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

By

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Running head: Bacterioplankton response to inputs of matter

Abstract

Anthropogenic pressures are changing the magnitude and nature of matter inputs into the ocean. The Ría de Vigo (NW Iberian Peninsula) is a highly productive and dynamic coastal system, likely affected by such alterations. Previous nutrient-addition microcosm experiments conducted during contrasting hydrographic conditions suggested that heterotrophic bacteria appear to be 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, where 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 with the relative phosphorous (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.

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 ground-water 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 10-20% 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 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 global increase of 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 known to be 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 indicate 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 allochtonous matter inputs, may determine the responses of the microbial communities to nutrient enrichment.

The Ría de Vigo is an eutrophic embayment located in the coastal system of the North-West 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 in the order of 1500 mg N m^{-2} yr^{-1} (Gago et al. 2005) associated with riverine discharge, and about 100-250 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 been also measured associated to 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; (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 three

experiments using different microbial plankton communities collected under contrasting hydrographic conditions (spring, summer and autumn) where 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 hypothesize that increasing amounts of riverine or atmospheric inputs will increasingly stimulate bacterial production, biomass and respiration in surface waters from the Ría de Vigo.

Methods

Natural seawater for the experiments was taken in the middle sector of the Ría de Vigo (42º14.09' N, 8º47.18' W) in spring, summer and autumn 2013. Vertical profiles of water column temperature, salinity and in situ fluorescence down to 25 m depth were obtained with a SBE 9/11 CTD probe and a Seatech fluorometer attached to a rosette sampler. Then, sub-surface seawater (3-4 m) was collected in 12 L acid-clean Niskin bottles and filtered through a 200 µm pore size mesh to remove larger zooplankton. Subsequently, 4 L UV transparent whirlpak 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 were 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 pre-washed (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 ºC, 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 carbon and nitrogen) substrates confirmed that the samples were concentrated quantitatively without any significant loss or gain.

A MTX rainwater sampler (model FAS005AB) and a high volume PM10 MCV PM1025 particle sampler (model CAV-A/MS) were installed at 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, the rainwater. Rainwater was collected from four weeks to one week 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 one 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 precombusted (450 ºC, 4 h) 140 mm GF/F filters. The particles were collected the week before the three addition experiments, operating during 48 h at a rate of 30 m^3 h^{-1}. Then, the water-soluble fraction (WSF) of one eighth of the filter was extracted in 400 mL of the corresponding rainwater concentrate by mechanic stirring during 40 min. For the case of the July experiment, the WSF was extracted in milli-Q water. These proportions (1/8 of the filter in 400 mL of water) were decided to obtain 10-fold the expected concentrations according to previous existing information on the composition of wet and dry deposition to the Ría de Vigo (Teira et al. 2013, Martínez-García et al. 2015). Final mixed extracts were filtered through precombusted (450 ºC, 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 fibber 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 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 independently of the amount of extract added. Seawater was

167 mixed with 1%, 2.5%, 4%, 5%, 7.5% and 10% of natural matter from riverine (riverine
168 discharge) and atmospheric (dry and wet deposition) origin. A control treatment (no
169 addition) was included for each type of input. Three replicates were included for the
170 control, 1%, 5% and 10% treatments, and one replicate for the 2.5%, 4% and 7.5%
171 treatments.

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

183 Controlled nutrient addition experiments were also conducted in order to
184 describe the limiting nutrient for bacterial growth during each sampled season. We used
185 the same controlled nutrient addition treatments as in Martínez-García et al. (2010): (1)
186 No addition treatment; (2) Inorganic nutrient treatment: 5 µmol L^{-1} nitrate (NO_3^-), 5
187 µmol L^{-1} ammonium (NH_4^+) and 1 µmol L^{-1} phosphate (HPO_4^{2-}); (3) Organic nutrient
188 treatment: 5 µmol L^{-1} glucose and 5 µmol L^{-1} equimolar mix of 18 aminoacids (all the
189 protein amino acids except cysteine and tyrosine); and (4) Mixed treatment: Inorganic
190 and organic nutrient treatments. Three replicates were included for each treatment. All
191 the experimental bags were incubated for 48 h under natural irradiance and temperature

conditions. Bacterial biomass, production and respiration were measured at time zero 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 nutrients determination (ammonium, nitrite, nitrate and phosphate) were collected in 50 mL polyethylene bottles and frozen at −20 ºC until analysis by standard colorimetric methods with an Alliance Futura segmented flow analyzer (Hansen and Grasshoff 1983). Water for the analysis of dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) was filtered through 0.2 µm filters (Pall, Supor membrane Disc Filter) in an all-glass filtration system under positive pressure of N_2 and collected into pre-combusted (450 ºC, 12 h) 10 mL glass ampoules acidified with H_3PO_4 to pH < 2. Samples were measured with a Shimadzu TOC-V total organic carbon analyzer fitted with a Shimadzu TNM-1 total nitrogen measurement unit. Dissolved organic nitrogen (DON) was obtained by subtracting ammonium + nitrite + 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 two 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 sulphuric acid following Nieto-Cid et al (2006) to convert fluorescence units to normalized fluorescence intensity units (NFIU).

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 ºC 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 in 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 x $BV^{0.72}$.

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 carbon conversion derived by Martínez-García et al. (2010) (2.6 ± 1.1 kg C mol Leu^{-1}).

Bacterial Respiration. INT ((iodo-phenyl)-3(nitrophenyl)-5(phenyl) tetrazolium chloride) reduction rate was used as estimator of bacterial respiration. Size-fractionated in vivo INT reduction rates were measured as described in 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 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 ºC 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 20-30 minutes in 50 ºC 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 13200 x g during 10 minutes at 18 ºC. 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 carbon respiration an R/ETS ratio of 12.8 (Martínez-García et al 2009) and a respiratory quotient (RQ) of 0.8 (Williams and 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 ºC until pigment extraction in 90% acetone at 4 ºC 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 PAR of 240 µE m^{-2} s^{-1}. After the incubation period, samples were filtered through 0.2 µm polycarbonate filters at

266 very low vacuum (<50 mm Hg). Filters were exposed to HCl fumes for 24 h to remove
267 unincorporated inorganic ^{14}C and radioassayed.
268
269 **Results**
270 **Initial conditions and chemical composition of natural inputs**
271 Initial conditions for each experiment are summarized in Table 1. Different
272 hydrographic conditions were found during each survey. In spring (May 2013), high
273 dissolved inorganic N (DIN= nitrate + nitrite + ammonium) and Chl *a* concentrations
274 occurred while in autumn (October 2013) lower Chl *a* concentration were observed
275 regardless of higher nutrient concentrations. In the summer experiment (July 2013)
276 inorganic nutrient and Chl *a* concentrations were the lowest. Primary production rates
277 were the highest in spring and lower values were measured in summer and autumn
278 (Table 1). Bacterial biomass was lower in summer than in spring and autumn, whereas
279 bacterial production and respiration rates did not notably varied among seasons (Table
280 1).The chemical composition of riverine and atmospheric (wet and dry) matter inputs
281 collected during the three studied seasons showed a high temporal variability (Table 2).
282 In general, riverine water collected in October 2013 contained higher DIN, phosphate
283 and DOC than that collected in spring and summer (Table 2). On the other hand,
284 atmospheric inputs in May 2013 contained higher DIN and DON but lower DOC
285 concentrations than that collected in autumn. Atmospheric deposition in summer
286 contained relatively low N concentrations (Table 2). Higher DOC and DON
287 concentrations and lower DOC:DIN ratios (except in spring) were measured in riverine
288 water compared to atmospheric inputs (Table 2). The relative contribution of nitrate to
289 total DIN was, on average, significantly higher in riverine (98%) than in atmospheric
290 (58%) inputs (t-test, $p < 0.001$). DON accounted on average 20% of total dissolved

nitrogen (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 values (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 bioavailability. The mean value of this ratio in the DOM concentrates was significantly higher for atmospheric (7.4 ± 2.5) 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 value ± standard deviation of the three different seasons) extrapolated from the concentrates were within the limits of previous reported values obtained in this area (Table 2), except for the 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 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 three experiments (Fig. 1B). Neither biomass nor production responded to inorganic addition alone (Fig. 1A, 1B).

Response of bacterial biomass and production to natural matter additions

In order to describe changes in biomass and production associated to 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 value in the control, versus the amount of DOC load associated to the different treatments (Fig. 2, 3). In spring, the bacterial biomass response significantly decreased with increasing

316 amounts of DOC associated to the riverine inputs (Fig. 2A), but did not change with
317 increasing DOC load associated to atmospheric inputs (Fig. 2B). In summer, the
318 biomass response did not change with increasing DOC load from riverine inputs (Fig.
319 2C), but was positively related to the total DOC load associated to atmospheric inputs
320 (Fig. 2D), which explained 46% of the observed variability. Mean (± SE) biomass
321 response was 1.06 ± 0.02-fold, ranging from 0.76-fold, for atmospheric inputs in spring,
322 to 1.29-fold for riverine inputs in summer.

323 Bacterial production showed a different pattern of response. The production
324 response associated to increasing amounts of riverine inputs significantly decreased in
325 spring and summer (Fig. 3A, 3C), explaining 34-36% of the variability. By contrast,
326 production significantly increased with increasing DOC load associated to atmospheric
327 inputs in summer and autumn, explaining 37-52% of the observed variability. The
328 ordinate intercepts of the significant regressions did not significantly differed from 1 (t-
329 test, $p>0.05$). Mean production response was 1.28 ± 0.05-fold, ranging from 0.76- to
330 1.97-fold, for riverine inputs in summer, and was significantly higher than the mean
331 biomass response (t-test, $p < 0.001$). Mean production response was significantly higher
332 for atmospheric (1.37 ± 0.07-fold) than for riverine inputs (1.19 ± 0.07-fold) (t-test, $p =$
333 0.039).

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

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

341 **Bacterial response compared to phytoplankton response**

342 In order to interpret the response of bacteria to the different natural matter
343 additions within the context of the microbial food web, we compared the response of
344 bacterial production to that of primary production (Fig. 5). A detailed description of
345 phytoplankton responses is described elsewhere (Fernández et al, in prep). Overall, the
346 mean bacterial production response was significantly lower (0.83-fold) than primary
347 production response for riverine inputs (t-test, $p = 0.044$) while no significant
348 differences were observed for atmospheric inputs. The bacterial to primary production
349 response decreased as the % of riverine addition increased in spring and summer (Fig.
350 5B), although the observed trend was not significant.

351

352 **Discussion**

353 The impact of natural matter inputs of riverine or atmospheric origin promoted
354 variable responses of heterotrophic bacteria depending on the initial physico-chemical
355 and ecological conditions of the water samples and the chemical composition of the
356 inputs.

357 Overall, the mean production response was higher than the mean biomass
358 response, as previously observed for natural rainwater additions (Teira et al. 2013),
359 natural dust additions (Bonnet et al. 2005, Marañón et al. 2010) and controlled additions
360 (Mills et al. 2008, Martínez-García et al. 2010), which is likely associated to top-down
361 processes (i.e. predation) (Zubkov et al. 2000) which prevent bacterial biomass
362 accumulation regardless of increments in bacterial production (Jürguens & Massana
363 2008). Discrepancies between biomass and production responses may also result from a
364 lag period between changes in production and biomass due to the heterogeneity of
365 population growth rates; if only a fraction of the total bacterial assemblage is growing

366 or actively incorporating leucine, production rates will increase faster than the total
367 biomass because the cells are “diluted” by the inactive or non-growing fraction
368 (Ducklow 2000). Previous studies in the same region showed that the effect of
369 controlled dissolved matter addition on bacterial production and biomass was most
370 apparent 24 h and 48 h after the amendments, respectively (Martínez-García et al.
371 2010). Nevertheless, due to the great number of replicates and different treatments in
372 the present study (i.e a total of 42 experimental units per experiment) we chose to
373 sample only after 48 h in order to capture the response of phytoplankton which typically
374 occur after 48-72 h (Martínez-García et al. 2010, 2015).

375 Overall, respiration did not significantly change with increasing nutrient loads in
376 agreement with previous experimental rainwater additions in the same sampling site
377 (Teira et al. 2013).

378

379 **Bacterial production response to atmospheric and riverine natural inputs**

380 The production response rate, estimated as the slope of the linear regression
381 between production response and DOC-load, varied from -0.10 to 0.14 μM DOC^{-1}
382 (Figures 3, 4) across the six experiments. As the ordinate intercepts did not significantly
383 differ from 1, a response rate of 0.14 implies that the production will increase 14% per
384 each μM of DOC-load increment. Even though production response rates were negative
385 for riverine and positive for atmospheric additions, both types of inputs had the greatest
386 effect on production when initial DIN concentrations were the lowest (summer) (Fig. 3,
387 Table 1), as shown before in rainfall addition experiments (Zou et al. 2000, Teira et al.
388 2013). As previously observed in the sampling area (Martínez-García et al. 2010), the
389 response of bacteria to controlled nutrient amendment experiments indicate that bacteria
390 were primarily limited by organic carbon during the three experiments as neither

biomass nor production responded to the addition of inorganic nutrients alone (Fig. 1A, 1B).

Since bacteria in our system are primarily limited by organic C, a higher production would be expected associated to increasing DOC-load. However, such response pattern was only observed in summer and autumn for the atmospheric inputs (Fig. 3D, 3F). A lower response of bacterial production associated to riverine compared to atmospheric inputs could be expected due to differences in DOM bioavailability. Previous studies indicate 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) suggest 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 (Figure 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). Despite the response rate was not correlated with the phosphorous load, the strong and significant relationship observed between the response rate and the P:DIN ratio of the inputs (Fig. 4), suggest 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, 3C), 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 support 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 to this, 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 (Marañón 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 is higher for treatments with low percentage of addition (Fig. 5B).

Even though the competitive advantage of bacteria over phytoplankton has been seen to hold for dissolved inorganic P uptake (Pengerud et al. 1987, Jansson 1993, Guerrini et al. 1998, Joint et al. 2002), there are no equally clear evidences for bacteria as 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 and 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 to nitrate uptake compared to ammonium or dissolved organic nitrogen (Vallino et al. 1996, Joint et al. 2002). Likewise, contrary to phytoplankton,

441 bacteria appear to be more commonly limited by P than by N in marine ecosystems
442 (Cotner et al. 1997, Church 2008, Zohary et al. 2005, Carlsson et al. 2012, Vadstein et
443 al. 2012), as it has been also suggested in our sampling area (Martínez-García et al.
444 2010). If bacteria are secondarily limited by inorganic P rather than by inorganic N, and
445 phytoplankton primarily limited by DIN, the negative bacterial production response rate
446 observed for riverine inputs in spring and summer (Fig. 3A, 3C), when P supply is
447 extremely low relative to DIN (Table 2, Fig. 4), suggests that the bacterial response
448 might be modulated by the phytoplankton response, which seems to profit from the
449 large nitrate concentration associated to the riverine inputs (Table 2, Fig. 5). A very low
450 P supply associated to riverine inputs have been previously reported elsewhere (Labry et
451 al. 2001).

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

460 In conclusion, we have shown that bacterial production response to increasing
461 DOC-load associated with atmospheric and riverine additions largely depends on the
462 P:DIN ratio of the inputs. Negative production response rates are associated to riverine
463 inputs that show extremely low P:DIN ratios, probably due to phytoplankton
464 outcompeting bacteria for P uptake when nitrate concentration is high. In a future global
465 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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675 **Figure legends**

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

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

690 Figure 3. Response (mean value in treatment relative to mean value in control after 48 h
691 of incubation) of bacterial production to increasing concentrations of natural riverine
692 (A, C, E) and atmospheric (B, D, F) matter inputs (expressed as total DOC-load) in
693 spring (A, B), summer (C, D) and autumn (E, F). Error bars represent the standard error;
694 where error bars are not visible, they are smaller than the size of the symbol. A
695 production response equal to 1 means no change relative to control. Regression line,
696 slope value (b) and determination coefficient (r^2) are represented if a significant increase
697 or decrease of the production response with increasing DOC-load was found.

698 Figure 4. Relationship between the production response rate (slope ± SE of the
699 regression between production response and DOC-load) and the P:DIN ratio of the
700 inputs.
701 Figure 5. (A) Response (mean value in treatment relative to mean value in control after
702 48 h of incubation) of primary production versus response of bacterial production to
703 natural continental and atmospheric inputs. The diagonal represents the 1:1 line where
704 bacterial and primary production equally responded to the inputs. (B) Bacterial to
705 primary production response along the gradient of increasing additions. Dashed line
706 indicates where bacterial and primary production equally responded to the inputs.

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

Variable	May-13	July-13	October-13
Temperature °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
Chlorophyll-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. 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. *The annual mean composition was extrapolated from the composition of the concentrates by dividing by the concentration factor (10). **Reference for riverine inputs is Gago et al 2005 (values for Eiras station during year 2002). Reference for atmospheric inputs is project IMAN (values for station Bouzas wet, during years 2008-2009).

		May-13	July-13	October-13	Calculated Annual Mean*	Reference Annual Mean**
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		

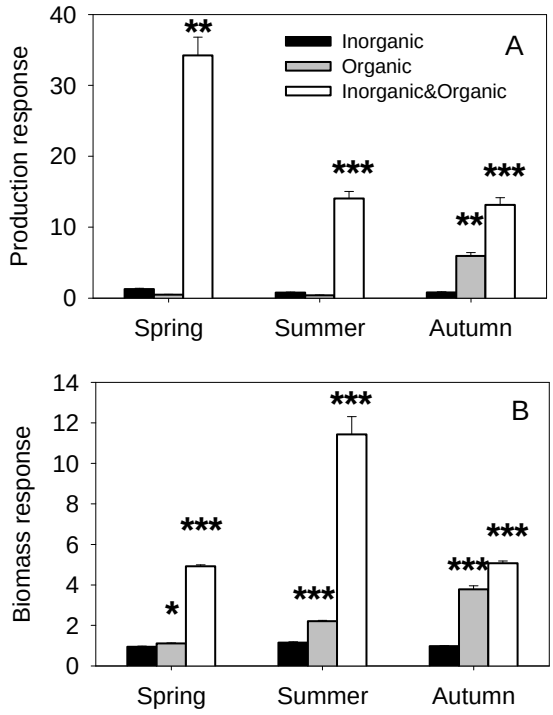
Production response
40
30
20
10
0
**
***
***
**
Inorganic
Organic
Inorganic&Organic
A
Spring
Summer
Autumn
Biomass response
14
12
10
8
6
4
2
0
*
***
***
***
***
B
Spring
Summer
Autumn

Figure 1

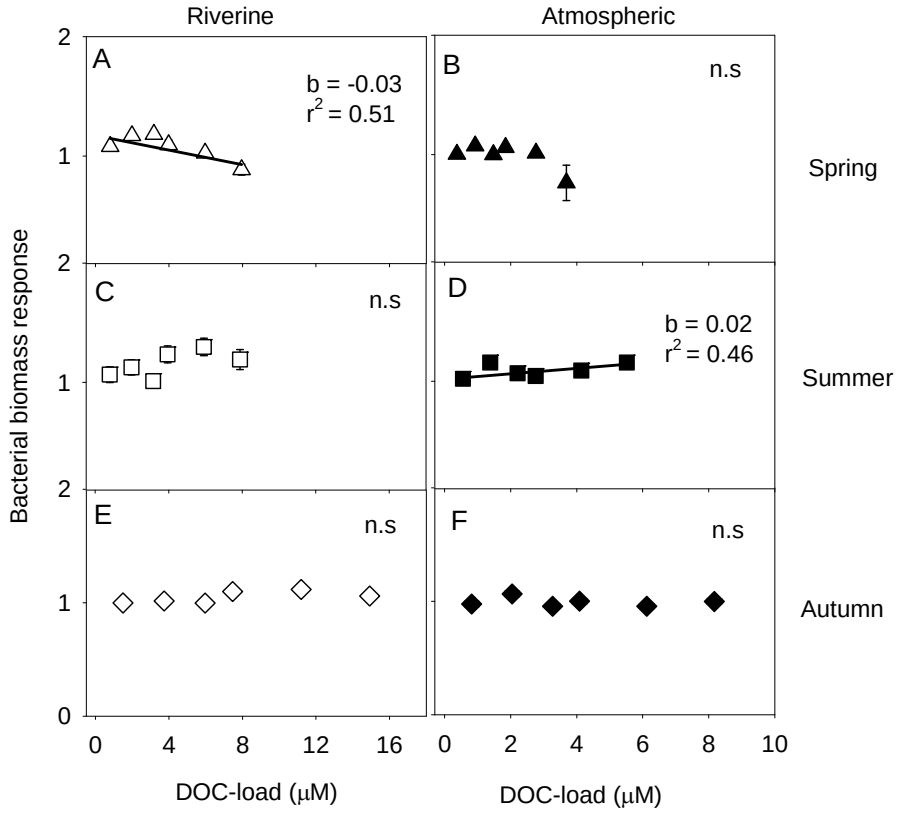
Riverine
Atmospheric
A
b = -0.03
r2 = 0.51
B
n.s
Spring
C
n.s
D
b = 0.02
r2 = 0.46
Summer
E
n.s
F
n.s
Autumn
Bacterial biomass response
DOC-load (μM)
DOC-load (μM)

Figure 2

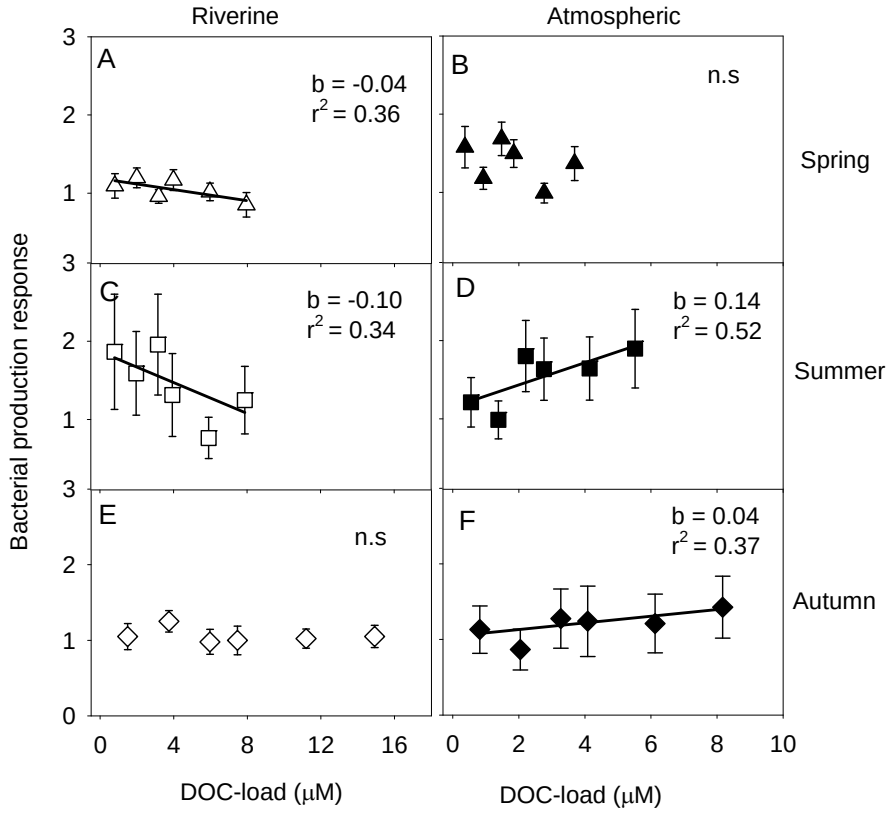
Riverine
Atmospheric
A
b = -0.04
r2 = 0.36
B
n.s
Spring
C
b = -0.10
r2 = 0.34
D
b = 0.14
r2 = 0.52
Summer
E
n.s
F
b = 0.04
r2 = 0.37
Autumn
Bacterial production response
DOC-load (μM)
DOC-load (μM)

Figure 3

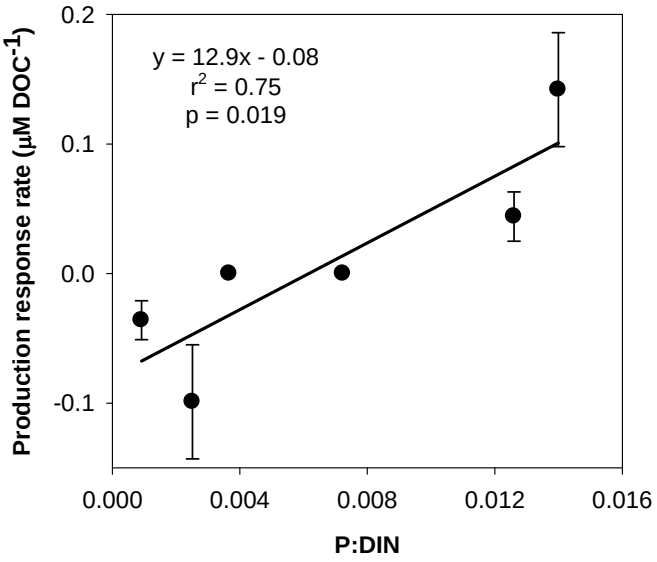
y = 12.9x - 0.08
r2 = 0.75
p = 0.019
Production response rate (µM DOC-1)
0.2
0.1
0.0
-0.1
0.000
0.004
0.008
0.012
0.016
P:DIN

Figure 4

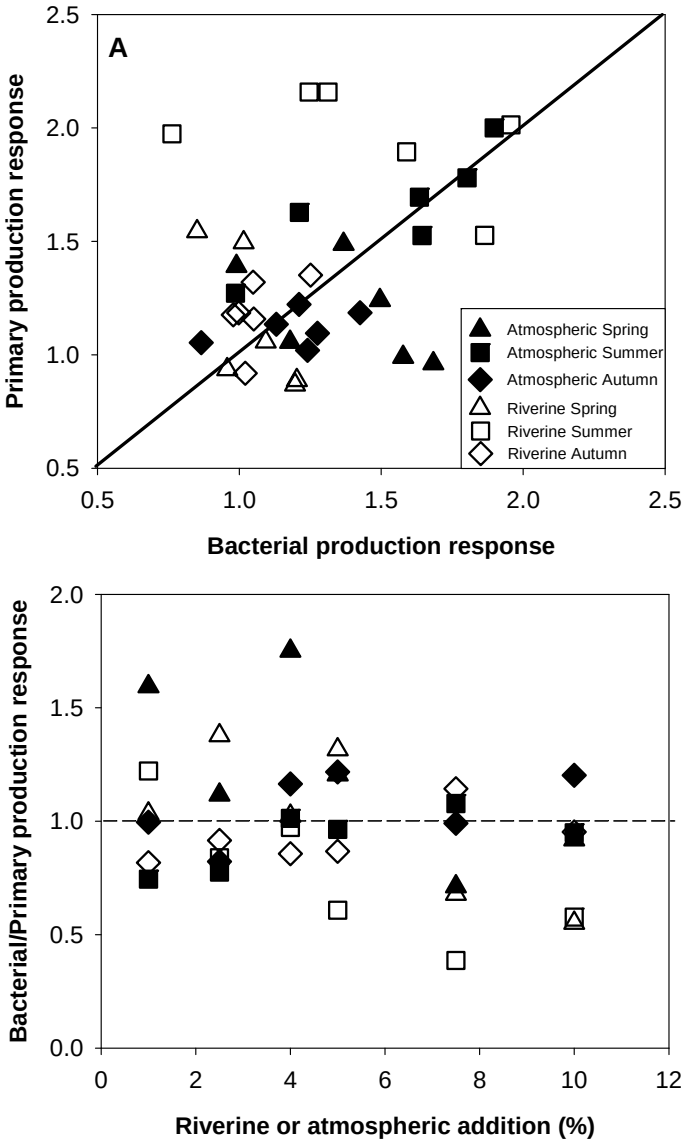
A
Primary production response
Bacterial production response
Atmospheric Spring
Atmospheric Summer
Atmospheric Autumn
Riverine Spring
Riverine Summer
Riverine Autumn
Bacterial/Primary production response
Riverine or atmospheric addition (%)

Figure 5