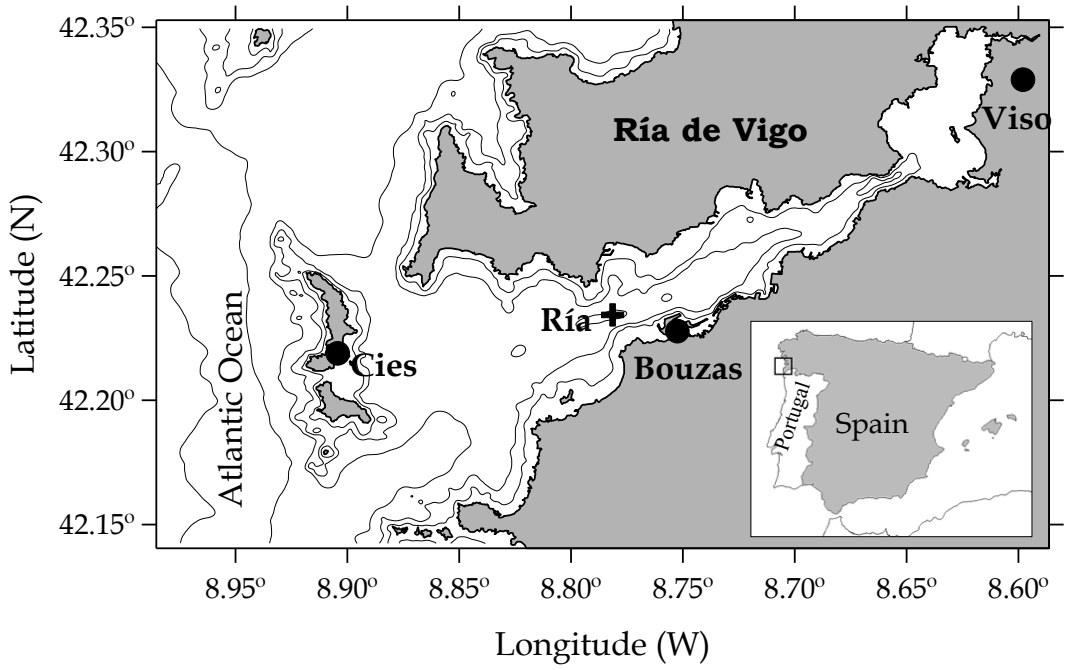

42.35º
42.30º
42.25º
42.20º
42.15º
Latitude (N)
8.95º
8.90º
8.85º
8.80º
8.75º
8.70º
8.65º
8.60º
Longitude (W)
Ría de Vigo
Viso
Ría
Bouzas
Cies
Atlantic Ocean
Portugal
Spain

Figure 2.

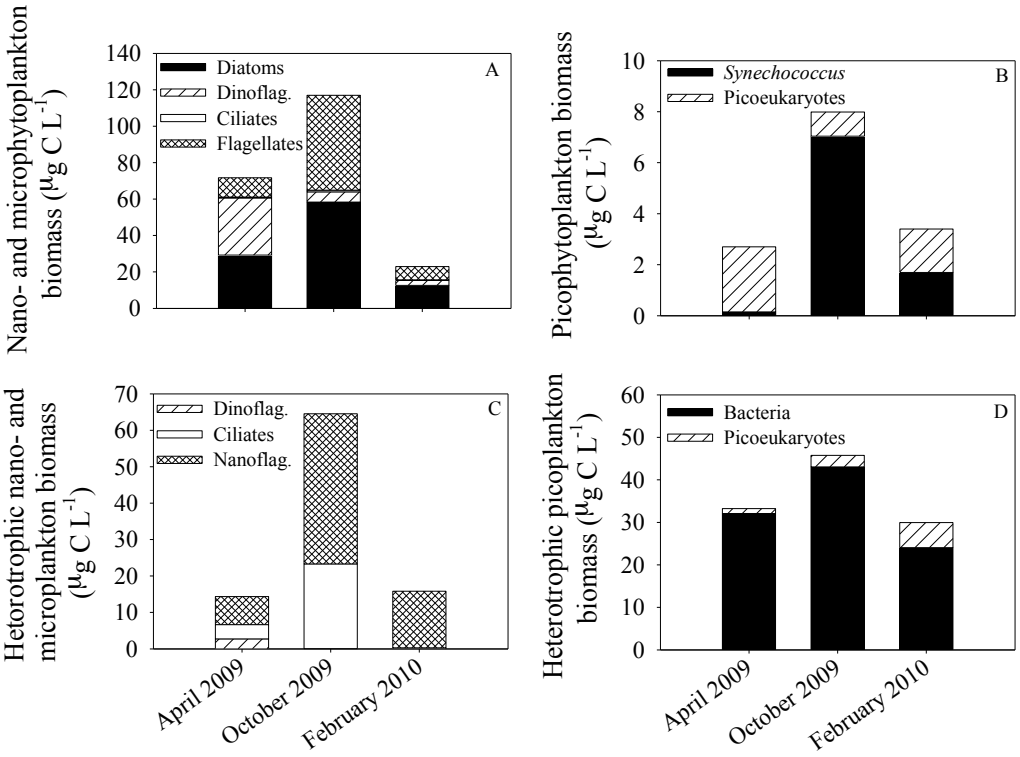

Nano- and microphytoplankton biomass (μg C L-1)
Diatoms
Dinoflag.
Ciliates
Flagellates
A
Picophytoplankton biomass (μg C L-1)
Synechococcus
Picoeukaryotes
B
Heterotrophic nano- and microplankton biomass (μg C L-1)
Dinoflag.
Ciliates
Nanoflag.
C
Heterotrophic picoplankton biomass (μg C L-1)
Bacteria
Picoeukaryotes
D
April 2009
October 2009
February 2010

Figure 3.

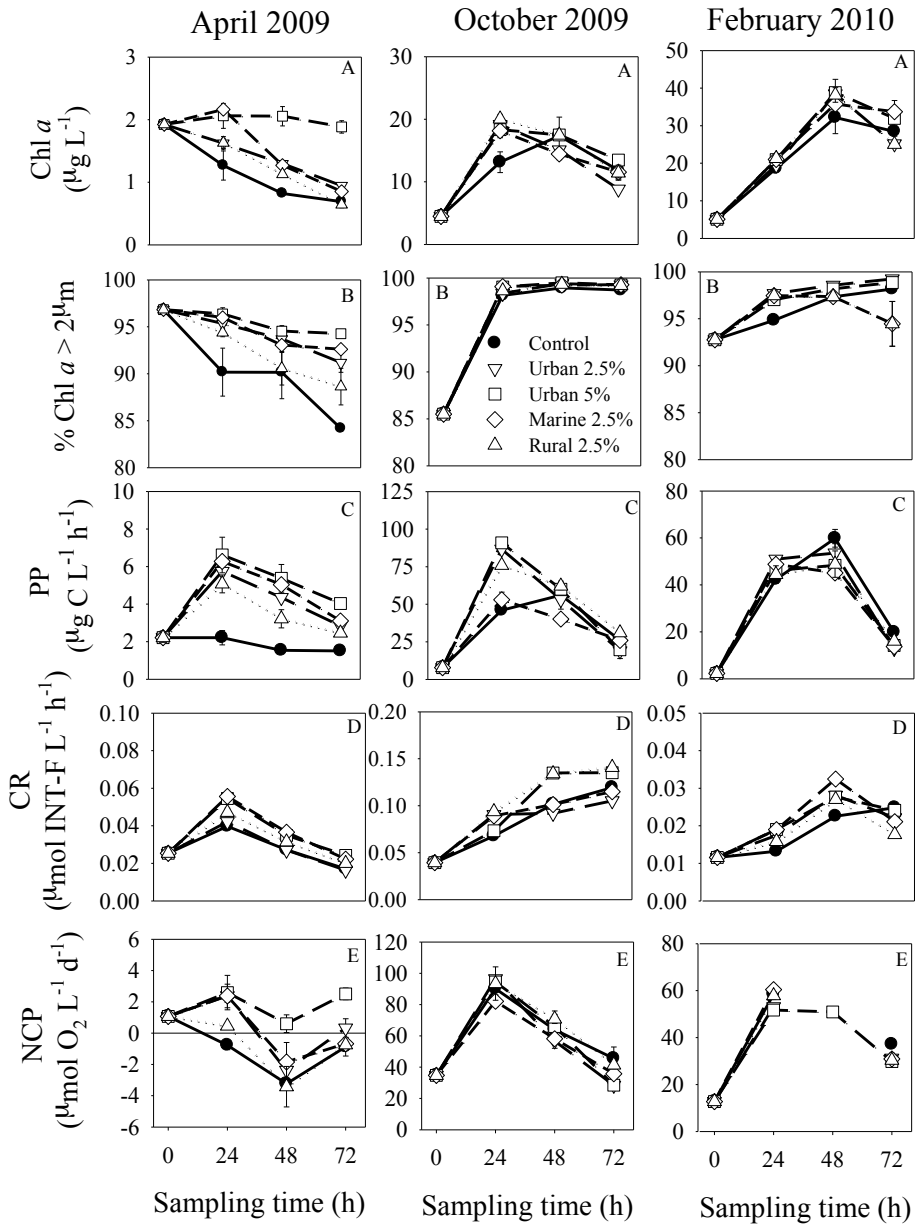

April 2009
October 2009
February 2010
Chl a (µg L-1)
% Chl a > 2µm
PP (µg C L-1 h-1)
CR (µmol INT-F L-1 h-1)
NCP (µmol O2 L-1 d-1)
A
B
C
D
E
Control
Urban 2.5%
Urban 5%
Marine 2.5%
Rural 2.5%
Sampling time (h)

Figure 4.

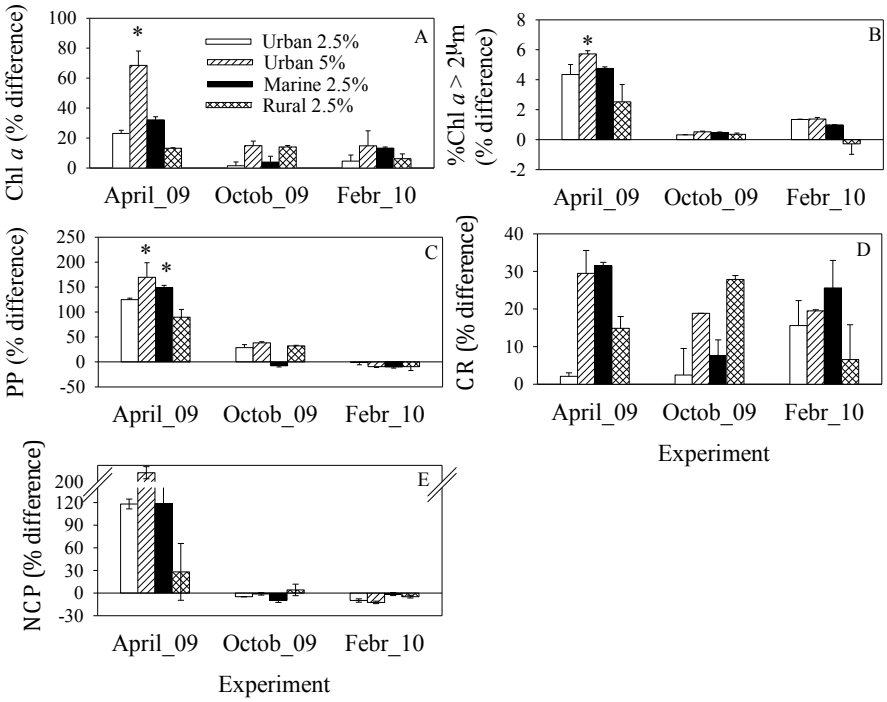
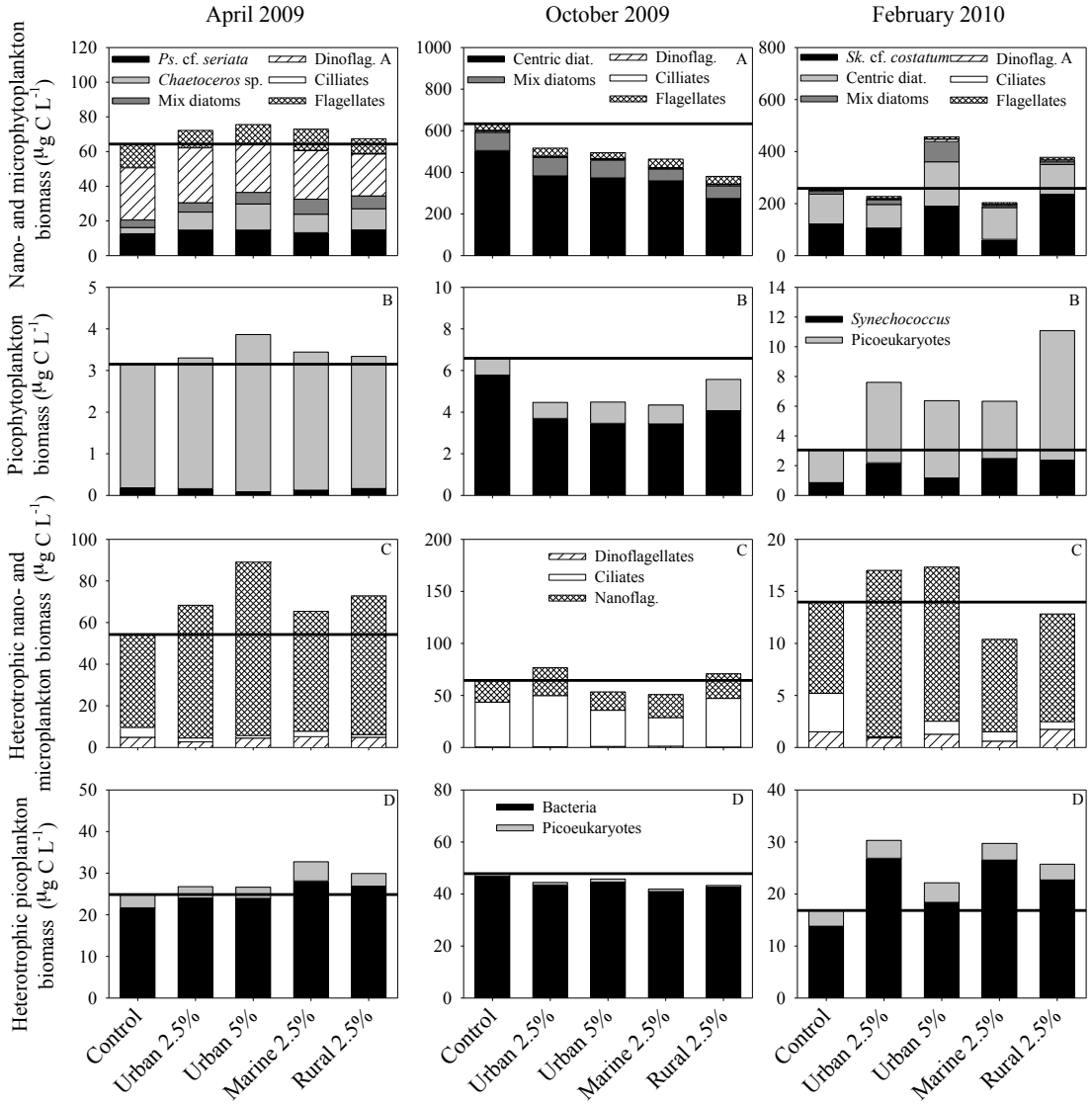

Chl a (% difference)
Urban 2.5%
Urban 5%
Marine 2.5%
Rural 2.5%
A
%Chl a > 2µm (% difference)
B
PP (% difference)
C
CR (% difference)
D
NCP (% difference)
E
April_09
Octob_09
Febr_10
Experiment

Figure5.


April 2009
October 2009
February 2010
Nano- and microphytoplankton biomass (µg C L-1)
Picophytoplankton biomass (µg C L-1)
Heterotrophic nano- and microplankton biomass (µg C L-1)
Heterotrophic picoplankton biomass (µg C L-1)
Ps. cf. seriata
Chaetoceros sp.
Mix diatoms
Dinoflag. A
Cilliates
Flagellates
Centric diat.
Dinoflag.
Sk. cf. costatum
Synechococcus
Picoeukaryotes
Dinoflagellates
Ciliates
Nanoflag.
Bacteria
A
B
C
D
Control
Urban 2.5%
Urban 5%
Marine 2.5%
Rural 2.5%