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Changes in bacterioplankton and phytoplankton community composition in response to nutrient additions in coastal waters off the NW Iberian Peninsula

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ABSTRACT: The short-term effects of inorganic N and P (nitrate, ammonium, phosphate) and organic C and N (glucose, amino acids) inputs, added separately as well as jointly, on phytoplankton and bacterioplankton community composition were studied in 6 microcosm experiments conducted in a eutrophic coastal embayment under contrasting hydrographic conditions. The responses of the different bacterioplankton and phytoplankton groups to the distinct nutrient inputs were highly variable among experiments, which was partially related to changes in the initial environmental conditions. Gammaproteobacteria and nanoflagellates were the most responsive groups to nutrient additions. Inorganic nutrients did not promote important changes in the microbial plankton community structure but did significantly reduce diatom diversity. In contrast, organic additions promoted changes mainly in bacterioplankton groups, whilst mixed additions provoked changes in both bacterial and phytoplankton groups. While nanoflagellates increased equally in abundance after inorganic and mixed additions (2.9-fold), dinoflagellates and diatoms increased their abundances more in the mixed treatment (2.3-fold and 2.2-fold, respectively) than in the inorganic treatment. Organic and mixed additions did not provoke changes in diatom or dinoflagellate diversity. The magnitude of response of Gammaproteobacteria largely explained changes in bulk bacterial biomass and activity, whereas changes in bulk phytoplankton biomass and primary production associated to nutrient enrichment were mostly explained by the response of diatoms and large picoeukaryotes. Our results demonstrate that the type of nitrogen inputs (inorganic and/or organic) strongly affects the microbial plankton community composition and functioning in this coastal ecosystem.

KEY WORDS: Nutrient additions · Organic nitrogen · Phytoplankton · Bacteria · Inorganic

INTRODUCTION

The coastal seas are among the most important areas of the world oceans from a human perspective (Jickells 1998). Coastal marine ecosystems are becoming increasingly affected by nutrient loadings from a variety of anthropogenic sources (Cloern 2001). Many studies on the effects of nutrient enrichment on microbial plankton communities focus on ecosystem-level properties, such as primary or bacterial production or phytoplankton and bacterial biomass (e.g. Caron et al. 2000, Joint et al. 2002, Olsen et al. 2006). However, shifts in the number and diversity of species associated with nutrient enrichment may severely impact a number of ecosystem characteristics (nutrient cycling, food web structure, water quality) (e.g. Klug & Cottingham 2001). Therefore, it is crucial to look for changes in species or functional groups with increasing nutrient

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inputs in order to better predict how microbial plankton communities will respond to environmental alterations. The influence of nutrient availability (mainly inorganic N and P and/or organic C) on species composition has been widely reported for both phytoplankton (Berg et al. 2003, Piehler et al. 2004, Jacquet et al. 2006) and bacterioplankton (Shäfer et al. 2001, Øvreås et al. 2003, Sipura et al. 2005, Pinhassi et al. 2006, Van Wambeke et al. 2009).

Nitrogen is the most common element limiting productivity in most marine ecosystems (Vitousek & Howarth 1991). Nitrogen sources to coastal waters include terrestrial runoff, riverine delivery, groundwater discharge, atmospheric deposition, biotic water column processes, upwelling and sediment remineralization (Capone 2000). Although organic nitrogen may constitute a relevant fraction of the total nitrogen inputs into coastal regions (Meybeck 1993, Peierls & Paerl 1997, Seitzinger & Sanders 1999), the effects of inorganic and/or organic nitrogen inputs on both phytoplankton and heterotrophic bacteria community composition remain poorly studied. Dissolved organic nitrogen can play an active role in supplying nitrogen nutrition directly or indirectly to phytoplankton and bacteria, and thus affect species composition of the microbial plankton community (Berman & Bronk 2003, Heil et al. 2007, Wawrik et al. 2009, Bradley et al. 2010). To the best of our knowledge, only the study by Davidson et al. (2007) has concurrently addressed the differential effect of inorganic versus organic nitrogen inputs on both phytoplankton and bacteria composition during one mesocosm experiment conducted with coastal waters. These authors found that the form of nitrogen (inorganic or organic) influences microbial plankton species composition.

The aim of our study was to assess the response of coastal microbial planktonic community composition to inorganic and/or organic nutrient additions. Differential responses of phytoplankton and heterotrophic bacteria to distinct nutrient inputs are expected depending on both the initial environmental conditions and microbial community structure and the interaction between both microbial compartments. To test this hypothesis, we evaluated the short-term response of both phytoplankton and heterotrophic bacteria to inorganic (N and P) versus organic (N and C) or mixed (N, P and C) nutrient additions under 6 contrasting environmental conditions.

MATERIALS AND METHODS

Survey area. The coastal system of the NW Iberian Peninsula is characterized by the intermittent upwelling of cold and inorganic nutrient-rich Eastern North Atlantic Central Water (Nogueira et al. 1997). Although upwelling-favourable northerly winds prevail from March to September and downwelling-favourable southerly winds prevail the rest of the year, out-of-season upwelling or downwelling events have been frequently recorded (Álvarez-Salgado et al. 2002, 2006). The Ría de Vigo is a eutrophic embayment located in this coastal area. Water exchange between the embayment and the adjacent shelf is determined by the balance between river discharge and shelf wind stress promoting upwelling or downwelling.

It has been estimated that ca. 10% of the inorganic nitrogen supply during the upwelling period comes from a continental source. This percentage is larger during downwelling periods, coinciding with higher precipitation and river flows, which additionally introduce significant amounts of organic nitrogen (Álvarez-Salgado et al. 1996, 2002). Several works have reported atmospheric deposition of nitrogen in this area (Vázquez et al. 2003, Rodriguez & Macías 2006). Wet atmospheric deposition has been calculated to introduce 10 to 25 kg N ha^{-1} yr^{-1} in the Ría de Vigo (Rodriguez & Macías 2006).

The Ría de Vigo and its adjacent shelf is, therefore, a highly productive and very dynamic coastal system where different microbial communities can be found in short time and space scales (Cermeño et al. 2006, Teira et al. 2008, Alonso-Gutiérrez et al. 2009).

Experimental design. Two different locations, one off the embayment on the shelf (Shelf, S) and one in the middle sector of the embayment (Ría, R) (Fig. 1), were sampled during 3 seasons (1, winter; 2, spring; and 3, summer), to cover a wide range of initial hydrographic and ecological conditions. We conducted 6 enrichment microcosm experiments: 2 in winter (S1 and R1, February 2008), 2 in spring (S2 and R2, April 2008) and 2 in summer (S3 and R3, June and July 2008 respectively).

Vertical profiles down to 80 and 25 m (Shelf and Ría stations respectively) of water column temperature, salinity and *in situ* fluorescence were obtained with a SBE 9/11 CTD probe and a Seatech fluorometer attached to a rosette sampler. Surface seawater samples (5 to 10 m) were collected in 12 l acid-clean Niskin bottles and filtered through a 200 µm pore size mesh to remove larger zooplankton in order to ensure good replication. Subsequently, 12 l acid-washed polycarbonate bottles were gently filled under dim light conditions.

The experimental design included duplicate bottles for a series of 4 treatment levels: (1) Control Treatment: no additions made; (2) Inorganic Addition Treatment: 5 µmol l^{-1} nitrate (NO_3^-), 5 µmol l^{-1} ammonium (NH_4^+), 1 µmol l^{-1} phosphate (HPO_4^{2-}); (3) Organic Addition Treatment: 5 µmol l^{-1} glucose and 5 µmol l^{-1} of an

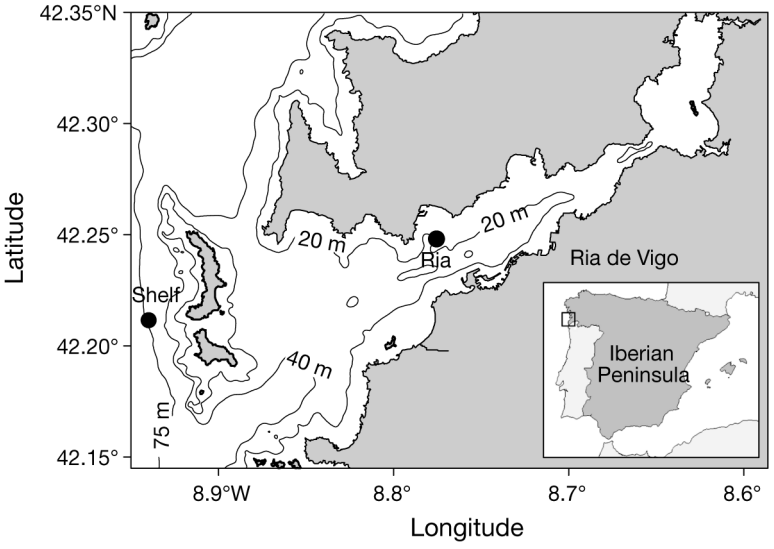

Fig. 1. Locations of the stations that were sampled in the Ría de Vigo and on the shelf off the NW Iberian Peninsula

equimolar mixture of 18 amino acids (all protein amino acids except cysteine and tyrosine); (4) Mixed Addition Treatment: combination of inorganic and organic additions. The N:P ratio of these additions was 10:1 to 15:1 depending on the addition made (inorganic or mixed addition treatment), thus avoiding P-limited additions in the inorganic and mixed treatment. The C:N ratio of these additions was ca. 3.3:1 to 10:1 depending on the addition made (mixed or organic addition). Organic additions were performed to simulate the increases of bioavailable organic nitrogen in atmospheric and/or continental discharge (Meybeck 1993, Peierls & Paerl 1997, Seitzinger & Sanders 1999). Furthermore, atmospheric deposition and river discharge have been shown to contain not only organic nitrogen but also labile dissolved organic carbon (Meybeck 1993, Jacobson et al. 2000, Jurado et al. 2008). We therefore added glucose and amino acids as they are among the most abundant organic labile substances identified in seawater (Bronk 2002). Despite its potentially important role, dissolved organic phosphorus (DOP) was not included, as DOP fluxes are less well documented than those of C and N (Meybeck 1993, Cauwet 2002).

The experimental bottles were maintained in a temperature-controlled room at *in situ* temperature (±0.1°C). Bottles were illuminated with cool white light from fluorescent tubes. The photoperiod ranged from 12 h light:12 h dark to 14 h light:10 h dark depending on the season of sampling and average photosynthetically active radiation was 240 µmol photons m^{-2} s^{-1}. To prevent cell sedimentation, bottles were placed on rollers which rotated them at ca. 6 rpm. After prescreening, samples were incubated overnight on the rollers, and single nutrient additions were performed before light was turned on. Experiments lasted 3 d and samples were taken every 24 h to monitor changes in microbial community structure. Changes in community metabolism were also analyzed and the results are described in Martínez-García et al. (2010b).

Chemical analysis. Aliquots for inorganic nutrients determination (ammonium, nitrite, nitrate, phosphate and silicate) were collected in 50 ml polyethylene bottles and frozen at –20 °C until analysis by standard colorimetric methods with an Alpkem segmented flow analyzer (Hansen & Grasshoff 1983). Water for the analysis of dissolved organic carbon (DOC) 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 in pre-combusted (450°C, 12 h) 10 ml glass ampoules acidified with H_3PO_4 to pH < 2. Samples were measured in a Shimadzu TOC-CVS analyzer (Pt-catalyst) following Álvarez-Salgado & Miller (1998). The fluorescence of dissolved protein-like substances (FDOM) was also determined on the 0.2 µm filtrated samples. Fluorescence measurements were performed at a constant room temperature of 25°C in a 1 cm quartz fluorescence cell in a Perkin Elmer LS 55 luminescence spectrometer, equipped with a xenon discharge lamp, equivalent to 20 kW for 8 µs duration. The detector was a red-sensitive R928 photomultiplier and a photodiode worked as reference detector. Slit widths were 10.0 nm for the excitation and emission wavelengths. Milli-Q water was used as a reference blank for fluorescence analysis. Fluorescence intensity was measured at a fixed excitation/emission wavelength of 280 nm/350 nm, which is characteristic of protein-like materials (Coble 1996). Following Nieto-Cid et al. (2006), the spectrofluorometer was calibrated daily with tryptophan in 0.1 N sulphuric acid. FDOM was expressed in µg l^{-1} equivalents of tryptophan, hereafter given as ppb Trp. Equiv. concentrations were determined by subtracting the average peak height from the Milli-Q blank height, and dividing by the slope of the standard curve.

Size-fractionated chlorophyll *a* (chl *a*). Size-fractionated chlorophyll *a* (chl *a*) concentrations were measured in 150 ml water samples which were filtered sequentially through 20, 5, 2 and 0.2 µm polycarbonate filters. After extraction with 90% acetone at 4°C overnight in dark, chl *a* fluorescence was determined, using the non-acidification technique (Welschmeyer 1994), with a TD-700 Turner Designs fluorometer calibrated with pure chl *a*.

Primary production (PP). Four 75 ml acid-cleaned polystyrene bottles (3 light and 1 dark) were filled with seawater and spiked with 185 kBq (5 µCi) $NaH^{14}CO_3$. Samples were incubated for 3 to 4 h in the same incubation chamber as the experimental bottles. After the incubation period, samples were sequentially filtered through 20, 5, 2 and 0.2 µm polycarbonate filters at very low vacuum (<50 mm Hg). Filters were processed to assess ^{14}C incorporation as described in Marañón et al. (2001).

Bacterial heterotrophic production (BP). The [^3H] leucine incorporation method (Kirchman et al. 1985), modified as described by Smith & Azam (1992), was used to determine leucine (Leu) incorporation rates (LIR). Leucine was added at 40 nM final concentration. Samples were incubated for 1 h in the same incubation chamber as the experimental bottles. Eight dilution experiments: 4 in the Ría (R3) and 4 in the Shelf (S3) were performed in order to determine empirical leucine to carbon conversion factors (CF) for the control, the inorganic, the organic and the mixed nutrient treatment, following the methods detailed elsewhere (Calvo-Díaz & Morán 2009). No significant differences in CFs were found between both sampling locations. The mean CFs derived were: 2.6 ± 1.1 kg C mol Leu^{-1} for the control, 1.6 ± 0.6 kg C mol Leu^{-1} for the inorganic treatment, 3.3 ± 0.9 kg C mol Leu^{-1} for the organic treatment and 4.4 ± 1.3 kg C mol Leu^{-1} for the mixed treatment.

***In vivo* electron transport system (ETS).** ETS activity rate was used as estimator of community respiration (CR). Size-fractionated *in vivo* ETS activity rates were measured using the *in vivo* ETS method (Martínez-García et al. 2009). 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. Fifteen minutes later, all the replicates were inoculated with a sterile solution of 7.9 mM INT ([iodo-phenyl]-3[nitrophenyl]-5[phenyl] tetrazolium chloride) to a final concentration of 0.2 mM. Samples were incubated at the same temperature as the microcosm bottles in dark conditions during 1 h. After incubation, samples were filtered sequentially through 0.8 and 0.2 µm pore size polycarbonate filters, which were stored at –20°C in 1.5 ml cryovials until further processing. The formed insoluble formazan crystals (INT-F) were extracted from the filters by adding 1 ml of propanol and sonicating for 20 to 30 min in 50°C water using an ultrasonic bath. One ml of the propanol extract containing the INT-F was transferred to 1.5 ml microfuge vials and centrifuged at 13 200 × *g* for 10 min at 18°C. The absorbance at 485 nm was then measured using a spectrophotometer (Beckman model DU640). Bacterial respiration (BR) was operationally defined as ETS activity <0.8 µm (Robinson 2008).

Autotrophic and heterotrophic picoplankton. The abundance of picoautotrophs (*Synechococcus*, small and large picoeukaryotes) and heterotrophic bacteria was determined with a Becton Dickinson FACSCalibur flow cytometer equipped with a laser emitting at 488 nm (Calvo-Díaz & Morán 2006). Picoplankton samples (1.8 ml) were preserved with 1% paraformaldehyde + 0.05% glutaraldehyde (Gasol & del Giorgio 2000) and frozen at –80°C until analysis. Aliquots of the same sample were used for the analysis of picophytoplankton (0.6 ml) and heterotrophic bacteria (0.4 ml). Prior to analysis, heterotrophic bacteria were stained with 2.5 mM SybrGreen DNA fluorochrome.

Picoplankton groups were identified on the basis of their fluorescence and light side scatter (SSC) signatures. *Synechococcus* cyanobacteria and 2 groups of eukaryotic cells (small and large picoeukaryotes) were identified in plots of SSC versus red fluorescence (FL3, >650 nm), and orange fluorescence (FL2, 585 nm) versus FL3. More details are given in Calvo-Díaz & Morán (2006).

The empirical calibrations between SSC and mean cell diameter described in Calvo-Díaz & Morán (2006) were used to estimate biovolume (BV) of picoautotrophs and heterotrophic bacteria

Biovolume (BV) was finally converted into biomass by using the volume-to-carbon conversion factors from Worden et al. (2004) for picophytoplankton and the allometric relationship of Norland (1993): fg C $cell^{-1}$ = $120 \times BV^{0.72}$ for heterotrophic bacteria

Nano- and microphytoplankton identification. For the analysis of larger cells under the microscope, 100 ml samples were preserved with Lugol's solution. After sedimentation of a subsample (5 to 50 ml), cells were counted and measured following the method of Utermöhl (Lund et al. 1958) at 100× and 200× on an Olympus IX50 inverted microscope connected to a NIS-Elements BR 3.0 image analysis program. Cell biovolume for each species was estimated from measurements of cell dimensions under the microscope following Olenina et al. (2006) and carbon-to-volume relationships were extracted from Menden-Deuer & Lessard (2000).

Bacterial community composition by CARD-FISH. Samples (10 to 15 ml) were fixed with 0.2-µm filtered paraformaldehyde (1% final conc.) and subsequently stored at 4°C in the dark for 12 to 18 h. Thereafter, each sample was filtered through a 0.2 µm polycarbonate filter (Millipore, GTTP, 25 mm filter diameter) supported by a cellulose nitrate filter (Millipore, HAWP, 0.45 µm), washed twice with Milli-Q water, dried and stored in a microfuge vial at –20°C until further processing in the laboratory.

The *in situ* abundance of different bacterial populations was determined using catalysed reported

deposition-fluorescence *in situ* hybridisation (CARD-FISH) with oligonucleotide probes specific for the domain Eubacteria (EUB338, 5′-GCT GCC TCC CGT AGG AGT-3′; EUB338II, 5′-GCA GCC ACC CGT AGG TGT-3′; and EUB338III, 5′-GCT GCC ACC CGT AGG TGT-3′) (Amann et al. 1990, Daims et al. 1999), Beta- and Gammaproteobacteria (BET42a, 5′-GCC TTC CCA CTT CGT TT-3′; GAM42a, 5′-GCC TTC CCA CAT CGT TT-3′) (Manz et al. 1992) subclasses, the Bacteroidetes group (CF319a, 5′-TGG TCC GTG TCT CAG TAC-3′) (Manz et al. 1996), the *Roseobacter* lineage (Ros537, 5′-CAA CGC TAA CCC CCT CC-3′) (Eilers et al. 2001), the SAR11 cluster (SAR11-441R, 5′-TAC AGT CAT TTT CTT CCC CGA C-3′) (Morris et al. 2002) and the SAR86 cluster (SAR86/1245, 5′-TTA GCG TCC GTC TGT AT-3′) (Zubkov et al. 2001). Filters for CARD-FISH were embedded in low-gelling-point agarose and incubated with lysozyme (Teira et al. 2008). Filters were cut in sections and hybridized at 35°C with horseradish peroxidase (HRP)-labeled oligonucleotide probes fo a minimum of 2 to 4 h. Tyramide-Alexa488 was used for signal amplification (30 to 40 min) as previously described (Pernthaler et al. 2002). We used 55% formamide for all probes except for SAR11-441R (45% formamide). The hybridization for all the probes was done overnight and cells were counter-stained with a DAPI-mix (5.5 parts of Citifluor [Citifluor, Ltd.], 1 part of Vectashield [Vector Laboratories, Inc.] and 0.5 parts of PBS with DAPI [final concentration 1 µg ml^{-1}]).

The slides were examined with an epifluorescence microscope equipped with a 100 W Hg-lamp and appropriate filter sets for DAPI and Alexa488. More than 800 DAPI-stained cells were counted per sample. For each microscope field, 2 different categories were enumerated: (1) total DAPI-stained cells and (2) cells stained with the specific probe. Negative control counts (hybridization with HRP-Non338) averaged 0.25% and were always below 1.0% of DAPI-stained cells. The counting error, expressed as the percentage of standard error between replicates (SE/mean × 100), was <2% for DAPI counts and <10% for FISH counts.

Statistical analysis. Repeated measures ANOVA (RMANOVA) was conducted to assess time (within-subjects factor), treatment (between-subjects factor, nutrient additions), and experiment (between-subjects factor, sampling location) effects. All data fitted a normal distribution (Kolmogorov-Smirnov test); however, even after log or arcsine data transformation, the homogeneity of covariance matrices failed for some datasets/variables. For the latter case we applied the Huynh-Feldt adjustment to correct the p-values (Scheiner & Gurevitch 1993). A Bonferroni 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 nutrient additions on the biomasses and abundances of the different groups, we calculated response ratios (RR) as AT/C, where AT and C are the time-averaged value of the variable in the Addition Treatment and the Control, respectively.

Principal component analysis (PCA) was used to reduce the complexity of multivariate data, including the RR of bacterial and phytoplankton groups to the different nutrient additions and a set of environmental and biotic variables.

RESULTS

Initial conditions

Initial conditions (measured in the controls at time 0) for each experiment are presented in Table 1. Different hydrographic conditions were found during each survey. In winter (S1 and R1), low surface temperature and high nutrient concentration indicate intense vertical mixing. Spring surveys (S2 and R2) were performed after an upwelling event. In R2 surface salinity was 33.7 indicating important continental inputs at this location by the time of sampling. Summer samplings (S3 and R3) coincided with an upwelling event.

Table 1. Summary of initial conditions for each experiment. Sampling depth was 5 to 10 m. FDOM: fluorescence of dissolved protein-like substances (ppb tryptophan). DOC: dissolved organic carbon

Experiment	Temperature	Salinity	Chl *a* (µg l^{-1})	Bacterial biomass (µg C l^{-1})	NO_3^- (µmol l^{-1})	NO_2^- (µmol l^{-1})	NH_4^+ (µmol l^{-1})	HPO_4^{-2} (µmol l^{-1})	SiO_2 (µmol l^{-1})	FDOM (ppb Trp)	DOC (µmol l^{-1})
S1	14.0	35.2	1.8	12.6	2.3	0.09	0.6	0.08	4.1	7.6	85
S2	14.6	35.4	0.3	20.4	2.4	0.03	1.4	0.04	0.5	6.9	74
S3	16.3	35.7	0.2	25.1	0.9	0.81	1.6	0.19	2.1	5.5	82
R1	13.5	35.0	0.7	17.2	9.6	0.32	5.5	1.19	5.4	8.9	86
R2	14.3	33.7	1.3	13.1	2.4	0.11	0.9	0.14	3.5	9.3	80
R3	15.2	35.6	2.1	32.0	3.6	0.25	1.5	0.52	4.4	7.9	73

Nitrate and nitrite concentrations varied one order of magnitude between stations and seasons (from 0.9 to 10 µmol l^{-1} and from 0.03 and 0.8 µmol l^{-1}, respectively). Phosphate initial concentrations ranged from 0.04 to 1.19 µmol l^{-1} and were always higher in the Ría than in Shelf waters. Silicate initial concentrations ranged from 0.5 to 5.4 µmol l^{-1}. DOC concentrations ranged from 73 to 86 µmol l^{-1}. Protein-like dissolved organic matter (as derived from fluorescence measurements) was overall high and rather stable (ranging from 7 to 9 ppb Trp) except in summer in Shelf waters. Chl *a* concentrations varied over 1 order of magnitude and ranged from 0.18 to 2.10 µg l^{-1}, being lowest (<0.3 µg l^{-1}) in April and June in Shelf waters (S2 and S3).

The initial bacterial community was rather constant in the Ría, where the group Bacteroidetes consistently dominated, followed by *Roseobacter*, within the Alphaproteobacteria subclass (Fig. 2A). By contrast, bacterial community structure changed considerably among sampling periods on the Shelf; SAR11, within the Alphaproteobacteria subclass, dominated in February (S1), Bacteroidetes and *Roseobacter* co-dominated in April (S2), and Bacteroidetes was the most abundant group in June (S3). The initial phytoplankton community composition was variable, although dinoflagellates tended to dominate in Shelf waters and diatoms dominated in the Ría (Fig. 2B). *Detonula pumila* dominated the diatom population on the Shelf in April. *Asterionellopsis glacialis, Detonula pumila* and *Thalassiosira rotula* dominated the diatom population of the Ría in February, April and July, respectively. Picoeukaryotes dominated phytoplankton biomass in February both in the Shelf (S1) and in the Ría (R1).

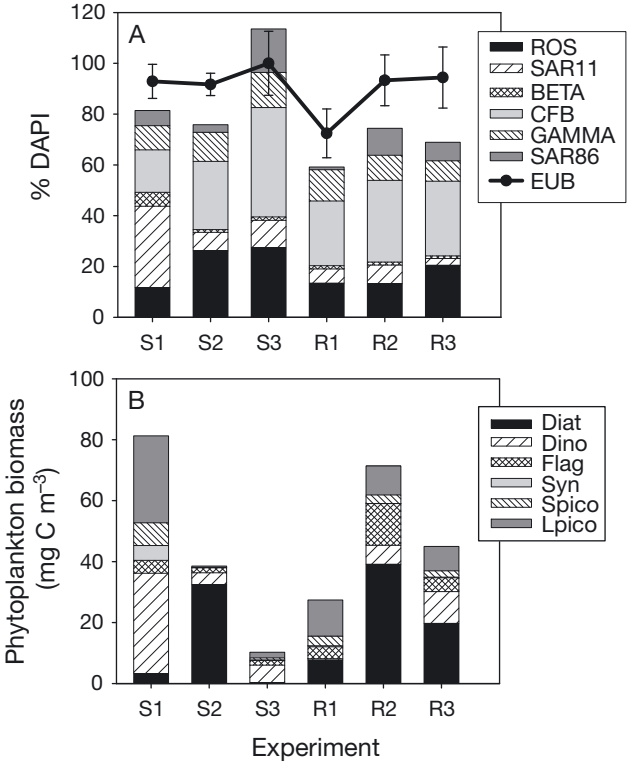

Fig. 2. Initial community composition for the 6 experiments measured as (A) % DAPI and (B) phytoplankton biomass. S1, S2, and S3 refer to the experiments conducted with water sampled in the shelf and R1, R2 and R3 refer to experiments conducted with water sampled in the Ría de Vigo. ROS, *Roseobacter*; BETA, Betaproteobacteria; CFB, Bacteroidetes; GAMMA, Gammaproteobacteria; EUB, Eubacteria; Diat, diatoms; Dino, dinoflagellates; Flag, nanoflagellates; Syn, *Synechococcus*; Spico, small picoeukaryotes; Lpico, large picoeukaryotes

Response of bacterial groups to nutrient additions

The time-course of the abundance of each bacterial group in the control and nutrient amended microcosms for the 6 experiments is represented in Fig. 3. The abundance of all the groups either remained stable, decreased or showed slight increases in the control microcosms. Overall, all the groups increased their abundance in response to the organic and/or mixed treatments, although the magnitude of the response was clearly variable both among groups and experiments. The groups reaching the highest abundances were Gammaproteobacteria (Fig. 3E), followed by *Roseobacter* and Bacteroidetes (Fig. 3A). SAR11 showed a general decreasing trend in abundance during the incubations (Fig. 3B). Betaproteobacteria (Fig. 3C) and SAR86 (Fig. 3F) showed very distinct dynamics among different experiments. In order to quantify and compare the response of bacterial groups to the different additions we calculated the response ratio (RR) (Fig. 4). None of the groups showed a significant response to inorganic nutrient additions (Fig. 4, Table 2). Within the Alphaproteobacteria, the groups *Roseobacter* and SAR11 showed contrasting patterns of response. *Roseobacter* significantly increased in abundance after organic and mixed additions (Table 2), although the response to the organic treatment was not significant in S1 and S2 (Fig. 4A), whereas SAR11 did not respond significantly to nutrient additions (Table 2). Only in R2 was a significant decrease in SAR11 abundance observed after mixed additions (Fig. 4B). Overall, Betaproteobacteria and SAR86 did not respond significantly to organic or mixed additions (Table 2), although significant responses were occasionally observed in S2 for Betaproteobacteria and in R1 for SAR86 (Fig. 4C,F). Bacteroidetes and Gammaproteobacteria showed the same patterns of response as *Roseobacter* (Fig. 4D,E). The magnitude of response of Gammaproteobacteria was clearly higher than that of the other groups (Fig. 4).

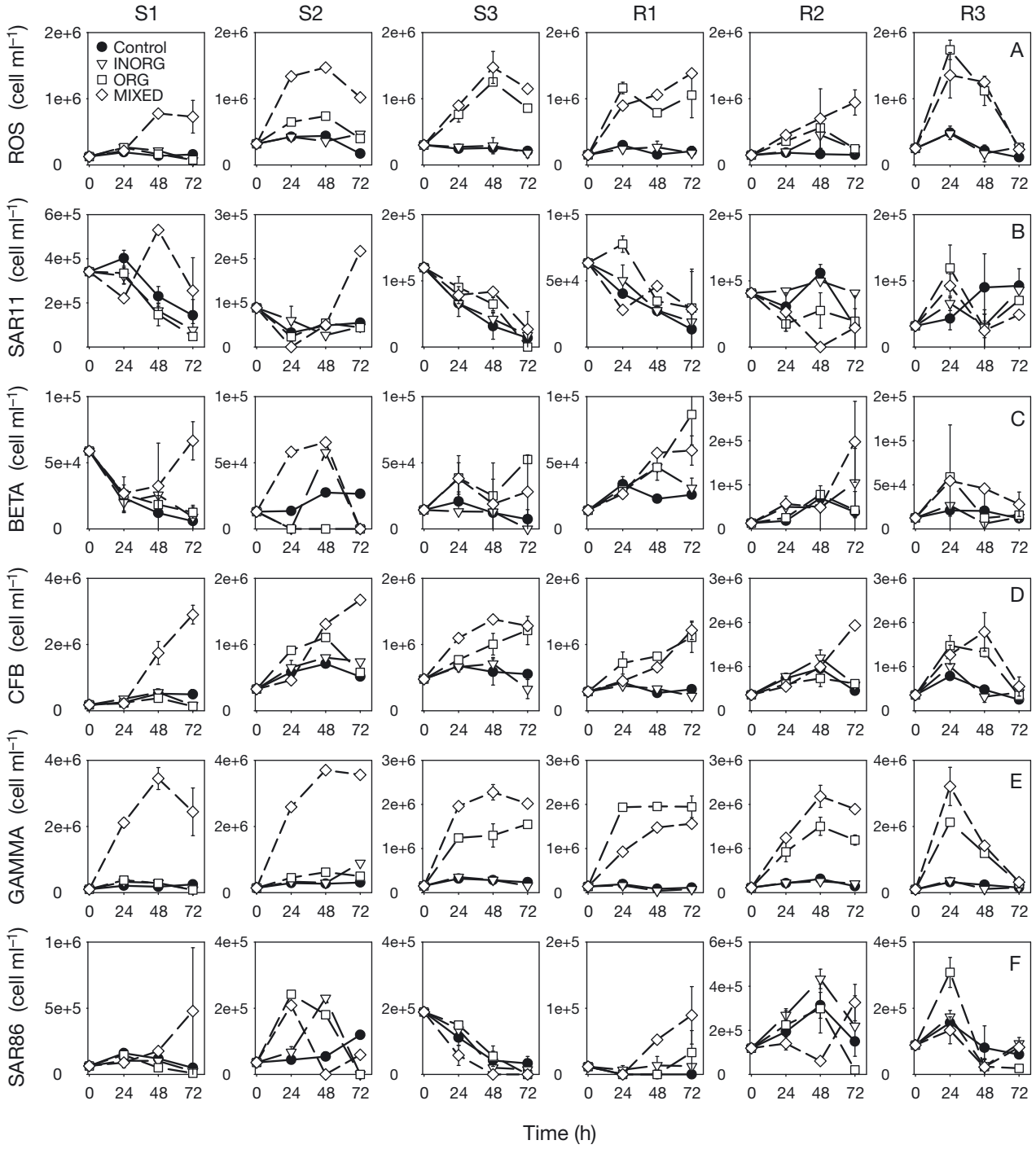

Fig. 3. Time course of the mean abundance (cell ml^{-1}) of (A) *Roseobacter*, (B) SAR11, (C) Betaproteobacteria, (D) Bacteroidetes, (E) Gammaproteobacteria, (F) SAR86. Control, no addition; INORG, inorganic addition; ORG, organic addition; MIXED, mixed addition. S1, S2, and S3 refer to the experiments conducted with water sampled in the shelf and R1, R2 and R3 refer to experiments conducted with water sampled in the Ría de Vigo. Error bars represent the standard error from duplicates; where error bars are not visible, they are smaller than the symbol size. Note that different scales were used

Using the data from the 6 experiments and all treatments (n = 36), we conducted a multivariate linear regression analysis using the response ratio of bacterial biomass, bulk bacterial production or respiration (described in Martínez-García et al. 2010b) as dependent variables and the response ratio of the abundance of the 6 bacterial groups as independent variables to explore the relationship between bacterial community

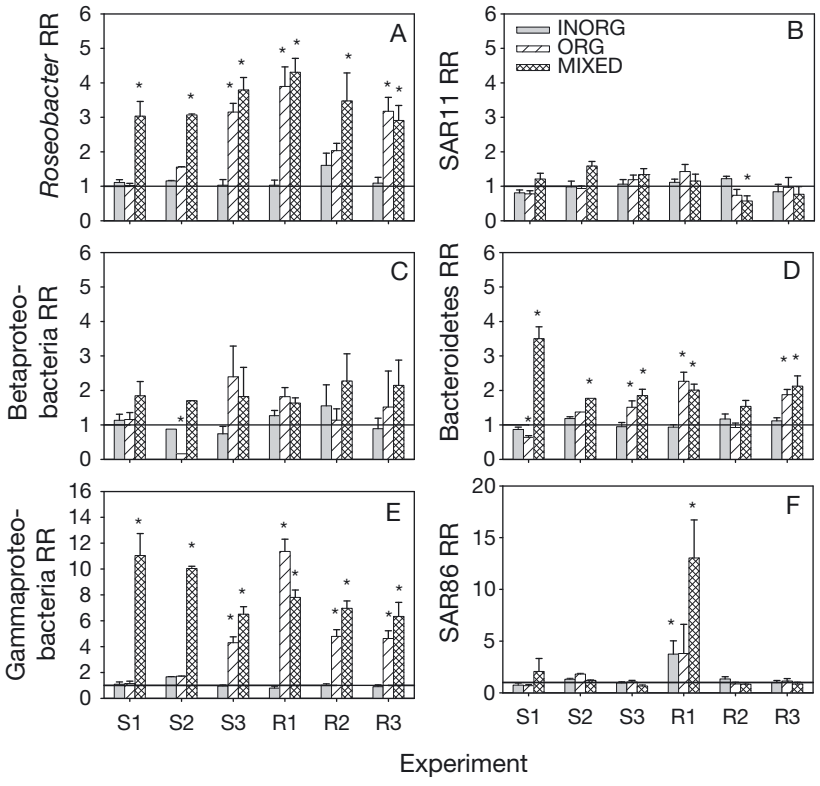

Fig. 4. Response ratio (RR) (relative to control) to different nutrient additions of (A) *Roseobacter*, (B) SAR11, (C) Betaproteobacteria (D) Bacteroidetes, (E) Gammaproteobacteria, (F) SAR86. INORG, inorganic addition; ORG, organic addition; MIXED, mixed addition. S1, S2, and S3 refer to the experiments conducted with water sampled in the shelf and R1, R2 and R3 refer to experiments conducted with water sampled in the Ría de Vigo. Note that different scales were used. Error bars represent the standard error from duplicates; where error bars are not visible, they are smaller than the thickness of the bar. The horizontal line represents RR = 1, i.e. no change relative to control. Asterisks indicate response ratios significantly different from 1 ($p < 0.05$). Note that different scales were used

Table 2. Summary of the global effect of the different additions on the different phytoplankton (diatoms, dinoflagellates, nanoflagellates, *Synechococcus*, small and large picoeukaryotes) and bacterioplankton (*Roseobacter*, SAR11, Betaproteobacteria, Bacteroidetes, Gammaproteobacteria, SAR86) groups (repeated measures ANOVA and Bonferroni post-hoc tests): 0, no significant effect; +, significant effect $p < 0.05$; ++, significant effect $p < 0.01$; +++, sig-nificant effect $p < 0.001$; +, stimulation; –, inhibition

Variable	Inorganic	Organic	Mixed
Diatoms	0	0	++
Dinoflagellates	0	0	+
Nanoflagellates	++	0	++
Synechococcus	–	0	+++
Small picoeukaryotes	0	0	0
Large picoeukaryotes	0	–	0
Roseobacter	0	+++	+++
SAR11	0	0	0
Betaproteobacteria	0	0	0
Bacteroidetes	0	+++	+++
Gammaproteobacteria	0	+++	+++
SAR86	0	0	0

structure and bacterial carbon fluxes (Table 3). The magnitude of response of Gammaproteobacteria has the greatest influence on bacterial carbon fluxes, explaining 99%, 97% and 94% of the variability in the magnitude of responses of bacterial biomass, bulk bacterial production and respiration, respectively.

Response of phytoplankton groups to nutrient additions

Most of the phytoplankton groups responded to the nutrient additions after 48 h of incubation (Fig. 5). The responses greatly varied among groups, although the most apparent responses occurred after mixed additions. Diatoms increased biomass in the mixed treatment in all the experiments (1.4 to 4.1-fold) except S3, whereas they only responded to inorganic nutrients in S2 (Figs. 5A & 6A). RMANOVA and post-hoc tests, including data from all the experiments, showed a significant positive effect of mixed additions on diatoms (Table 2). A similar pattern of response was observed for dinoflagellates, which mostly increased after mixed additions (1.5 to 4.6-fold) (Figs. 5B & 6B, Table 2). The nanoflagellates showed a significant increase (1.2 to 9.6 fold) in biomass in response to both inorganic and mixed additions (Figs. 5C & 6C, Table 2). *Synechococcus* showed a significant positive response to mixed additions (1.2 to 2.2-fold) (Table 2) although higher biomass was measured after organic additions in some experiments (Figs. 5D & 6D). *Synechococcus* and large picoeukaryotes biomass showed a significant decrease in response to inorganic and organic nutrients (Figs. 5D,F & 6D,F, Table 2). The response of small picoeukaryotes to distinct nutrient additions was not statistically significant (Table 2).

Using the data from the 6 experiments and all treatments (n = 36), we conducted a multivariate linear regression analysis using the response ratio of chl *a* concentration or bulk primary production (described in Martínez-García et al. 2010b) as dependent variables and the response ratio of the biomass of the 6 phytoplankton groups as independent variables to explore the relationship between phytoplankton community structure and activity (Table 4). The magnitude of

Table 3. Linear regression analysis of the contribution of the response ratios of major bacterial groups to the variability in the response ratios of bacterial biomass (BB), bacterial production (BP) and bacterial respiration (BR). NS, not significant. Standardized coefficients measure the contribution by the bacterial group to explaining variation in the dependent variables. N = 36

Dependent variable	Standardized coefficients						Adjusted	
	Ros	SAR11	Beta	CFB	Gamma	SAR86	r^2	p
BB	NS	NS	NS	−0.12	0.99	NS	0.96	<0.001
BP	NS	NS	NS	NS	0.97	NS	0.94	<0.001
BR	NS	NS	NS	NS	0.94	NS	0.87	<0.001

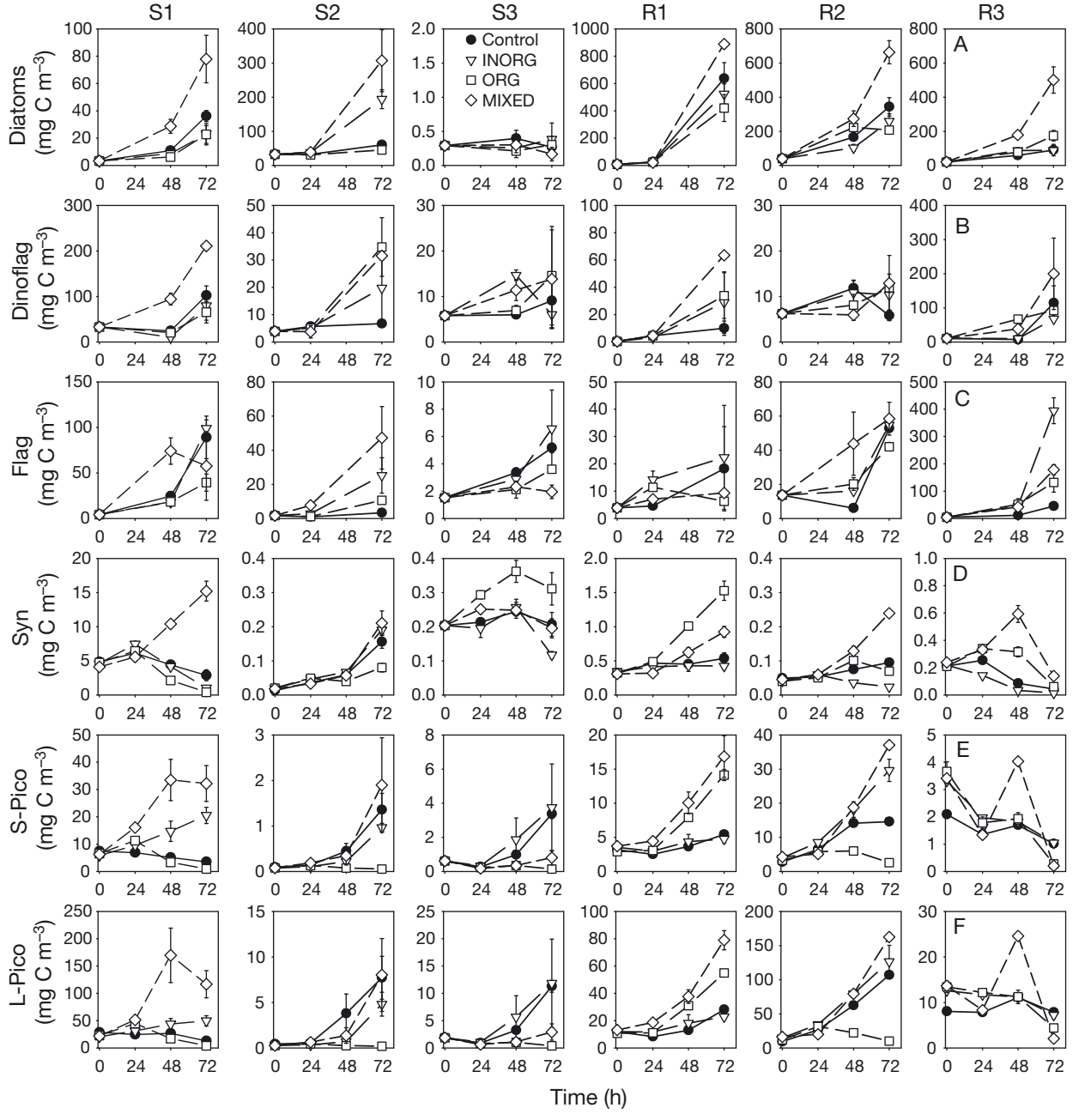

Fig. 5. Time course of the mean biomass (mg C m^{-3}) of (A) diatoms, (B) dinoflagellates, (C) nanoflagellates, (D) *Synechococcus*, (E) small picoeukaryotes, (F) large picoeukaryotes. Control, no addition; INORG, inorganic addition; ORG, organic addition; MIXED, mixed addition. S1, S2, and S3 refer to the experiments conducted with water sampled in the shelf and R1, R2 and R3 refer to experiments conducted with water sampled in the Ría de Vigo. Error bars represent the standard error from duplicates; where error bars are not visible, they are smaller than the size of the symbol. Note that different scales were used

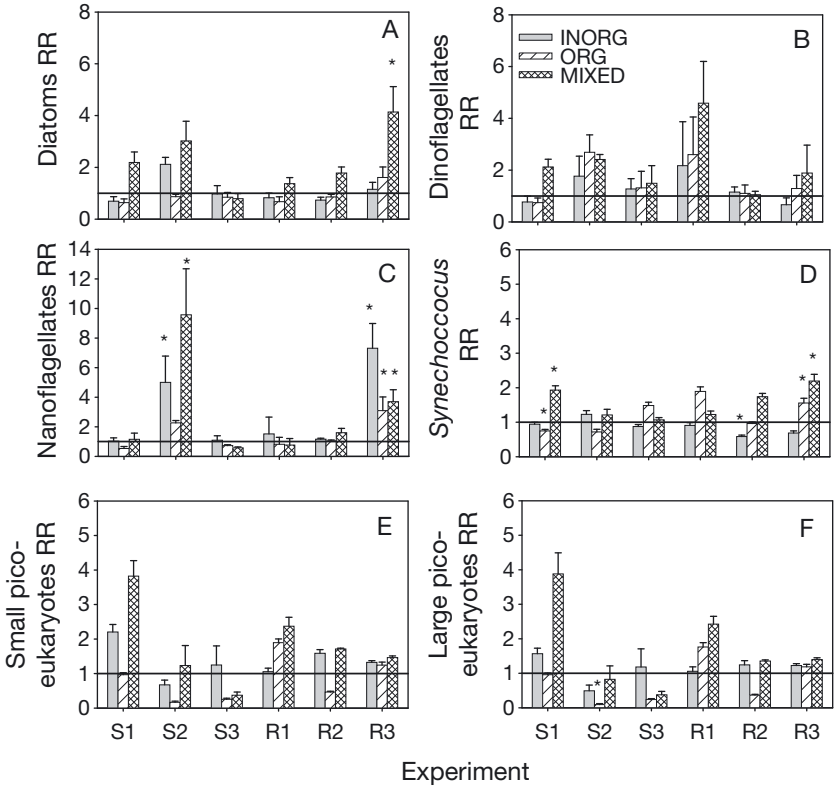

Fig. 6. Response ratio (RR) (relative to control) to different nutrient additions of (A) diatoms, (B) dinoflagellates, (C) nanoflagellates, (D) *Synechococcus*, (E) small picoeukaryotes, (F) large picoeukaryotes. INORG, inorganic addition; ORG, organic addition; MIXED, mixed addition. S1, S2, and S3 refer to the experiments conducted with water sampled in the shelf and R1, R2 and R3 refer to experiments conducted with water sampled in the Ría de Vigo. Error bars represent the standard error from duplicates; where error bars are not visible, they are smaller than the thickness of the bar. The horizontal line represents RR = 1, i.e. no change relative to control. Asterisks indicate response ratios significantly different from 1 ($p < 0.05$). Note that different scales were used

response of diatoms had the greatest influence on phytoplankton dynamics, explaining 89 and 63% of the variability in the phytoplankton biomass (chl *a* concentration) and primary production response ratio, respectively. The response ratio of large picoeukaryotes biomass also significantly contributed (37%) to the observed variability in primary production response ratio.

Changes in microbial plankton community structure

We evaluated the effect of confinement itself on both the bacterioplankton and phytoplankton. No significant differences were found in the relative contribution of each bacterial or phytoplankton group between the initial and final sampling time in the control (paired t-test, $p > 0.06$, $n = 6$). The distinct patterns of response of different bacterial and phytoplankton groups to nutrient additions resulted in changes in the plankton community structure (Fig. 7). Overall, the organic and mixed treatments provoked more changes in both bacterial and phytoplankton community structure than the inorganic treatment, with some exceptions. The bacterial community structure did not change importantly after organic additions in S1 and S2. In the case of phytoplankton, major changes in the community structure occurred in response to inorganic nutrients in R3, where nanoflagellates comprised 70% of the total final phytoplankton biomass (Fig. 7).

We also explored whether the nutrient addition treatments caused changes in diatoms and dinoflagellates species richness and diversity (Fig. 8). The confinement in 12 l microcosms and incubation during 3 d under controlled conditions did not affect species richness or diversity of diatoms and dinoflagellates. The diversity of dinoflagellates tended to decrease (from 1.4 to ca. 1) as a consequence of the confinement and experimental conditions, irrespective of the nutrient additions, although the differences were not statistically significant (paired *t*-test, $p > 0.05$, $n = 6$). The diatoms diversity significantly decreased compared to the control in response to inorganic nutrient addition (paired *t*-test, $p = 0.04$, $n = 6$).

Table 4. Linear regression analysis of the contribution of the response ratios of major phytoplankton groups to the variability in the response ratios of chlorophyll a concentration (chl *a*) and primary production (PP). NS, not significant. Standardized coefficients measure the contribution by the bacterial group to explaining variation in the dependent variables. Diat, diatoms; Dino, dinoflagellates; Flag, nanoflagellates; Syn, *Synechococcus*; Spico, small picoeukaryotes; Lpico, large picoeukaryotes; N = 36

Dependent variable	Standardized coefficients						Adjusted	
	Diat	Dino	Flag	Syn	Spico	Lpico	r^2	p
Chl *a*	0.89	NS	NS	NS	NS	NS	0.78	<0.001
PP	0.63	NS	NS	0.22	NS	0.37	0.92	<0.001

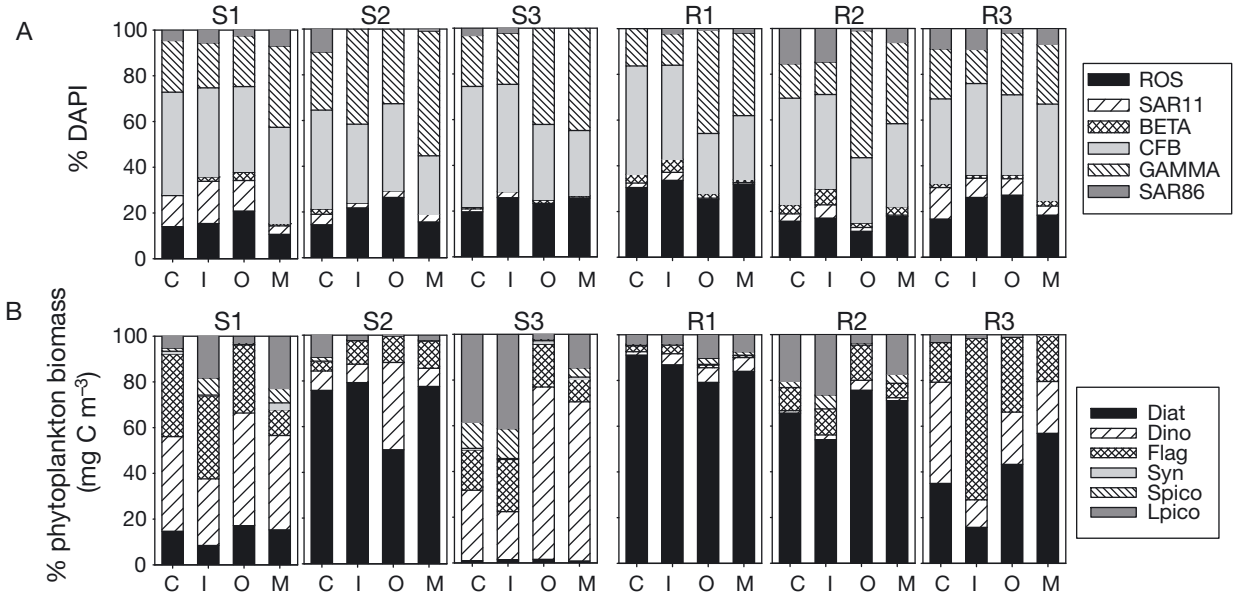

Fig. 7. Final community composition, measured as (A) % DAPI and (B) % phytoplankton biomass at the 6 sampling stations in the control (C), inorganic treatment (I), organic treatment (O), and mixed treatment (M) microcosm. S1, S2, and S3 refer to the experiments conducted with water sampled in the shelf and R1, R2 and R3 refer to experiments conducted with water sampled in the Ría de Vigo. ROS, *Roseobacter*; BETA, Betaproteobacteria; CFB, Bacteroidetes; GAMMA, Gammaproteobacteria; Diat, diatoms; Dino, dinoflagellates; Flag, nanoflagellates; Syn, *Synechococcus*; Spico, small picoeukaryotes; Lpico, large picoeukaryotes

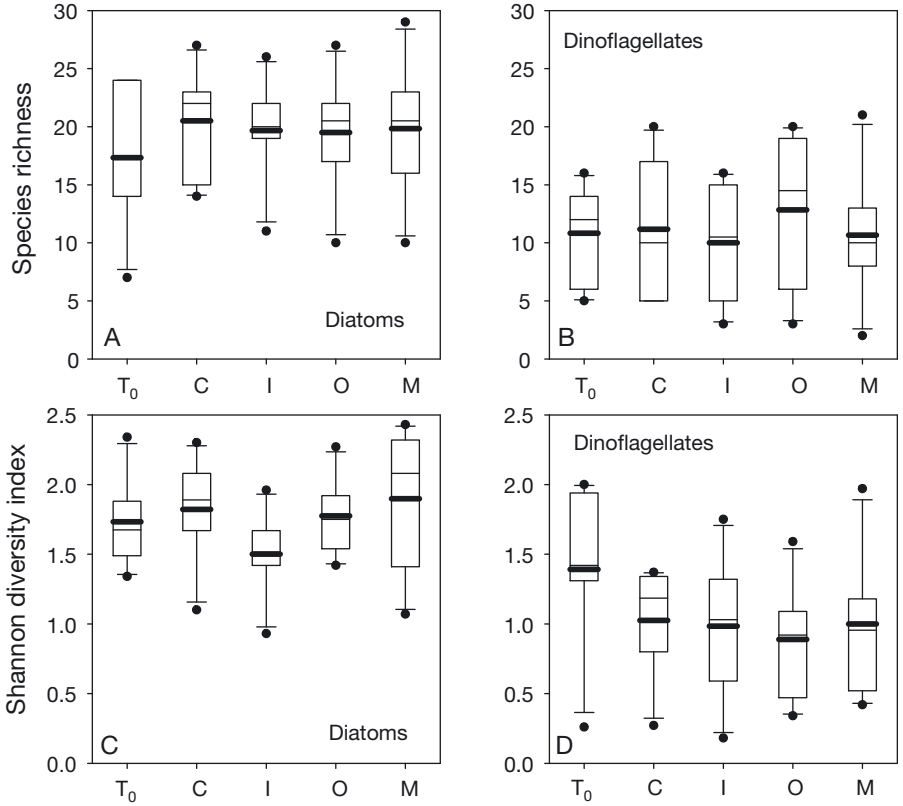

Fig. 8. Box and whisker plots showing changes associated with the incubation and the different addition treatments for (A) diatom species richness, (B) dinoflagellate species richness, (C) diatom diversity, and (D) dinoflagellate diversity. The boxes represent the 25th and 75th percentiles and the whiskers represent the 10th and 90th percentiles. The thick black line represents the mean for the 6 experiments, while the thinner line represents the median (50th percentile). The black dots are data beyond the 10th and 90th percentiles T_0, initial time; C, final time in the control; I, final time in the inorganic treatment; O, final time in the organic treatment; M, final time in the mixed treatment

Response of microbial plankton groups and environmental variables

In order to link the observed differential responses of bacterioplankton and phytoplankton groups to distinct nutrient additions with initial environmental conditions and initial community structure, we conducted several principal component analyses (Fig. 9). We only included in the analysis the response ratios of those groups that were statistically significant (see Table 2).

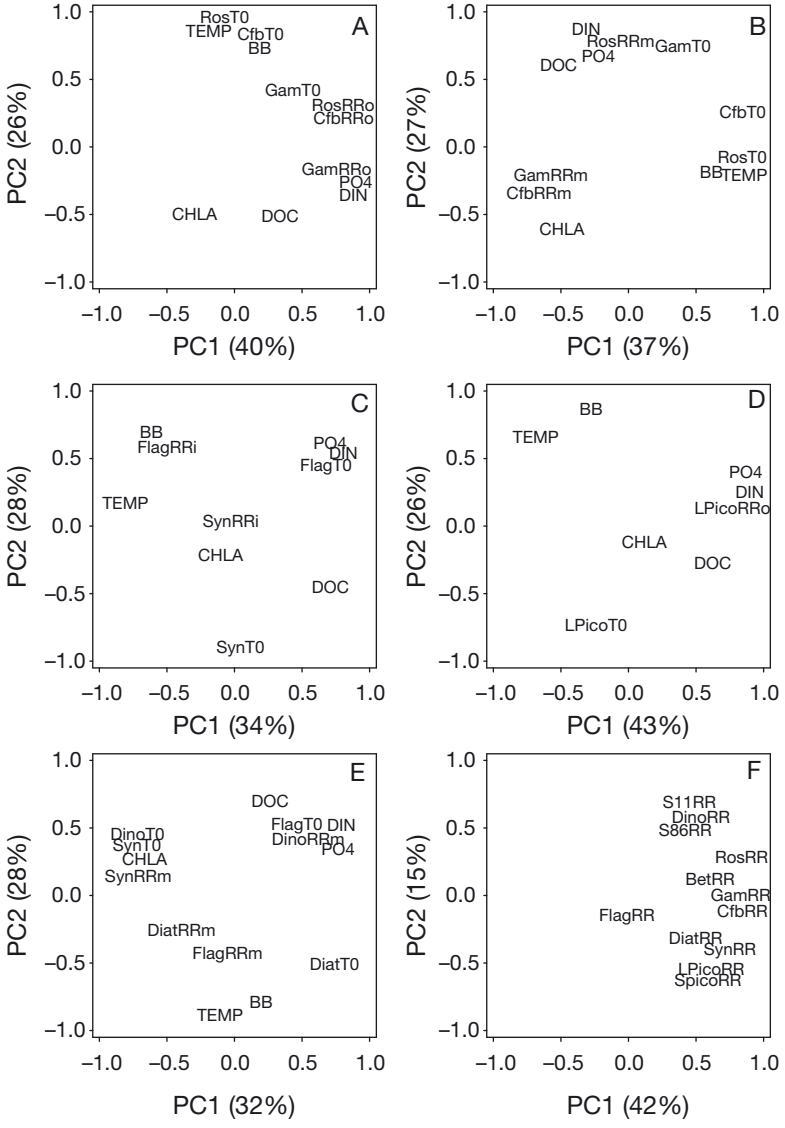

Fig. 9. Principal component analysis (PCA) showing the clustering among different combinations of environmental variables and responses of (A) bacterioplankton to organic additions, (B) bacterioplankton to mixed additions, (C) phytoplankton to inorganic additions, (D) phytoplankton to organic additions, and (E) phytoplankton to mixed additions. (F) PCA showing the clustering of the responses of phytoplankton and bacterioplankton groups. See text for abbreviations

The response of *Roseobacter*, Bacteroidetes and Gammaproteobacteria to organic additions (RosRRo, CfbRRo, and GamRRo, respectively) was positively correlated to dissolved inorganic nitrogen (DIN) and phosphate (PO4) concentrations (Fig. 9A). The response of Bacteroidetes and Gammaproteobacteria to mixed nutrient additions (CfbRRm and GamRRm, respectively) was only negatively correlated to the initial abundance of Bacteroidetes (CfbT0), whilst the response of *Roseobacter* to the mixed treatment (RosRRm) was positively correlated with DIN concentration and the initial abundance of Gammaproteobacteria (GamT0) (Fig. 9B).

The response of nanoflagellates to inorganic nutrient additions (FlagRRi) was negatively correlated to the initial dissolved organic carbon (DOC) concentration and positively to the initial bacterial biomass (BB), whereas the response of *Synechococcus* to the inorganic treatment (SynRRi) was negatively correlated with initial chl *a* (CHLA) concentration (Fig. 9C). The response of large picoeukaryotes to organic nutrient additions (LPicoRRo) was positively correlated with DIN and phosphate concentration and negatively with water temperature (TEMP) (Fig. 9D). The response of *Synechococcus* and diatoms to mixed additions (SynRRm and DiatRRm, respectively) was positively correlated with initial chlorophyll concentration, whereas the response of diatoms and nanoflagellates (FlagRRm) to mixed additions was negatively correlated with DOC (Fig. 9E). The response of dinoflagellates to mixed additions (DinoRRm) was positively correlated to the initial DIN and phosphate concentrations and negatively with temperature (Fig. 9E). The initial biomass of diatoms (DiatT0) and dinoflagellates (DinoT0) was negatively and positively correlated, respectively, with the response of *Synechococcus* to mixed additions (SynRRm).

Overall, the responses of the bacterioplankton and phytoplankton groups to the different nutrient additions clustered together (Fig. 9F). Exceptions were the response of dinoflagellates, which correlated positively only with the responses of the bacterioplankton groups, and the response of nanoflagellates, which only correlated with the response of diatoms.

DISCUSSION

Response of bacterioplankton and phytoplankton groups to confinement

The methodology used in enclosure experiments (bottle, microcosm or even mesocosm confinement) may provoke significant changes in the microbial plankton community structure (Schäfer et al. 2000, Massana et al. 2001, Allers et al. 2007, Van Wambeke et al. 2009). Therefore, in an ideal enrichment experiment aimed at testing changes in community composition, the effects of the incubation itself should be minor. We did not find significant changes in the relative abundance of any of the 6 bacterial and 6 phytoplankton groups in the control microcosms during our 3 d incubations, in agreement with some other studies (Carlson et al. 2002, Teira et al. 2008, Teira et al. 2009). Most of the studies detecting changes in bacterial composition upon confinement are based on very small volume bottle incubations (<0.5 l) (e.g. Van Wambeke et al. 2009) or long-term incubations (>4 d) (e.g. Massana et al. 2001). Nevertheless, our results do not exclude potential changes in other non-detected groups or at a lower phylogenetic level within each of the considered major groups. We could test for changes in species richness and diversity only for diatoms and dinoflagellates. For both groups, we also did not find significant changes over time in the control (Fig. 8), although the Shannon-Wiener diversity index for dinoflagellates decreased from 1.4 to 1.0. A similar constancy in diatom diversity and a decrease in dinoflagellate diversity over time were observed by Davidson et al. (2007) in 1.5 m^3 mesocosms with coastal waters.

Response of bacterioplankton groups to nutrient enrichment

Phylogenetic analysis based on 16S rRNA genes indicates that 80% of bacterioplankton in surface waters consists of about 10 abundant clades (Giovannoni & Rappé 2000). Thus, the response of the 6 selected groups, which accounted for 60–100% of the total prokaryotic community, to the nutrient additions appears to be representative of changes in bacterial community composition at the level of major groups.

The response to nutrient enrichment greatly differed among experiments, as reflected by the great variability observed both in time-courses (Fig. 3) and in the estimated response ratios (Fig. 4). Such variability in the responses could be partially related to spatial and temporal changes in grazing pressure over different bacterial groups or in competitive abilities. Caron et al. (2000) clearly demonstrated that grazing pressure might indeed modulate the response of bacteria to nutrient enrichment. On the other hand, Teira et al. (2009) showed considerable temporal variability in growth rates for most of the bacterial groups studied here, suggesting that variation in the competitive abilities of the different bacterial groups could also modulate their response to the additions.

None of the 6 considered bacterial groups responded to inorganic nutrients (Table 2), which we attributed to an overall carbon limitation of the bacterioplankton inhabiting this coastal area (Martínez-García et al. 2010b). A very similar result was found by Carlson et al. (2002) in seawater cultures conducted in the Sargasso Sea and in mesocosms at a coastal site by Øvreås et al. (2003). The limited change in bacterioplankton community composition was accompanied by a limited response of bulk bacterial biomass, bacterial production, and bacterial respiration (see detailed discussion in Martínez-García et al. 2010b). Thus, the bacterioplankton community composition in this eutrophic area seems to be mainly governed by the availability and the diversity of organic substrates. Alonso-Sáez et al. (2009) observed that different bacterial community structures evolved in seawater cultures amended with glucose, leucine, or a yeast extract. Important changes in the bacterial community structure were found when organic nutrients were added, either alone or in combination with inorganic nutrients (Figs. 4 & 7). The most responsive groups were Gammaproteobacteria (on average, 6.4-fold increase in abundance), followed by *Roseobacter* (on average, 3-fold increase) and Bacteroidetes (on average, 1.7-fold increase), whereas the other 3 groups, SAR11, Betaproteobacteria, and SAR86, did not show a statistically significant response to the additions. Interestingly, the mean magnitude of response of the 6 groups to organic additions positively correlated with the mean growth rates recently reported by Teira et al. (2009) in the same coastal area ($r = 0.89$, $p = 0.04$, $n = 6$). Therefore, the most responsive groups are also the ones with the highest growth rates. The limited response of bacterial groups to organic additions in S1 and S2 is rather striking for this eutrophic area. Nevertheless, these 2 experiments, conducted with water collected in the shelf, coincide with the lowest levels of initial phosphate concentration and the highest inorganic N:P ratios (>38) (Table 1). Given that the organic additions do not include P, the lack of response suggests a secondary P limitation. Indeed, the magnitude of response of the different groups to the organic additions was positively correlated with phosphate concentration (Fig. 9A). A reduced response of bacterioplankton to organic C and N amendment has also been associated with phosphate availability in the north central Atlantic Ocean (Martínez-García et al. 2010a).

A prominent response of Gammaproteobacteria to labile organic matter additions has been previously demonstrated in both oceanic and coastal waters (Harvey et al. 2006, Davidson et al. 2007, Alonso-Sáez et al. 2009, Teira et al. 2010). The growth of this group associated to nutrient enrichment mostly explained concurrent increments in bacterial production and biomass (Martínez-García et al. 2010b) (Table 3). Teira et al. (2010) also found that the magnitude of response of Gammaproteobacteria abundance explained 89% of the variability in the magnitude of response of bacterial production. The limited response of SAR86 upon enrichment has been also observed by Eilers et al. (2000).The response of *Roseobacter* to nutrient amendments was previously reported by Allers et al. (2007) who concluded that this group was essentially profiting from the concurrent positive response of phytoplankton in the amended mesocosms. However, this does not seem to be the case in our experiments given that, in most of the cases, the maximum *Roseobacter* abundance in response to nutrient enrichment occurred immediately (after 24 to 48 h) which indicates that these bacteria are likely using the added labile substrates. A positive response of Bacteroidetes to labile organic matter enrichment in coastal waters was also reported by Davidson et al. (2007), which contrasts with the significantly negative response observed in the oligotrophic central Atlantic (Teira et al. 2010). The great genetic diversity of the group Bacteroidetes could explain this contrasting pattern of response to nutrient enrichments. It has been demonstrated that members of the group Bacteroidetes are particularly efficient in the use of complex organic matter such as protein (Cottrell & Kirchman 2000), as they are highly responsive to decaying phytoplankton blooms (Pinhassi et al. 2004, Alderkamp et al. 2006, Teira et al. 2008). This agrees with our finding that the maximum response of this group tended to occur by the end of the experiment (after 72 h), which suggested that the response is not directly linked to the added labile substrates. It is expected that the utilization of complex organic substrates will render lower growth efficiencies. This is in agreement with the relatively important contribution of the group Bacteroidetes in explaining the response of bacterial respiration to the additions (Table 3).

The lack of response of SAR11 also contrasts with the negative effect of organic nutrient additions previously reported in oligotrophic waters (Teira et al. 2010). Stingl et al. (2007) speculated that the higher nutrient concentrations in coastal waters compared to oligotrophic waters could facilitate ecotype speciation of the SAR11 clade. A higher microdiversity of SAR11 in coastal versus open ocean areas could explain the observed different patterns of response to organic nutrient inputs. On the other hand, the limited response of the group Betaproteobacteria in this marine ecosystem contrasts greatly with the prominent responses encountered in freshwater ecosystems (Simek et al. 2005), where Betaproteobacteria are numerically dominant and show higher growth rates (Simek et al. 2006) than mean growth rates reported for this coastal area (Teira et al. 2009).

Response of phytoplankton groups to nutrient enrichment

Nutrient quality, particularly the proportion of inorganic and organic nitrogen sources, strongly affects phytoplankton community composition (Berg et al. 2003, Heil et al. 2007, Bradley et al. 2010), which agrees with the changes in phytoplankton composition associated with our different nutrient additions (Fig. 7). The 6 phytoplankton groups showed greater responses to the mixed than to the inorganic or organic additions. The overall lower response of phytoplankton groups to inorganic nutrients appears to be consistent with the constancy in bacterial community structure in the inorganic treatment. If phytoplankton had responded, the released DOC would likely have promoted changes in bacterial community structure, as observed by Allers et al. (2007).

Contrary to the expected preferential response of large phytoplankton cells to nutrient enrichment (Agawin et al. 2000, Joint et al. 2002, Jacquet et al. 2006) nanoflagellates, on average, responded 2.4-fold more than microphytoplankton, such as dinoflagellates or diatoms, and 2.6-fold more than picophytoplankton, such as *Synechococcus* or picoeukaryotes, to inorganic nutrient additions (Fig. 6). The magnitude of the response of nanoflagellates to inorganic additions was negatively correlated with initial DOC concentration (Fig. 9), which suggests that nanoflagellates are good competitors for inorganic nutrients when bacteria are C-limited. The response of nanoflagellates to inorganic additions was also positively correlated to the initial bacterial biomass (Fig. 9). Many marine phototrophic flagellates are mixotrophs, deriving their nutrition from both photosynthesis and bacteria ingestion (Sanders 1991). Therefore, some of these nanoflagellates could also be feeding on bacteria, as we were unable to unequivocally classify them as autotrophs, heterotrophs or mixotrophic.

Synechococcus biomass was significantly reduced in the inorganic treatment (Table 2), likely as a result of competition with larger cells (Jacquet et al. 2002, 2006). Jacquet et al. (2002) also found that larger-celled autotrophic populations were favoured after inorganic N and P additions. Both *Synechococcus* and di-

noflagellates showed a globally higher response to organic than to inorganic additions and increased significantly in mixed additions treatments; these increases likely reflected their mixotrophic capabilities (Stoecker 1999, Glibert & Legrand 2006, Wawrik et al. 2009).

Large autotrophic picoeukaryotes showed a significantly negative response to organic additions (Table 2), which could result from competition for inorganic nutrients with bacteria. The response of this phytoplankton group to organic additions was positively correlated with the initial DIN and phosphate concentration (Fig. 9), which suggests that competition with bacteria was less intense as the availability of inorganic nutrients increased.

The magnitude of the response of diatoms to experimental treatments mostly explained changes in chl *a* concentration and primary production rates associated to nutrient additions (Table 4). Diatoms did not show a significant response to inorganic additions, which contrasts with previous field observations and suggests that inorganic nutrient enrichment favours large diatoms (Berg et al. 2003, Jacquet et al. 2006, Heil et al. 2007). The limited response of diatoms to inorganic nutrients could be related to a Si limitation, however the levels of silicate, except in S2, were high enough (>2 µM) to sustain considerable nitrate consumption in the inorganic treatments (Table 1). Moreover, the same potential Si limitation might have existed in the mixed treatment, where diatoms were found to be highly responsive. The positive response of diatoms to mixed additions (Table 2) suggested organic nutrient requirements for growth. However, diatoms were not significantly affected by organic additions, suggesting a complex control of their growth by diverse nitrogen sources. Wawrik et al. (2009) recently demonstrated that diatoms from coastal waters are able to incorporate N from nitrate, ammonium and urea. On the other hand, Armbrust et al. (2004) demonstrated, from genome sequencing, that the diatom *Thalassiosira pseudonana* has the potential for the uptake and use of organic forms of N, including amino acids and purines. The dominant diatom species were the same regardless of the nutrient treatment; thus, the apparent complex N nutrition seems to be not directly linked with changes in diatom community composition. A significant reduction of diatom diversity associated with inorganic additions (Fig. 8) has been previously reported by Davidson et al. (2007) and appeared to be associated to changes in evenness rather than richness (Fig. 8). Decreases in phytoplankton diversity due to massive blooms of single species have been reported as a consequence of nutrient pulses (Beman et al. 2005, Spatharis et al. 2007). The higher resource diversity in the organic and mixed treatments could have promoted the coexistence of several species and maintained the diversity of both diatoms and dinoflagellates (Fig. 8).

We have shown that the magnitudes of responses of different bacterioplankton and phytoplankton groups to distinct nutrient inputs are highly variable among experiments. This variability is likely related to changes in the initial environmental conditions, including biotic factors (grazing, competition). Overall, the most prominent changes in microbial community structure occurred after organic or mixed additions. Only the nanoflagellates significantly increased abundance after inorganic nutrient additions, suggesting important organic requirements by diatoms, dinoflagellates and *Synechococcus,* which mainly responded to the mixed additions. The most responsive bacterial group was Gammaproteobacteria, followed by *Roseobacter* and Bacteroidetes. The magnitudes of responses of the different bacterial groups to organic and mixed additions appear to be positively related to their respective growth characteristics.

Our results show that the type of nitrogen input (inorganic and organic) strongly affects the community composition of both bacterioplankton and phytoplankton. Therefore, considering that an important fraction of the external nitrogen entering the ocean is in organic form, the combined effect of organic and inorganic nitrogen inputs over microbial plankton populations must be explored in detail over a wide range of marine ecosystems if we aim to understand the impact of the anthropogenic alteration of the nitrogen cycle on the marine microbial diversity.

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