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Differential response of microbial plankton to nutrient inputs in oligotrophic versus mesotrophic waters of the North Atlantic

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ORIGINAL ARTICLE

Differential response of microbial plankton to nutrient inputs in oligotrophic versus mesotrophic waters of the North Atlantic

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Abstract
The effects of inorganic (DIN + PO43- ) and/or organic (glucose + AAs) inputs on phytoplankton and heterotrophic bacteria were assessed, using a microcosm approach, in two contrasting marine environments: an open ocean oligotrophic site (North Atlantic Subtropical Gyre) and a highly productive coastal embayment (Ría de Vigo, NW Spain). Overall, changes in microbial plankton biomass were smaller than those of metabolic rates. The largest increases in primary production, bacterial production and community respiration were measured in response to mixed (DIN + PO43- + glucose + AAs) nutrient additions in both sites. Primary production responded to DIN + PO43- additions only in oligotrophic waters. The distinct autotrophic responses to nutrient additions measured in these environments were related to the different initial composition of phytoplankton populations and, presumably, also to differences in grazing pressures in both marine ecosystems. Heterotrophic bacteria were limited by organic substrates in both ecosystems, although mixed additions further enhanced bacterial growth in the subtropical gyre. The differences detected in bacterial responses to nutrient additions may be related to differences in nutrient limitations and to the prevalence of different relationships between components of the microbial food web (e.g. coupling between heterotrophic bacteria and phytoplankton and predation pressure) in both environments. We found a more relevant role of inorganic nutrients in controlling the efficiency of bacterial growth in oligotrophic regions as compared with highly productive systems. Our results suggest that organic matter inputs into both ecosystems might result in a tendency towards heterotrophy and in increases in bacterial growth efficiency.

Key words: Microbial plankton, nutrients inputs, bacteria, phytoplankton, organic nitrogen, microcosms

Introduction
Human activities (e.g. fossil fuel combustion, and changes in land use) have considerably increased the quantity of reactive nitrogen (Nr) entering the world’s oceans in recent decades (Falkowski et al. 1998; Galloway & Cowling 2002; Mathews 2006; Duce et al. 2008). On a global scale, nitrogen input into marine systems has increased two- to threefold from the 1860s and is expected to further increase over future decades (Galloway et al. 2004).

It has been widely demonstrated that nitrogen (N) controls ecosystem productivity over ecological time scales in coastal (Nixon & Pilson 1983; Oviatt et al. 1995) and open ocean (Graziano et al. 1996; Mills et al. 2004, 2008; Moore et al. 2008) waters. Enhanced productivity eventually results in an accumulation of organic matter which promotes microbial activity and the consumption of dissolved oxygen (Nixon 1995; Cloern 2001; Diaz & Rosenberg 2008; Gruber & Galloway 2008).

The forms of reactive nitrogen that affect aquatic ecosystems include inorganic dissolved compounds (nitrate, ammonium), a variety of dissolved organic compounds (amino acids, urea, and composite dissolved organic nitrogen) and particulate nitrogen. Microbial plankton populations utilize different forms of nitrogen preferentially, and both the magnitude and the composition of the nitrogen

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input may modify the structure and metabolism of microbial plankton communities (Antia et al. 1991; Peierls & Paerl 1997; Seitzinger & Sanders 1999; Bradley et al. 2010).

An important fraction of nitrogen inputs into both open-ocean and coastal waters is organic. About one-third of the total quantity of atmospheric nitrogen entering the world’s oceans annually is organic and largely bioavailable (>45%) (Peierls & Paerl 1997; Seitzinger & Sanders 1999). It is also known that about 40–50% of nutrients in fluvial inputs are organic (Meybeck 1993).

Nutrient availability in the upper layers of the global ocean depends on the physical and chemical properties of the water column, which widely change through different environments (Longhurst 2006). The rate of nutrient supply into the upper layer of the ocean largely controls the size of dominant phytoplankton species, thereby modifying the structure of the planktonic food web (e.g. Legendre & Le Fèvre 1989; Kiorboe 1993) and the associated patterns of organic matter circulation (Legendre & Rassoulzadegan 1996; Calbet & Laundry 2004).

When nutrient availability is low, the relative importance of small phytoplankton cells is high, total primary producer biomass and photosynthetic carbon fixation are low, and the microbial food web dominates (Azam et al. 1983; Platt et al. 1983; Sherr & Sherr 2000; Fenchel 2008). Larger phytoplankton cells, which dominate as we move towards more eutrophic conditions, have higher potential for growth when nutrients are available (Thingstad & Sakshaug 1990; Agawin et al. 2000; Cermenó et al. 2005).

As a consequence of the contrasting characteristics of the diverse marine ecosystems, the factors limiting productivity of phytoplankton and heterotrophic bacteria in the ocean are likely to change over a variety of spatial and temporal scales (Cullen et al. 1992; Arrigo 2005; Church 2008; Saito et al. 2008). Hence, the microbial responses to inorganic and organic nutrient inputs and the underlying ecological processes are expected to differ in distinct marine ecosystems. However, only a few studies have addressed the effect of inorganic and organic inputs on autotrophic and heterotrophic communities and little is known about the similarities or differences in the responses of open-ocean and coastal microbial communities to the increasing nutrient enrichment of the ocean. Furthermore, no direct comparison of open-ocean vs. coastal autotrophic and heterotrophic responses to the same inorganic and organic nutrient enrichments has ever been published. The present work has two aims: (1) to achieve a better understanding of microbial communities functioning in the ocean through the analysis of their responses to nutrient inputs; and (2) to compare the responses to qualitatively similar inorganic nutrients and/or organic substrate inputs of microbial communities characteristic from two different environments (i.e. an open-ocean and a coastal location) subjected to increasing nutrient enrichment in a changing world.

Here, we assess the differential effect of inorganic versus organic nitrogen inputs on autotrophic and heterotrophic microbial communities in two contrasting environments: an oligotrophic open-ocean site and a mesotrophic coastal site. We discuss some methodological constraints associated with the experimental approach that must be taken into consideration when comparing results from bioassays implying different enrichment levels. This comparative study allows us to infer some insights into ecological processes regulating the microbial plankton responses to nitrogen loading in coastal and open-ocean ecosystems.

**Materials and methods**

**Survey areas**

Sampling sites (open ocean site: 23.12°N, 26.31°W, November 2006, coastal site: 42.24°N, 8.77°W, February 2008) are shown in Figure 1. Vertical profiles down to 300 and 25 m (open-ocean and Ria 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.

The Eastern North Atlantic Subtropical Gyre is characterized by strong thermal stratification and nutrient depletion in the upper mixed layer for most of the year, which translates into very low levels of chlorophyll a and primary production (Teira et al. 2006; Marañón et al. 2007). This experiment was performed in the framework of the CARPOS project on board B.O. Hespérides in November 2006.

The coastal system of the northwestern Iberian Peninsula is characterized by periodic upwelling (usually from March to September) of cold and nutrient-rich North Atlantic Central Waters (NACW) (Nogueira et al. 1997). The Ría de Vigo (Spain), an embayment located in this upwelling area, is a highly productive and dynamic coastal ecosystem (Cermenó et al. 2006). Three experiments were performed in this ecosystem in different seasons in order to cover the temporal variability of microbial community structure and functioning (Martínez-García et al. 2010a). Similar general biological initial conditions and the same patterns and magnitude of response to the nutrient additions were found in the three experiments (response ratios in the experiment shown here are within the same
range of the other two experiments). In order to simplify the comparison of the two systems, only the experiment performed in winter in the Rías presented as it was performed in the same season as the one in the open ocean, although under contrasting initial environmental conditions (i.e. water column structure, surface temperature and nutrient concentrations).

**Experimental design**

Surface seawater samples (5–10 m) were collected in 12- or 15-litre acid-clean Niskin bottles and filtered through 150- and 200-μm pore size meshes (open ocean and Rías stations, respectively) to remove larger zooplankton. Subsequently, 12-litre acid-washed polycarbonate bottles were gently filled under dim light conditions.

Following sample collection, nutrients were added to the 12-litre microcosm bottles. The experimental design included duplicate bottles for a series of four treatments: (1) control treatment – no additions made; (2) inorganic addition treatment (DIN+PO₄³⁻) – 0.5 and 5 μmol l⁻¹ nitrate (NO₃⁻), 0.5 and 5 μmol l⁻¹ ammonium (NH₄⁺), 0.1 and 1 μmol l⁻¹ phosphate (HPO₄²⁻) (open ocean and Ría stations, respectively); (3) organic addition treatment (Glucose + AAs): 1 and 5 μmol l⁻¹ glucose and 1 and 5 μmol l⁻¹ of an equimolar mixture of 18 amino acids (all protein amino acids, except cysteine and tyrosine) (open ocean and Ría stations, respectively); (4) mixed addition treatment: combination of inorganic and organic additions. Glucose and amino acids were included, as they are the organic labile identified substances more abundant in seawater and both atmospheric and riverine inputs have been reported to contain organic carbon and nitrogen (Meybeck 1993; Jacobson et al. 2000; Jurado et al. 2008). The added inorganic nutrients concentrations were 10-fold higher in the coastal experiment than in the open-ocean experiment, in accordance with the differences in the mean initial nutrient concentrations, standing stocks and metabolic rates between both ecosystems (see Initial conditions, Table I and Figure 2). The magnitude of the additions was chosen to be in excess relative to the mean concentrations measured at the surface waters of each ecosystem (Álvarez-Salgado et al. 1996; Marañón et al. 2001).

In both experiments, temperature was maintained within ±0.1°C of in-situ values. An in-door incubation chamber and a temperature-controlled incubation room were used in the open-ocean and coastal experiments, respectively. Bottles were illuminated with cool white light from fluorescent tubes (photoperiod 12L : 12D and average PAR was 240 μE m⁻² s⁻¹). Experiments lasted 3 days.
a Alpkem segmented-flow analyser (Hansen & Grasshoff 1983).

**Size-fractionated chlorophyll a**

Size-fractionated chlorophyll a (chl a) concentrations were measured in 250 and 150 ml water samples (in the open-ocean and the Ría experiments, respectively) which were filtered sequentially through 2- and 0.2-µm polycarbonate filters. After extraction with 90% acetone at 4°C overnight in the dark, chlorophyll a fluorescence was determined, using the non-acidification technique, with a TD-700 Turner Designs fluorometer calibrated with pure chl a.

**Photosynthetic efficiency**

FRR fluorescence measurements were made using a Chelsea Instruments FastTracka FRR fluorometer. Fluorescence variables $F_0$ (initial fluorescence) and $F_m$ (maximal fluorescence) were obtained by fitting the model of Kolber et al. (1998) to the FRR fluorescence using the FRS program. Photosynthetic efficiency ($F_v/F_m$) was calculated as $F_v/F_m = (F_m - F_0)/F_m$.

**Primary production**

Four 75-ml acid-cleaned polystyrene bottles (3 light and 1 dark) were filled with seawater and spiked with 555 and 185 kBq (15 and 5 µCi) NaH$_{14}$CO$_3$ (in the open-ocean and the Ría experiments, respectively). Samples were incubated for 3 and 12 h (in the Ría and the open-ocean experiments, respectively) in the same incubation chamber as the experimental bottles. After the incubation period, samples were sequentially filtered through 2- and 0.2-µm polycarbonate filters under low vacuum pressure (<50 mmHg). Filters were processed to assess $^{14}$C incorporation as described in Marañón et al. (2001). The daily primary production rates in the Ría experiments were obtained by applying the photoperiod in the microcosms to the hourly primary production (PP) rates. Gross primary production (GPP) per day, used to compute the PP to community respiration (P/R) ratio, was estimated assuming that (i) phytoplankton respiration amounts to 20% of the carbon fixed during the light period, (ii) the percentage of extracellular release (PER) of phytoplankton is 20% (Marañón et al. 2004).

**Bacterial heterotrophic production**

The $[^{1}H]$leucine incorporation method (Kirchman et al. 1985), modified as described by Smith & Azam...
(1992), was used to determine Leu incorporation rates (LIR). Samples were incubated for 1 and 2 h (in the Ria and the open-ocean experiments, respectively) in the same incubation chamber as the experimental bottles. Dilution experiments (4 in the Ria and 1 in the open-ocean experiments) were performed in order to determine empirical leucine incorporation rates (LIR). Samples were incubated for 1 and 2 h (in the Ria and open-ocean experiments, respectively) in the same incubation chamber as the experimental bottles. Differences between the CFs measured in open ocean and coastal bacterial communities have been previously reported in several studies (Morán et al. 1999; Pedrós-Alió et al. 1999; Sherr et al. 2001; del Giorgio et al. 2011) and may have been increased by nutrient additions in the present investigation. Bacterial growth efficiency (BGE) was calculated as: \( \frac{BP}{BP + BR} \), where BP is bacterial productivity and BR is bacterial respiration.

In vivo electron transport system (ETS)

ETS activity rate was used as an estimator of community respiration (CR). Size-fractionated in-vivo ETS activity rates were measured using the in-vivo INT method (Martínez-García et al. 2009), which is based on the reduction of the tetrazolium salt 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) to INTformazan (INT-F) by ETS dehydrogenase enzymes. Four 100- and 250-ml (in the open-ocean and Ria experiments, respectively) dark bottles were filled from each microcosm bottle. One bottle was immediately fixed by adding formaldehyde (2% w/v final concentration) and used as a killed-control.

Samples were incubated at the same temperature as the microcosm bottles and in dark conditions for 6 and 1 h (in the open-ocean and Ria experiments, respectively). After incubation, samples were filtered sequentially through 0.8- and 0.2-m\( \mu \)m pore size polycarbonate filters. The reduced INT-F was extracted from the filters using propanol and its concentration determined colorimetrically using an Ultra Violet-2401 PC Shimadzu spectrophotometer. BR was operationally defined as ETS activity <0.8 \( \mu \)m (Robinson 2008). In order to transform ETS activity in carbon respiration, a R/ETS ratio of 12.8 (Martínez-García et al. 2009) and a respiratory quotient (RQ) of 0.8 (Williams & del Giorgio 2005) were used. Daily ETS activity rates were calculated by multiplying the hourly rates by 24.

Flow cytometry

Samples for heterotrophic bacteria (1.8 ml) were preserved with 1% paraformaldehyde + 0.05% glutaraldehyde and frozen at \(-80^\circ\)C until analysis on board (NASG) or in the laboratory, within 6 months of collection, with a FACSCalibur flow cytometry (Becton-Dickinson) equipped with a laser emitting at 488 nm. Prior to analysis, heterotrophic bacteria were stained with 2.5 mM DMSO-diluted SybrGreen I DNA fluorochrome (Molecular Probes). Two groups of heterotrophic bacteria were distinguished by their green fluorescence (FL1, 530 nm) after SybrGreen staining and side scatter (SSC) signals: high (HNA) and low (LNA) nucleic acid content bacteria.

Empirical calibrations specific to these data sets between flow cytometry light scatter (SSC or forward scatter, FSC) and mean cell size [biovolume (BV) or cell diameter], as explained in Calvo-Díaz & Morán (2006), were used: \( BV = 0.06 \times FSC + 0.01 \); \( r^2 = 0.60 \), \( n = 13 \) in the Ria de Vigo; diameter = \( 0.79 \times SSC + 0.47 \); \( r^2 = 0.60 \), \( n = 16 \) in the open ocean, using the sequential filtration method as described in Zubkov et al. (1998) to estimate bacterial BV.

Heterotrophic bacterial biomass was finally calculated by using the allometric relationship of Gundersen et al. (2002): bacterial biomass (fg C cell\(^{-1}\)) = 108.8 \( \times BV^{0.898} \) for open-ocean waters and that of Norland (1993): fg C cell\(^{-1} \) = 120 \( \times BV^{0.72} \) for the Ria de Vigo.

Data analysis

In order to compare the effect of different nutrient additions on the standing stocks and rates, we calculated response ratios (RR), by dividing the time-integrated value in the addition treatment by the time-integrated value in the control. In the case of standing stocks and P/R ratio, time-averaged values were used. Values presented in this work were integrated (or averaged in the case of standing stocks) from 0 to 72 h incubation. Different amounts of nutrients were added in the two experiments (open-ocean and coastal ocean, see Experimental design).

Methodological constraints

Although nutrients were added well above background concentrations, we cannot guarantee that nutrient concentrations in the experimental bottles...
just after nutrients were added were above saturation levels in both open and coastal ocean experiments. We are aware that some previous works comparing addition experiments assume saturation levels of the added nutrients to allow the magnitude of the effects promoted by the additions in different experiments to be compared (Downing et al. 1999; Elser et al. 2007). Nevertheless, we decided not to derive conclusions from potentially underestimated response ratios and therefore avoided direct comparisons of response ratios between experiments except when the variable itself is a ratio between variables (i.e. BGE and P/R ratio).

Statistical analysis

A repeated-measure ANOVA (RMANOVA) was conducted to assess conductivity (within subject factor), and treatment (between subject factor, nutrient additions) effects. A Least Significance Difference (LSD) post-hoc test was conducted to assess the effect of the addition treatments on the microbial parameters. For those data sets that did not fit a normal distribution (Kolmogorov–Smirnov test) a log transformation was applied.

Results

Initial conditions

Initial conditions for both experiments are presented in Table I and Figure 2. The Ria station was sampled during an intense winter mixing period with low surface temperature and high surface nutrient concentrations. Chl a concentration and primary production (PP) rate were 0.7 μg Chl a l−1 and 21.6 μg C l−1 d−1, respectively (Table I and Figure 2). The phytoplankton community was dominated by >2 μm cells (>80%) and the photosynthetic efficiency (Fv/Fm) was 0.4. Bacterial biomass (BB) was 17.2 μg C l−1 and was dominated by HNA bacteria (68%) (Table I and Figure 2). The bacterial production (BP) rate was 14.8 μg C l−1 day−1 (Table I and Figure 2). Community respiration (CR), estimated from in-vivo ETS activity, was 25.8 μg C l−1 day−1 and BR (<0.8 μm fraction) represented 42% of CR, rendering a high bacterial growth efficiency (BGE) of 0.56. Initial PP to CR ratio (P/R) was 0.8 (Table I and Figure 2).

As expected, in the open-ocean station, surface temperature was higher than in the Ria station, causing strong thermal stratification and much lower nutrient levels than those in the Ria station (one and two orders of magnitude in the case of nitrate and phosphate, respectively). Chl a concentration and PP rate were 0.18 μg Chl a l−1 and 1.3 μg C l−1 day−1, respectively (Table I and Figure 2). Small phytoplankton (<2 μm) dominated chl a concentration (63%), whereas >2-μm phytoplankton dominated PP (57%). The Fv/Fm was slightly higher (0.48) than that of coastal phytoplankton (Table I and Figure 2). BB was 6.3 μg C l−1 and was also dominated by HNA bacteria (59%) and the BP rate was 0.02 μg C l−1 day−1. CR was one order of magnitude lower than in the Ria (4.7 μg C l−1 day−1), bacteria contributed to 58% of CR and BGE was very low (0.01). The initial P/R ratio was considerably lower than in the Ria (0.3) (Table I and Figure 2).

Responses of coastal microbial communities to nutrient additions

Although nutrient additions did not have a statistically significant effect on autotrophic biomass (chl a) and production (PP) in the Ria experiment (RMANOVA F-test, p > 0.05), chl a concentration significantly (LSD post-hoc test, p < 0.05, Table II) increased at the end of the incubation after mixed additions (Figure 3a).

Primary production responses to the additions in the Ria paralleled those of chl a (Figure 3b). Phytoplankton community size structure did not change after the additions (i.e. PP due to phytoplankton >2 μm dominated before and after the additions) (Figure 3c). An increase in Fv/Fm was observed in all treatments, including the control (we lack Fv/Fm data from some bottles at t = 48 and 72 h due to saturation of the FRR fluorescence in this experiment) (Figure 3d).

A statistically significant effect of nutrient additions on bacterial abundance (BB), production (BP), respiration (BR) and growth efficiency (BGE) was found (RMANOVA F-test, p < 0.01).

BB significantly (LSD post-hoc test, p < 0.05, Table II) increased during the first 48 h of incubation after glucose + AAs and mixed additions (Figure 3c). Maximum BP rates were registered after 24 h incubation (Figure 3f). BP significantly (LSD post-hoc test, p < 0.05, Table II) decreased after DIN + PO4−3 additions, while it significantly (LSD post-hoc test, p < 0.05, Table II) increased after glucose + AAs and mixed additions (Figure 3f). The magnitude of response of BP was higher than that of BB. BR significantly (LSD post-hoc test, p < 0.05, Table II) decreased after DIN + PO4−3 additions in the Ria and increased after glucose + AAs and mixed additions (Figure 3g). BGE significantly (LSD post-hoc test, p < 0.05, Table II) responded during the first incubation day to glucose + AAs and mixed additions (Figure 3h).
Table II. Summary of the effect of the different additions on biological variables (RMANOVA and LSD post-hoc tests): 0, no significant effect; +, significant stimulation $p < 0.05$; −, significant inhibition, $p < 0.05$. Chl a, chlorophyll $a$ concentration; PP, primary production; %PP $> 2 \mu m$, contribution of $> 2 \mu m$ to total PP; Fv/Fm, photosynthetic efficiency; BB, heterotrophic bacterial biomass; BP, bacterial production; BR, bacterial respiration (BR due to the fraction $< 0.8 \mu m$); BGE, bacterial growth efficiency; CR, community respiration; P/R, production to respiration ratio.

<table>
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<tr>
<th>Variable</th>
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<th>Open ocean experiment</th>
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<td>Chl a</td>
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A statistically significant effect of nutrient additions on community respiration (CR) and P/R ratio ($P/R = \text{daily GPP/daily CR}$) was found (RMANOVA F-test, $p < 0.01$). CR significantly (LSD post-hoc test, $p < 0.05$, Table II) increased after glucose + AAs and mixed additions (Figure 3i).

P/R ratios significantly (LSD post-hoc test, $p < 0.05$, Table II) higher than the control after DIN + PO$_4^{3-}$ additions (Figure 3j). This can be interpreted as a tendency towards short-term autotrophy after DIN + PO$_4^{3-}$ amendment and towards short-term heterotrophy after glucose + AAs and mixed additions (Figure 3j).

Responses of open-ocean microbial communities to nutrient additions

Nutrient additions did not have a statistically significant effect on autotrophic biomass (chl a) and production in the open-ocean experiment (PP)
(RMANOVA F-test, \( p > 0.05 \)). Minor changes in chl \( a \) concentration were observed in all treatments (Figure 3k) although significant (LSD post-hoc test, \( p < 0.05 \), Table II) enhancements of PP rates in the DIN + PO\(_4^3-\) and mixed treatments were registered (Figure 3l). Significant (LSD post-hoc test, \( p < 0.05 \), Table II) increases in the relative contribution of phytoplankton >2 \( \mu \)m to total PP (%PP >2 \( \mu \)m) were found after mixed additions (Figure 3m). No responses of photosynthetic efficiency (F\(_v\)/F\(_m\)) to the additions were registered (Figure 3n).

A statistically significant effect of nutrient additions on bacterial abundance (BB), production (BP), respiration (BR) and growth efficiency (BGE) was found in the open-ocean experiment (RMANOVA F-test, \( p < 0.05 \)). Significant (LSD post-hoc test, \( p < 0.05 \), Table II) increases in BB were only measured after the mixed addition (Figure 3o) and BP significantly increased (LSD post-hoc test, \( p < 0.05 \), Table II) after glucose + AAs and mixed additions (Figure 3p). The magnitude of response of BP was higher than that of BB.

BR significantly (LSD post-hoc test, \( p < 0.05 \), Table II) decreased after DIN + PO\(_4^3-\) additions (Figure 3q). By contrast, BR significantly (LSD post-hoc test, \( p < 0.05 \), Table II) increased after mixed additions (Figure 3q). BGE significantly (LSD post-hoc test, \( p < 0.05 \), Table II) increased after DIN + PO\(_4^3-\) and glucose + AAs additions (Figure 3r).

A statistically significant effect of nutrient additions on community respiration (CR) and P/R ratio (P/R = daily GPP/daily CR) was found in the open-ocean (RMANOVA F-test, \( p < 0.01 \)). CR significantly (LSD post-hoc test, \( p < 0.05 \), Table II) increased after glucose + AAs and mixed additions (Figure 3s). P/R ratios significantly (LSD post-hoc test, \( p < 0.05 \), Table II) decreased after glucose + AAs and mixed additions and maintained significantly higher values (LSD post-hoc test, \( p < 0.05 \), Table II) than the control after DIN + PO\(_4^3-\) additions (Figure 3t). Consequently, a tendency towards short-term autotrophy after the DIN + PO\(_4^3-\) treatment and towards short-term heterotrophy after the glucose + AAs and mixed treatments was registered (Figure 3t).

**Discussion**

Our results indicate that the responses of phytoplankton and heterotrophic bacteria to nutrient additions, both in terms of biomass and metabolism, are different in coastal and oceanic environments. They also suggest that, in general, phytoplankton responses to nutrient amendments are smaller than those of heterotrophic bacteria, irrespective of markedly contrasting initial conditions.

In the experiment performed in the Ria de Vigo, autotrophic biomass and production were co-limited by DIN + PO\(_4^3-\) and glucose + AAs (Figure 4a,b). These increases were similar to those registered in similar experiments performed in this area throughout different seasons (Martinez-Garcia et al. 2010a) and might be explained by direct utilization of organic...
substrates by the phytoplankton community or by the additional input of inorganic nutrients derived from remineralization by heterotrophic bacteria in the mixed treatments. In contrast, in the open ocean experiment phytoplankton primary production was apparently solely limited by $\text{DIN} + \text{PO}_4^{3-}$ and no response was observed after the addition of glucose + AAs (Figure 4a,b). Martínez-García et al. (2010b) reported similar responses of primary producers after $\text{DIN} + \text{PO}_4^{3-}$ and mixed inputs in two similar experiments performed in oligotrophic waters of the central North Atlantic Ocean in the same season. Mills et al. (2004) and Moore et al. (2006, 2008) found higher primary production responses in the subtropical North Atlantic after inorganic (N and P) nutrient additions, possibly due to the higher final concentrations of the nutrients added (twofold higher for N and P). The differences in phytoplankton nutrient limitation in both environments may be related to differences in the phytoplankton community composition, dominated by picoeukaryotes, *Prochlorococcus* and *Synechococcus* in the open-ocean experiment and by diatoms and dinoflagellates in the coastal experiment (data not shown). In this regard, mixotrophy and auxotrophy of coastal phytoplankton communities have been widely reported (Antia et al. 1991; Bronk et al. 2007; Burkholder et al. 2008).

Contrary to our observation in the coastal site, increases in PP were not paralleled by increases in phytoplankton biomass in the open ocean, suggesting substantial grazing pressure at this site (Quevedo & Anadón 2001). Previous addition experiments in oligotrophic areas have related the lack of significant increases in phytoplankton biomass, despite clear increases in primary production after nutrient additions, with top-down processes (Moore et al. 2008; Marañón et al. 2010). In the present work prefiltration (150- and 200-μm pore size mesh in open-ocean and coastal experiments, respectively) of the samples could have released ciliates and heterotrophic flagellates from predators to some extent. In the open-ocean site this may enhance grazing pressure by microbial protists on small phytoplankton and bacteria (Martínez-García et al. 2010b). In contrast, in the coastal experiment a strong grazing control can be ruled out because the phytoplankton community in this experiment was mostly dominated by large phytoplankton, which presumably would not be severely grazed in this 200-μm prefiltered water (Martínez-García et al. 2010a).

The relative contribution of $>2$-μm phytoplankton cells to PP increased after the additions in the open-ocean experiment (Figure 4c), which was related to the higher storage abilities, photosynthetic efficiencies and maximum potential growth rates of large phytoplankton cells compared to small phytoplankton cells when nutrients concentrations are high (Thingstad & Sakshaug 1990; Agawin et al. 2000; Cermeño et al. 2005). This pattern of response was not evident in the Ría experiment as $>2$-μm cells initially dominated phytoplankton biomass and production in the coastal experiment (Figure 4c).

Therefore, the distinct autotrophic responses to nutrient additions measured in these environments were related with different composition of phytoplankton populations and also likely with differences in grazing pressures in both marine ecosystems. Heterotrophic bacteria were limited by organic substrates in both experiments and a secondary limitation by $\text{DIN} + \text{PO}_4^{3-}$ nutrients was registered in the open-ocean site (Figure 4e,f). The supply of $\text{DIN} + \text{PO}_4^{3-}$ alone had none and negative effects in BP in the open-ocean experiment and coastal experiments, respectively, suggesting no primary limitation of BP by $\text{DIN} + \text{PO}_4^{3-}$ in any of the experiments and a probable competition between phytoplankton and bacteria for $\text{DIN} + \text{PO}_4^{3-}$ in the coastal experiment. Limitation of heterotrophic bacteria biomass and activity by organic carbon in coastal areas is well known (Jacquet et al. 2002; Joint et al. 2002; Davidson et al. 2007). In the open-ocean experiment, the responses of BB and BP were larger in the mixed than in the glucose + AAs treatment, suggesting a secondary limitation by inorganic nutrients: more organic matter was utilized by bacteria in the mixed than in the glucose + AAs treatment, fuelled by the extra $\text{DIN} + \text{PO}_4^{3-}$ added. The supply of inorganic nutrients has been seen to limit the ability of bacteria to utilize organic matter (Rivkin & Anderson 1997; Thingstad et al. 1997; Gasol et al. 2009; Tanaka et al. 2009). The larger bacterial responses to mixed (including N and P) as compared to glucose + AAs (including N) additions observed in the open-ocean experiment might be explained by two different processes: (a) the previously reported phosphorus limitation in the North Atlantic (Fanning 1992; Mather et al. 2008; Martínez-García et al. 2010b). It is important to note here that because the type of nitrogen substrate added in both treatments is different (i.e. DIN and AAs, and AAs only in the mixed and organic treatments, respectively) caution must be exercised when trying to make conclusions about a single limiting nutrient (i.e. phosphorous). Also (b) the close coupling between heterotrophic bacteria and phytoplankton, i.e. enhanced bacterial growth associated with the release of extra labile photosynthetically produced dissolved organic carbon, increasing offshore (Morán et al. 2002). Therefore, the differences detected in bacterial responses to nutrient additions in the studied environments may be related...
to changes in nutrient limitations (i.e. high inorganic nutrient limitation in the oligotrophic site compared to the coastal site) and to the prevalence of different relationships between components of the microbial food web in both environments (i.e. tight coupling between bacteria and phytoplankton in oligotrophic offshore areas). The response of BP was higher than that of BB in glucose + AAs and mixed treatments in both experiments (Figure 4e,f) suggesting an important and similar grazing pressure on bacterioplankton in these two contrasting ecosystems. Previous studies have reported reduced BB relative to BP responses after nutrient additions in oligotrophic and eutrophic environments associated to top-down control processes (Caron et al. 2000). The increase in bacterial predation pressure after glucose + AAs additions has been previously reported and related to enhanced bacterial abundance and cell size that may increase edibility (Alonso-Sáez et al. 2009). BB and BP decreased (response ratios <1) after the DIN + PO₄³⁻ treatment in the Ria experiment (Figure 4e,f). This could be explained by the competition for inorganic nutrients between heterotrophic bacteria and phytoplankton in this experiment.

Organic carbon limitation of BGE in the Ria experiment was shown by marked increases in BGE measured after glucose + AAs and mixed additions but not when adding DIN + PO₄³⁻ alone (Figure 4h). Several authors have also reported negligible effects of inorganic nutrient additions on BGE in coastal systems (Jorgensen et al. 1993; Zweifel et al. 1993; Daneri et al. 1994). In the open-ocean experiment, BGE significantly increased after DIN + PO₄³⁻ additions (Figure 4h), again suggesting a more relevant role of inorganic nutrients in controlling bacterial growth efficiency (Gasol et al. 2009) in oligotrophic versus highly productive systems. On the other hand, the high increase of BGE after glucose + AAs additions in open-ocean areas is probably related to a low quality of the available organic substrates at this site, where the initial BGE was extremely low (0.02; Figure 2). Contrastingly, when mixed additions were performed in the open-ocean experiment no significant increases in BGE were observed as a consequence of the disproportionate increase of BR in the mixed compared to the DIN + PO₄³⁻ and glucose + AAs treatments (Figure 4g). In general, changes in the growth efficiency of heterotrophic bacteria after nutrient additions were more important in the open ocean as compared to the coastal experiment (Figure 4h), as initial oligotrophic populations characterized by low growth efficiency greatly responded to the availability of new nutrients. This finding suggests a higher capacity of starved bacteria from oligotrophic environments to increase their growth efficiency in response to nutrient inputs compared to coastal bacteria. A higher BGE implies that a higher amount of the carbon processed by bacteria is transformed into biomass, which translates into a higher carbon flow towards higher trophic levels, which may be exported to subsurface waters (Azam et al. 1983; del Giorgio & Cole 2000; Ducklow 2000).

The response of CR to the glucose + AAs and mixed treatments exceeded that of PP in both experiments (Figure 4b,i). This result has been repeatedly observed in similar nutrient addition experiments performed in both areas (Martínez-García et al. 2010a,b). This leads to decreases in the photosynthesis to respiration ratio of microbial planktonic communities after glucose + AAs input in both systems while a tendency towards autotrophy was found after DIN + PO₄³⁻ inputs in both experiments (Figure 4j). It could be expected that the effect of nutrient enrichment on the net metabolism of the planktonic microbial communities in both experiments would differ according to differences in the initial metabolic balance, the phytoplankton nutrient limitation (i.e. inorganic nutrients in both experiments would differ according to differences in the initial metabolic balance, the phytoplankton nutrient limitation (i.e. inorganic nutrients in both systems whereas grazing pressure over phytoplankton appears to be relevant only in the open-ocean experiment; (2) the qualitative differences in phytoplankton nutrient limitation (i.e. inorganic nutrients and/or organic substrates limitation) in both environments may be related to the composition of the contrasting phytoplankton communities and their different nutritional requirements; (3) bacterial responses to nutrient additions appear to be related to a different magnitude of the coupling between bacteria and phytoplankton and the more important inorganic limitation in the open ocean compared to coastal ocean; and (4) surprisingly, despite the different microbial responses and the distinct underlying ecological processes shown to control these responses in the two contrasting marine environments studied, the response pattern
of the metabolic balance to inorganic and/or organic enrichment was similar in both environments.

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