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Bacterioplankton responses to riverine and atmospheric inputs in a coastal upwelling system (Ría de Vigo, NW Spain)

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ABSTRACT: Anthropogenic pressures are changing the magnitude and nature of matter inputs into the ocean. The Ría de Vigo (NW Spain) is a highly productive and dynamic coastal system that is likely affected by such alterations. Previous nutrient-addition microcosm experiments conducted during contrasting hydrographic conditions suggested that heterotrophic bacteria are limited by organic carbon (C) and occasionally co-limited by inorganic nutrients in this coastal area. In order to assess short-term responses in biomass, production, and respiration of heterotrophic bacteria from the Ría de Vigo to increasing amounts of natural inputs of matter, we conducted 6 microcosm experiments, wherein surface seawater collected in spring, summer, and autumn was mixed with increasing amounts of dissolved natural matter concentrates from riverine and atmospheric origin. Simultaneous experiments with controlled inorganic and/or organic additions indicated that bacteria were co-limited by inorganic nutrients and C in spring and summer and primarily limited by C in autumn. Production responded more than biomass to increasing inputs of matter, whereas respiration did not change. The bacterial production response to increasing dissolved organic C load associated with riverine and atmospheric inputs was strongly related to the relative phosphorus (P) content of the dissolved matter concentrates. Our data suggest that bacterial production might decrease with the increase of P-deficient allochthonous matter inputs, which would have important biogeochemical consequences for C cycling in coastal areas.

KEY WORDS: Bacterioplankton · Production · Biomass · Riverine water · Atmospheric deposition · Spain · Galicia · Ría de Vigo

INTRODUCTION

Nutrient inputs to the ocean have increased over the past decades as a result of human activity (Galloway & Cowling 2002) and are expected to further increase in the future (Galloway et al. 2004, Duce et al. 2008). Atmospheric deposition, surface run-off, and groundwater effluents introduce inorganic and organic nutrients and pollutants from anthropogenic origin into the coastal ocean (Jickells 1998, Doney 2010, Statham 2012, Jickells et al. 2014). The flux of reactive nitrogen to the coastal oceans through atmospheric deposition and riverine discharge is expected to increase 10 to 20% by 2050, primarily due to the intensification of fertilizer and energy production (Howarth et al. 2012, Moore et al. 2013). Anthropogenic alterations of global biogeochemical cycles are changing not only the magnitude but also

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the nature of matter inputs into the ocean. For instance, the relatively higher increase in anthropogenic carbon (C) and nitrogen (N) compared to phosphorus (P) inputs appears to result in a general increase in the C:P and N:P supply ratios to the global biosphere (Peñuelas et al. 2012, 2013). These altered nutrient inputs will likely affect microbial plankton dynamics (Grover 2000, Danger et al. 2007, Hitchcock & Mitrovic 2013) and suggest a global scenario of increasing P limitation in marine ecosystems (Peñuelas et al. 2012), where N is currently the major limiting nutrient (Elser et al. 2007).

When bacteria are not limited by organic C, they are predicted to outcompete phytoplankton for mineral nutrient uptake (Grover 2000). Many studies have indicated that bacteria are better competitors than phytoplankton for P uptake (Pengerud et al. 1987, Jansson 1993, Guerrini et al. 1998, Joint et al. 2002, Danger et al. 2007, Vadstein et al. 2012), particularly at very low concentrations (Thingstad et al. 1993). Therefore, the distinct nutrient requirements and uptake efficiencies of bacteria and phytoplankton, as well as the magnitude and composition of allochthonous matter inputs, may determine the responses of the microbial communities to nutrient enrichment.

The Ría de Vigo is a eutrophic embayment located in the coastal system of the northwestern Iberian Peninsula, characterized by the intermittent upwelling of inorganic nutrient-rich water (Fraga 1981, Tenore et al. 1995). Water exchange between this embayment and the adjacent shelf is determined by the balance between river discharge and on-shelf wind stress (Álvarez-Salgado et al. 2000). Nutrient delivery in this area has been reported to be ~1500 mg N m^{-2} yr^{-1} (Gago et al. 2005) associated with riverine discharge, and about 100 to 250 mg N m^{-2} yr^{-1} associated with wet atmospheric deposition (Rodríguez & Macías 2006). Significant inputs of organic C in the Ría de Vigo have also been associated with riverine (Gago et al. 2005) and atmospheric (Teira et al. 2013) matter. Previous experimental studies on the effect of controlled inorganic and organic nutrient additions (Martínez-García et al. 2010) and natural additions of rainwater (Teira et al. 2013, Martínez-García et al. 2015) on coastal microbial planktonic communities in this coastal area showed that (1) microbial plankton responses to nutrient enrichment are highly variable; (2) phytoplankton is more responsive to natural rainwater additions than heterotrophic bacteria; and (3) heterotrophic bacteria are primarily limited by organic C and occasionally co-limited by inorganic nutrients.

In the present study, we aimed at further investigating the response of microbial plankton to dissolved natural matter inputs in the Ría de Vigo. The type of inputs studied included not only atmospheric but also riverine dissolved matter entering through fluvial discharge, as the latter introduces higher amounts of inorganic and organic nutrients into this highly productive ecosystem (Gago et al. 2005, Rodríguez & Macías 2006). In order to improve our predictive capability, we conducted 3 experiments using different microbial plankton communities collected under contrasting hydrographic conditions (spring, summer, and autumn) in which we evaluated the response of heterotrophic bacterial biomass, production, and respiration to increasing amounts of matter inputs from riverine and atmospheric sources. As riverine discharge and atmospheric deposition introduce organic C to the Ría de Vigo, and bacteria are primarily limited by C in this region, we hypothesized that increasing amounts of riverine or atmospheric inputs will increasingly stimulate bacterial production, biomass, and respiration in surface waters from the Ría de Vigo.

MATERIALS AND METHODS

Natural seawater for the experiments was taken in the middle sector of the Ría de Vigo (42° 14.09′ N, 8° 47.18′ W) in spring, summer, and autumn of 2013. Vertical profiles of water column temperature, salinity, and *in situ* fluorescence down to 25 m depth were obtained with an SBE 9/11 CTD probe and a Seatech fluorometer attached to a rosette sampler. Sub-surface seawater (3–4 m) was then collected in 12 l acid-cleaned Niskin bottles and filtered through a 200 µm pore size mesh to remove larger zooplankton. Subsequently, 4 l UV-transparent Whirl-pak® bags were gently filled with 2 l of seawater under dim light conditions.

Preparation of natural concentrates for the addition experiments

River and rainwater samples and <10 µm atmospheric particles were collected and processed to obtain natural concentrates of the riverine and atmospheric inputs to the Ría de Vigo. Our aim was to reduce the volume of the original water samples 10-fold without altering their chemical composition, i.e. trying to avoid the addition or the loss of any component in the natural samples.

The River Oitabén-Verdugo was sampled in April, July, and October 2013, a week before the addition experiments. We collected the water samples just upstream of the freshwater–seawater interface, to ensure that the chemical composition of the river samples was representative of the riverine water that mixes with the seawater of the Ría de Vigo. Five liters of each sample were gravity filtered through a prewashed (with 10 l of ultrapure water) dual-stage (0.8 and 0.2 µm) filter cartridge (Pall-Acropak supor Membrane), and the filtrate was concentrated 10-fold by using rotatory evaporation with a Buchi R215 evaporator. This concentration was performed under mild conditions (bath temperature: 25°C, vacuum: 13 mbar, condenser: acetone/CO_2) to avoid breakage of any organic compound present in the original water samples. Measurements of the concentration of inorganic (ammonium, nitrite, nitrate, and phosphate) and organic (dissolved organic C and N) substrates confirmed that the samples were concentrated quantitatively without any significant loss or gain.

An MTX rainwater sampler (model FAS005AB) and a high-volume PM10 MCV PM1025 particle sampler (model CAV-A/MS) were installed on the rooftop of the Instituto de Investigaciones Marinas (CSIC) to collect samples of wet and dry deposition to the Ría de Vigo. The MTX sampler was equipped with a humidity sensor to open the system only when it was raining, allowing the sampling of just the wet fraction of the atmospheric deposition, i.e. the rainwater. Rainwater was collected from 4 to 1 wk before the addition experiment. Samples were taken on a daily basis and frozen immediately after collection. A week before the experiment, the daily samples were thawed at ambient temperature, mixed in 1 volume (6 l), and quantitatively concentrated following the same procedure as for the riverine samples. Rainwater was collected only for the experiments conducted in April and October 2013 because wet deposition was very scarce the weeks before the July experiments (36 mm accumulated from 11 June to 10 July collected in the meteorological station at the Vigo city hall). The high-volume sampler was used to gather atmospheric particles (1–10 µm) on pre-combusted (450°C, 4 h) 140 mm GF/F filters. The particles were collected the week before the 3 addition experiments, operating for 48 h at a rate of 30 m^3 h^{-1}. The water-soluble fraction (WSF) of 1/8 of the filter was extracted in 400 ml of the corresponding rainwater concentrate by mechanical stirring over 40 min. For the July experiment, the WSF was extracted in Milli-Q water. These proportions (1/8 of the filter in 400 ml of water) were chosen in order to obtain 10-fold the expected concentrations according to previous existing information on the composition of wet and dry deposition in the Ría de Vigo (Teira et al. 2013, Martínez-García et al. 2015). Final mixed extracts were filtered through pre-combusted (450°C, 4 h) 47 mm diameter Whatman GF/F filters in an acid-cleaned glass filtration system, under low N_2 flow pressure, to be chemically characterized and used in the experiments as atmospheric concentrate. As for the riverine concentrates, quantitative concentration was observed except for the silicate, since the reduction of the rainwater volume was carried out in a glass rotary evaporator and the atmospheric particles were collected onto a glass-fiber filter.

Natural and controlled addition experiments

Increasing aliquots of riverine and atmospheric concentrates were added to surface seawater collected in the middle Ría de Vigo in spring, summer, and autumn of 2013. The concentrates were supplemented in proportions ranging from 1% (0.1% concentrate: 0.9% ultrapure water: 99% seawater) to 10% (1% concentrate: 99% seawater) of the original (previous to concentration) riverine and atmospheric materials, ensuring that the final salinity of the samples was kept constant independent of the amount of extract added. Seawater was mixed with 1, 2.5, 4, 5, 7.5, and 10% of natural matter from riverine (riverine discharge) and atmospheric (dry and wet deposition) origin. A control treatment (no addition) was included for each type of input. Three replicates were included for the control, 1, 5, and 10% treatments, and 1 replicate for the 2.5, 4, and 7.5% treatments.

With this procedure, we aimed to test the impact of natural additions of riverine and atmospheric materials over a wide range of realistic concentrations. To calculate the current average riverine and atmospheric inputs to the Ría de Vigo, we used the average river flow of the River Oitabén-Verdugo (17 m^3 s^{-1}; Gago et al. 2005) and the average precipitation to the Ría de Vigo (7.7 mm d^{-1}). The surface area of the ría is 174 km^2, the mean surface mixing layer is 2 m, and the average flushing time of this layer is 5 d; thus, the surface mixing layer of the ría contains about 2% of riverine water and 2% of rainwater. Therefore, the additions up to 10% would serve as a test for the response of the Ría de Vigo to future global change scenarios in which human activities increase the quantity without modifying the quality of riverine and atmospheric substrates.

Controlled nutrient addition experiments were also conducted in order to describe the limiting

nutrient for bacterial growth during each sampled season. We used the same controlled nutrient addition treatments as in Martínez-García et al. (2010): (1) no addition treatment; (2) inorganic nutrient treatment: 5 µmol l^{-1} nitrate (NO_3^-), 5 µmol l^{-1} ammonium (NH_4^+), and 1 µmol l^{-1} phosphate (HPO_4^{2-}); (3) organic nutrient treatment: 5 µmol l^{-1} glucose and 5 µmol l^{-1} equimolar mix of 18 amino acids (all of the protein amino acids except cysteine and tyrosine); and (4) mixed treatment: inorganic and organic nutrient treatments. Three replicates were included for each treatment. All experimental bags were incubated for 48 h under natural irradiance and temperature conditions. Bacterial biomass, production, and respiration were measured at time 0 and after 48 h incubations. In the nutrient-controlled experiments, only biomass and production were measured after 48 h.

Inorganic and organic nutrients

Aliquots for inorganic nutrient determination (ammonium, nitrite, nitrate, and phosphate) were collected in 50 ml polyethylene bottles and frozen at –20°C until analysis by standard colorimetric methods with an Alliance Futura segmented flow analyzer (Hansen & Grasshoff 1983). Water for the analysis of dissolved organic C (DOC) and total dissolved N (TDN) was filtered through 0.2 µm filters (Pall, Supor membrane Disc Filter) in an all-glass filtration system under positive pressure of N_2 and collected into precombusted (450°C, 12 h) 10 ml glass ampoules acidified with H_3PO_4 to pH < 2. Samples were measured with a Shimadzu TOC-V total organic C analyzer fitted with a Shimadzu TNM-1 total N measurement unit. Dissolved organic N (DON) was obtained by subtracting ammonium + nitrite + nitrate from TDN.

Dissolved organic matter (DOM) fluorescence

The fluorescence of dissolved protein-like ($FDOM_T$) and humic-like substances ($FDOM_A$) was also determined on the concentrates. Measurements were performed in a Perkin Elmer LS 55 luminescence spectrometer at 2 fixed pairs of excitation/emission wavelengths: 280/350 nm for $FDOM_T$ and 250/435 nm for $FDOM_A$ (Coble 1996). Calibration was done using a mixed standard of quinine sulfate and tryptophan in 0.1 N sulfuric acid following Nieto-Cid et al. (2006) to convert fluorescence units to normalized fluorescence intensity units.

Bacterial biomass

The abundance of heterotrophic bacteria was determined with a BD FACSCalibur flow cytometer equipped with a laser emitting at 488 nm. Picoplankton samples (1.8 ml) were preserved with 1% paraformaldehyde + 0.05% glutaraldehyde and frozen at –80°C until analysis. Prior to analysis, heterotrophic bacteria were stained with 2.5 mM SybrGreen DNA fluorochrome. The empirical calibrations between side scatter and mean cell diameter described by Calvo-Díaz & Morán (2006) were used to estimate biovolume (BV) of heterotrophic bacteria. BV was finally converted into biomass by using the allometric relationship of Norland (1993): fg C $cell^{-1}$ = 120 × $BV^{0.72}$.

Heterotrophic bacterial production

The [^{3}H]leucine incorporation method (Kirchman et al. 1985), modified as described by Smith & Azam (1992), was used to determine leucine (Leu) incorporation rates. Leucine was added at 40 nM final concentration. Samples were incubated for 1 h simulating *in situ* temperature conditions in a dark incubation chamber. We used the empirical leucine to C conversion derived by Martínez-García et al. (2010) (2.6 ± 1.1 kg C mol^{-1} Leu).

Bacterial respiration

The reduction rate of iodo-phenyl-3(nitrophenyl)-5(phenyl) tetrazolium chloride (INT) was used as an estimator of bacterial respiration. Size-fractionated *in vivo* INT reduction rates were measured as described by 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 a killed-control. After 15 min, all replicates were inoculated with a sterile solution of 7.9 mM INT 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 then centrifuged at 13 200 × *g* (10 min at 18°C). The absorbance at 485 nm was then measured using a spectrophotometer (Beckman model DU640). Bacterial respiration was operationally defined as INT reduction rates in the <0.8 µm size fraction (Robinson 2008). In order to transform INT reduction rates into C respiration, a ratio of 12.8 mol O_2/mol INT-F (Martínez-García et al. 2009) and a respiratory quotient (RQ) of 0.8 (Williams & del Giorgio 2005) were used.

Chlorophyll *a* (chl *a*) concentration

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

Primary production

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

RESULTS

Initial conditions and chemical composition of natural inputs

Initial conditions for each experiment are summarized in Table 1. Different hydrographic conditions were found during each survey. In spring (May 2013), high dissolved inorganic N (DIN = nitrate + nitrite + ammonium) and chl *a* concentrations were recorded, whereas in autumn (October 2013), lower chl *a* concentrations were observed regardless of higher nutrient concentrations. In the summer experiment (July 2013), concentrations of inorganic nutrients and chl *a* were the lowest. Primary production rates were highest in spring, and lower values were measured in summer and autumn (Table 1). Bacterial biomass was lower in summer than in spring and autumn, whereas bacterial production and respiration rates did not notably vary among seasons (Table 1). The chemical composition of riverine and atmospheric (wet and dry) matter inputs collected during the 3 studied seasons showed a high temporal variability (Table 2). In general, riverine water collected in October 2013 contained higher DIN, phosphate, and DOC than that collected in spring and summer (Table 2). On the other hand, atmospheric inputs in May 2013 contained higher DIN and DON but lower DOC concentrations than in autumn. Atmospheric deposition in summer contained relatively low N concentrations (Table 2). Higher DOC and DON concentrations and lower DOC:DIN ratios (except in spring) were measured in riverine water compared to atmospheric inputs (Table 2). The relative contribution of nitrate to total DIN was, on average, significantly higher in riverine (98%) than in atmospheric (58%) inputs (*t*-test, $p < 0.001$). DON accounted on average for 20% of TDN (Table 2) both in riverine and atmospheric inputs. Phosphate concentration was relatively low in both atmospheric and riverine inputs, resulting in P:DIN ratios largely below the Redfield value (0.0625; Table 2). Considering that, in general, protein-like substances are labile and humic-like compounds are recalcitrant, the ratio $FDOM_T$:$FDOM_A$ can be used as a proxy for DOM

Table 1. Summary of the physicochemical and biological conditions of seawater at the sampling station in spring, summer, and autumn of 2013. DOC: dissolved organic carbon; DON: dissolved organic nitrogen; DIN: dissolved inorganic nitrogen

Variable	May	Jul	Oct
Temp. (°C)	14.3	16.6	18.5
Salinity	35.35	35.62	33.40
Nitrate (µM)	2.80	0.22	3.48
Nitrite (µM)	0.11	0.05	0.31
Ammonium (µM)	1.05	0.96	2.02
Phosphate (µM)	0.13	0.16	0.33
DOC (µM)	65.2	76.2	84.9
DON (µM)	4.7	6.3	7.6
P:DIN	0.033	0.130	0.057
Chl *a* (mg m^{-3})	13.5	0.6	1.4
Primary production (mg C m^{-3} h^{-1})	17.4	1.2	3.6
Bacterial biomass (mg C m^{-3})	26.8	11.1	27.0
Bacterial production (mg C m^{-3} h^{-1})	0.17	0.18	0.18
Bacterial respiration (mg C m^{-3} h^{-1})	0.87	0.60	0.93

Table 2. Summary of the chemical characteristics of dissolved matter concentrates from riverine water and atmospheric wet (spring and autumn) or dry deposition (summer) collected in spring, summer, and autumn of 2013. DOC: dissolved organic carbon, DON: dissolved organic nitrogen, DIN: dissolved inorganic nitrogen, $FDOM_T$ and $FDOM_A$: protein-like and humic-like fluorescence of dissolved organic matter, respectively

	May	Jul	Oct	Calculated annual mean[a]	Reference annual mean[b]
Riverine					
DOC (µM)	795	788	1492	102 ± 40	89 ± 12
DON (µM)	64	50	39	5.1 ± 1.2	7.7 ± 1.5
Nitrate (µM)	151	186	330	22.3 ± 9.5	6.0 ± 4.2
Nitrite (µM)	0.43	0.50	1.28	0.07 ± 0.05	0.11 ± 0.04
Ammonium (µM)	1.5	4.6	8.9	0.50 ± 0.37	0.62 ± 0.34
Phosphate (µM)	0.14	0.48	2.46	0.10 ± 0.13	0.15 ± 0.08
DOC:DIN	5.2	4.1	4.4		
P:DIN	0.0009	0.0025	0.0072		
$FDOM_T/FDOM_A$	2.5	3.7	3.8		
Atmospheric					
DOC (µM)	369	552	817	58 ± 23	56 ± 34
DON (µM)	41	18	22	2.7 ± 1.2	7.5 ± 26
Nitrate (µM)	105	28	71	6.8 ± 3.9	9.1 ± 10.5
Nitrite (µM)	0.04	0.03	0.61	0.02 ± 0.03	0.05 ± 0.07
Ammonium (µM)	74.7	22.5	49.2	4.9 ± 2.6	9.7 ± 8.4
Phosphate (µM)	0.66	0.70	1.52	0.10 ± 0.05	0.09 ± 0.18
DOC:DIN	2.1	11.0	6.8		
P:DIN	0.0037	0.0139	0.0127		
$FDOM_T/FDOM_A$	6.3	5.8	10.3		

[a]The annual mean composition was extrapolated from the composition of the concentrates by dividing by the concentration factor (10)

[b]The reference for riverine inputs is Gago et al. (2005) (values for Station Eiras during 2002). The reference for atmospheric inputs is project IMAN (values for Station Bouzas-wet, during 2008–2009)

bioavailability. The mean value of this ratio in the DOM concentrates was significantly higher for atmospheric (7.4 ± 2.5 SD) than for riverine (3.3 ± 0.7) inputs (*t*-test, p = 0.05) (Table 2). We also confirmed that the annual average nutrient concentrations of riverine and atmospheric inputs (mean ± SD of the 3 different seasons) extrapolated from the concentrates were within the limits of previous reported values obtained in this area (Table 2), except for riverine nitrate, which is very unpredictable and variable.

Bacterial response to controlled nutrient additions

The response of heterotrophic bacteria to controlled nutrient additions differed among experiments. Enhanced bacterial production relative to the control (up to 34-fold) was observed after mixed additions in the 3 experiments and also after organic addition in autumn (Fig. 1A). Bacterial biomass significantly increased after mixed addition (5- to 13-fold) and to a lesser extent after organic addition (3.8- to 6-fold) in the 3 experiments (Fig. 1B). Neither biomass nor production responded to inorganic addition alone (Fig. 1).

Response of bacterial biomass and production to natural matter additions

In order to describe changes in biomass and production associated with increasing amounts of riverine or atmospheric inputs, we represented the response, calculated as the ratio between the mean value in the treatment and the corresponding mean

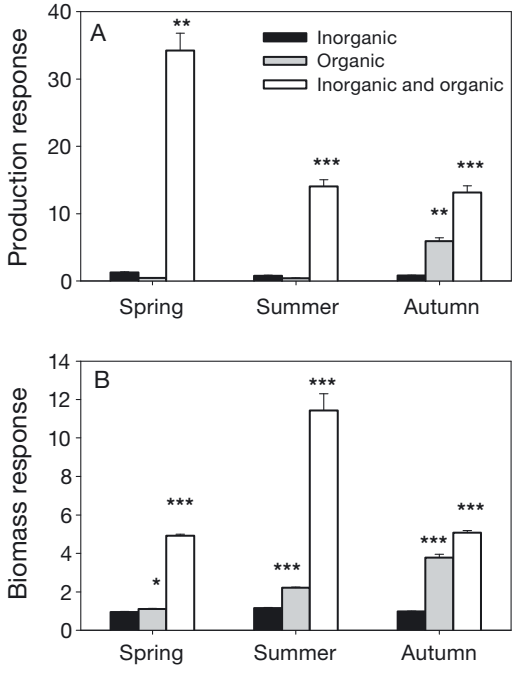

Fig. 1. Response (mean value in treatment relative to mean value in control after 48 h of incubation) of (A) bacterial production and (B) bacterial biomass to controlled nutrient additions (see 'Materials and methods' for details) in spring, summer, and autumn of 2013. Error bars represent the standard error; where error bars are not visible, they are smaller than the size of the symbol. A response equal to 1 means no change relative to the control. Asterisks indicate a response significantly >1 (*t*-test; *p < 0.05, **p < 0.01, ***p < 0.001)

value in the control, versus the amount of DOC load associated with the different treatments (Figs. 2 & 3). In spring, the bacterial biomass response significantly decreased with increasing amounts of DOC associated with the riverine inputs (Fig. 2A), but did not change with increasing DOC load associated with atmospheric inputs (Fig. 2B). In summer, the biomass response did not change with increasing DOC load from riverine inputs (Fig. 2C), but was positively related to the total DOC load associated with atmospheric inputs (Fig. 2D), which explained 46% of the observed variability. Mean (±SE) biomass response was 1.06- ± 0.02-fold, ranging from 0.76-fold for atmospheric inputs in spring to 1.29-fold for riverine inputs in summer.

Bacterial production showed a different pattern of response. The production response associated with increasing amounts of riverine inputs significantly decreased in spring and summer (Fig. 3A,C), explaining 34 to 36% of the variability. By contrast, production significantly increased with increasing DOC load associated with atmospheric inputs in summer and autumn, explaining 37 to 52% of the observed variability. The ordinate intercepts of the significant regressions did not significantly differ from 1 (*t*-test, $p > 0.05$). Mean production response was 1.28- ± 0.05-fold, ranging from 0.76- to 1.97-fold, for riverine inputs in summer, and was significantly higher than the mean biomass response (*t*-test, $p < 0.001$). Mean production response was significantly higher for atmospheric (1.37- ± 0.07-fold) than for riverine inputs (1.19- ± 0.07-fold; *t*-test, $p = 0.039$).

The slopes of the regressions between the production response (i.e. the production response rates) and the DOC load (a 0 value was assigned for the atmospheric inputs in spring and the riverine inputs in autumn) were significantly correlated with the P:DIN ratio of the inputs ($r = 0.87$, $p = 0.025$, $n = 6$). The P:DIN ratio of the inputs explained 75% of the response rate variability (Fig. 4).

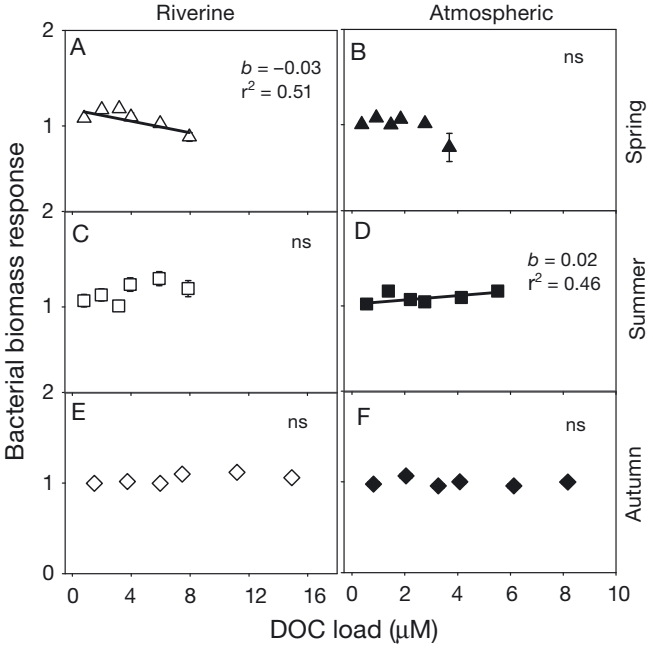

Fig. 2. Response (mean value in treatment relative to mean value in control after 48 h of incubation) of bacterial biomass to increasing concentrations of (A,C,E) natural riverine and (B,D,F) atmospheric matter inputs (expressed as total dissolved organic carbon, DOC, load) in spring (A,B), summer (C,D), and autumn (E,F) of 2013. Error bars represent the standard error; where error bars are not visible, they are smaller than the size of the symbol. A biomass response equal to 1 means no change relative to control. Regression line, slope value (*b*), and determination coefficient (r^2) are represented if a significant increase or decrease of the biomass response with increasing DOC load was found. ns: not significant

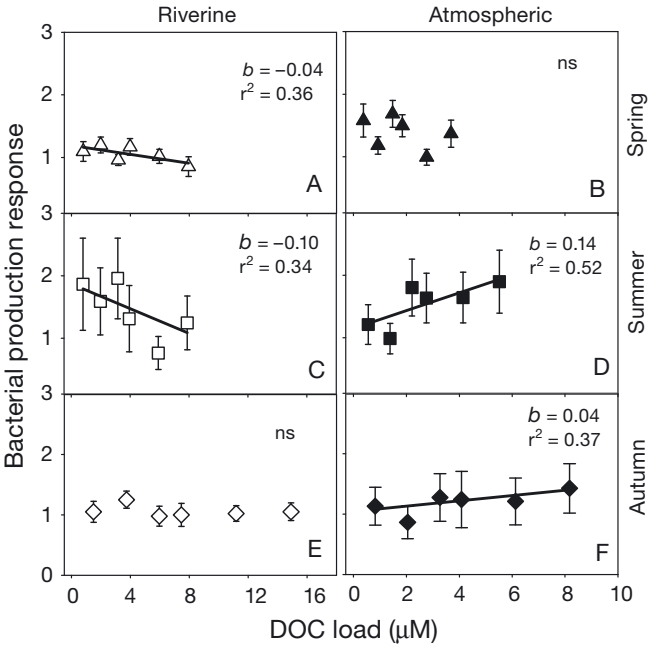

Fig. 3. Response (mean value in treatment relative to mean value in control after 48 h of incubation) of bacterial production to increasing concentrations of (A,C,E) natural riverine and (B,D,F) atmospheric matter inputs (expressed as total dissolved organic carbon, DOC, load) in spring (A,B), summer (C,D), and autumn (E,F) of 2013. Error bars represent the standard error; where error bars are not visible, they are smaller than the size of the symbol. A biomass response equal to 1 means no change relative to control. Regression line, slope value (*b*), and determination coefficient (r^2) are represented if a significant increase or decrease of the biomass response with increasing DOC load was found. ns: not significant. See Fig. 2 for further details

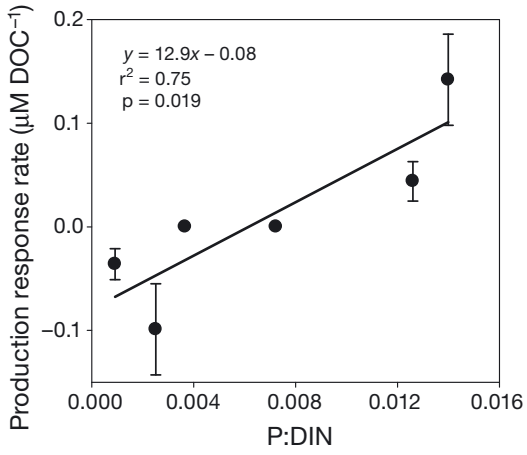

Fig. 4. Relationship between the production response rate (slope ± SE of the regression between production response and dissolved organic carbon, DOC, load) and the phosphorus:dissolved inorganic nitrogen (P:DIN) ratio of the inputs

We did not find any significant response in bacterial respiration to increasing matter additions in any of the 6 experiments (data not shown).

Bacterial response compared to phytoplankton response

In order to interpret the response of bacteria to the different natural matter additions within the context of the microbial food web, we compared the response of bacterial production to that of primary production (Fig. 5). A detailed description of phytoplankton responses will be provided in a forthcoming publication (E. Fernández et al. unpubl.). Overall, the mean bacterial production response was significantly lower (0.83-fold) than the primary production response for riverine inputs (*t*-test, p = 0.044), while no significant differences were observed for atmospheric inputs. The bacterial to primary production response decreased as the percentage of riverine addition increased in spring and summer (Fig. 5B), although the observed trend was not significant.

DISCUSSION

The impact of natural matter inputs of riverine or atmospheric origin promoted variable responses of heterotrophic bacteria depending on the initial physicochemical and ecological conditions of the water samples and the chemical composition of the inputs.

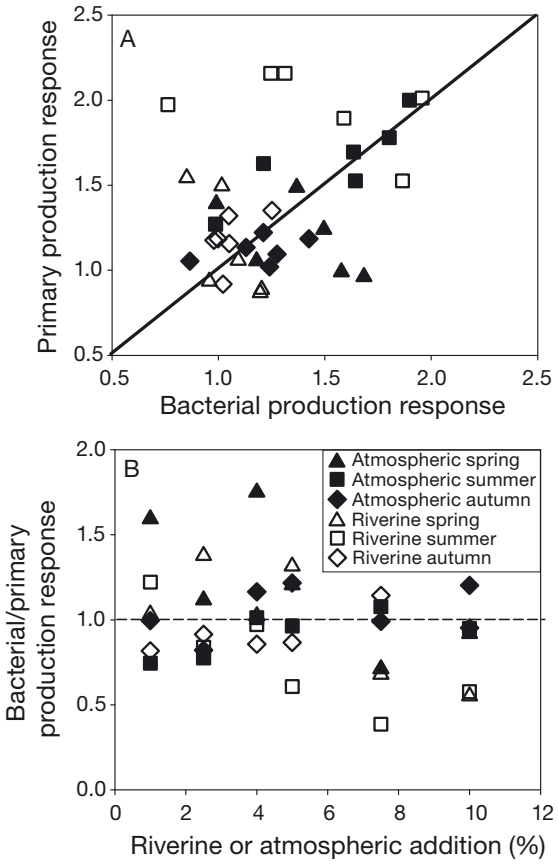

Fig. 5. (A) Response (mean value in treatment relative to mean value in control after 48 h of incubation) of primary production versus response of bacterial production to natural continental and atmospheric inputs. The diagonal represents the 1:1 line where bacterial and primary production equally responded to the inputs. (B) Bacterial to primary production response along the gradient of increasing additions. Dashed line indicates where bacterial and primary production equally responded to the inputs

Overall, the mean production response was higher than the mean biomass response, as previously observed for natural rainwater additions (Teira et al. 2013), natural dust additions (Bonnet et al. 2005, Marañén et al. 2010), and controlled additions (Mills et al. 2008, Martínez-García et al. 2010), which is likely associated with top-down processes (i.e. predation; Zubkov et al. 2000) that prevent bacterial biomass accumulation regardless of increments in bacterial production (Jürgens & Massana 2008). Discrepancies between biomass and production responses may also result from a lag period between changes in production and biomass due to the heterogeneity of population growth rates; if only a fraction of the total bacterial assemblage is growing or actively incorporating leucine, production rates

will increase faster than the total biomass because the cells are 'diluted' by the inactive or non-growing fraction (Ducklow 2000). Previous studies in the same region showed that the effect of controlled dissolved matter addition on bacterial production and biomass was most apparent 24 and 48 h after the amendments, respectively (Martínez-García et al. 2010). Nevertheless, due to the great number of replicates and different treatments in the present study (i.e. a total of 42 experimental units per experiment) we chose to sample only after 48 h in order to capture the response of phytoplankton, which typically occurs after 48 to 72 h (Martínez-García et al. 2010, 2015). Overall, respiration did not significantly change with increasing nutrient loads, in agreement with previous experimental rainwater additions at the same sampling site (Teira et al. 2013).

Bacterial production response to atmospheric and riverine natural inputs

The production response rate, estimated as the slope of the linear regression between production response and DOC load, varied from −0.10 to 0.14 μM^{-1} DOC (Figs. 3 & 4) across the 6 experiments. As the ordinate intercepts did not significantly differ from 1, a response rate of 0.14 implies that the production will increase 14 % for each µM of DOC-load increment. Even though production response rates were negative for riverine and positive for atmospheric additions, both types of inputs had the greatest effect on production when initial DIN concentrations were the lowest (summer; Fig. 3, Table 1), as shown before in rainfall addition experiments (Zou et al. 2000, Teira et al. 2013). As previously observed in the sampling area (Martínez-García et al. 2010), the response of bacteria to controlled nutrient amendment experiments indicates that bacteria were primarily limited by organic C during the 3 experiments, as neither biomass nor production responded to the addition of inorganic nutrients alone (Fig. 1A,B).

Since bacteria in our system are primarily limited by organic C, a higher production would be expected associated with increasing DOC load. However, such a response pattern was only observed in summer and autumn for the atmospheric inputs (Fig. 3D,F). A lower response of bacterial production associated with riverine compared to atmospheric inputs could be expected due to differences in DOM bioavailability. Previous studies have indicated that riverine DOM is largely refractory (Søndergaard & Middelboe 1995, Moran et al. 1999) compared to rainwater DOM (Avery et al. 2003). The significantly higher $FDOM_T$:$FDOM_A$ ratio (used as a proxy for DOM bioavailability) of atmospheric inputs compared to those of riverine inputs (Table 2) suggests a higher availability of atmospheric than riverine DOC; however, the $FDOM_T$:$FDOM_A$ ratio did not significantly explain the variability observed in the response rates.

Bacterial response to the additions might be also constrained by the mineral components of the inputs. Particularly, the lack of bacterial production response to our controlled organic additions, containing C and N but not P (Fig. 1A), strongly suggests a P deficiency for bacterial growth in spring and summer, as previously observed on certain occasions in the sampling area (Martínez-García et al. 2010). Although the response rate was not correlated with the P load, the strong and significant relationship observed between the response rate and the P:DIN ratio of the inputs (Fig. 4) suggests that the balance between inorganic P and N forms largely regulate the response of bacteria to riverine and atmospheric nutrient fluxes. Furthermore, the intriguing negative bacterial production response rate associated with riverine matter inputs observed in spring and summer (Fig. 3A,C) suggests that bacteria may be competing with phytoplankton for inorganic nutrients. The higher response of primary production compared to bacterial production after riverine inputs, particularly in spring and summer (Fig. 5), partially supports this possibility.

Considering that bacteria typically have lower C:N and C:P ratios than primary producers (Chrzanowski et al. 1996, Cotner et al. 2000, Cotner & Biddanda 2002, Vrede et al. 2002, Carlsson et al. 2012), they may exhibit high inorganic nutrient demands and, thus, directly compete with phytoplankton for the uptake of mineral N and P. Bacteria, due to their smaller size, are expected to outcompete phytoplankton for the uptake of limiting inorganic nutrients (Thingstad et al. 1993, Cotner & Biddanda 2002, Joint et al. 2002). In accordance with this expectation, bacterial production response relative to phytoplankton production response to dust inputs has been shown to increase as the degree of oligotrophy increases in the Atlantic Ocean (Marañén et al. 2010), likely due to the superior ability of bacteria to take up inorganic nutrients at very low concentrations. We also observed that the production response of bacteria relative to that of phytoplankton after the natural matter inputs was higher for treatments with a low percentage of addition (Fig. 5B).

Even though the competitive advantage of bacteria over phytoplankton has been observed for dissolved inorganic P uptake (Pengerud et al. 1987, Jansson 1993, Guerrini et al. 1998, Joint et al. 2002), there is no equally clear evidence that bacteria are better competitors than phytoplankton for DIN (Danger et al. 2007, Vadstein et al. 2012). Although heterotrophic bacteria significantly contribute to both ammonium and nitrate uptake (Kirchman & Wheeler 1998, Zehr & Ward 2002, Fouilland et al. 2007), they are not expected to outcompete phytoplankton for nitrate due to the higher energetic cost associated with nitrate uptake compared to ammonium or DON (Vallino et al. 1996, Joint et al. 2002). Likewise, contrary to phytoplankton, bacteria appear to be more commonly limited by P than by N in marine ecosystems (Cotner et al. 1997, Zohary et al. 2005, Carlsson et al. 2012, Vadstein et al. 2012), as has also been suggested in our sampling area (Martínez-García et al. 2010). If bacteria are secondarily limited by inorganic P rather than by inorganic N, and phytoplankton is primarily limited by DIN, the negative bacterial production response rate observed for riverine inputs in spring and summer (Fig. 3A,C), when P supply is extremely low relative to DIN (Table 2, Fig. 4), suggests that the bacterial response might be modulated by the phytoplankton response, which seems to profit from the large nitrate concentration associated with the riverine inputs (Table 2, Fig. 5). A very low P supply associated with riverine inputs has been reported previously (Labry et al. 2002).

The hypothesis of a P-mediated bacterial response to natural matter inputs in the Ría de Vigo is further supported by controlled nutrient addition experiments conducted by our research group at the same sampling site. When pooling all available data from such experiments, including the present and 2 previous studies (Martínez-García et al. 2010, Prieto et al. 2015), we found a significant and positive correlation between the production response to organic additions (containing C and N but not P) and the ambient phosphate concentration in the Ría de Vigo ($r^2 = 0.75$, $p = 0.011$, $n = 7$; data not shown).

In conclusion, we have shown that bacterial production response to increasing DOC load associated with atmospheric and riverine additions largely depends on the P:DIN ratio of the inputs. Negative production response rates are associated with riverine inputs that show extremely low P:DIN ratios, probably due to phytoplankton outcompeting bacteria for P uptake when nitrate concentration is high. In a future global change scenario, where both the riverine and atmospheric nutrient fluxes are expected to increase and their associated relative P content is expected to decrease as a consequence of anthropogenic activities (Galloway et al. 2004, Peñuelas et al. 2013), autotrophic production would likely benefit more than heterotrophic bacterial production. Moreover, the limited response of bacteria to increasing P-limited inputs to coastal waters may have further implications for ocean C cycling, as the unused allochthonous DOC might be eventually transported to open ocean waters where it could be utilized by bacteria or exported to the ocean interior.

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