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Role of vitamin B_{12} in the microbial plankton response to nutrient enrichment

Esther Barber-Lluch[1,2,*], Marta Hernández-Ruiz[1,2], Antero Prieto[1,2], Emilio Fernández[1,2], Eva Teira[1,2]

[1]Grupo de Oceanografía Biolóxica, Departamento de Ecoloxía e Bioloxía Animal, 36310 Universidade de Vigo, Spain
[2]Estación de Ciencias Mariñas de Toralla, ECIMAT, 36331 Universidade de Vigo, Spain

ABSTRACT: In order to evaluate the role of vitamin B_{12} availability in microbial plankton communities, 10 microcosm experiments were performed in which inorganic nutrients (nitrate, ammonium and phosphate) and vitamin B_{12} were added, separately or in combination, to surface seawater samples drawn from an on-shelf station over an annual cycle. The responses of both autotrophic and heterotrophic microbial plankton were highly variable. Vitamin B_{12} addition enhanced phytoplankton biomass in early spring (March and April) and autumn (October) and bacterial biomass in winter (January) and summer (June and September). Our data reinforce the idea that inorganic nutrient limitation experienced by autotrophic and heterotrophic microbial communities is mostly dependent on the initial background nutrient conditions. The single (addition of vitamin B_{12} alone) or secondary vitamin B_{12} limitation (combined with inorganic nutrients) of phytoplankton growth implies that the availability of this molecule might modulate the increase in primary production associated with enhanced nitrogen loads to the ocean derived from human activities. These findings may have significant implications for our understanding of carbon and nutrient cycling through the planktonic microbial compartments of coastal ecosystems.

KEY WORDS: Vitamin B_{12} · Nutrient addition · Limitation · Competition · Phytoplankton · Bacterioplankton

1. INTRODUCTION

Understanding the chemical factors limiting phytoplankton production in marine ecosystems has been the scope of multiple studies over recent decades (e.g. de Baar 1994, Morel et al. 1994, Moore et al. 2001) due to its implications for the energy transfer through the pelagic food web and the biogeochemical cycling of matter. Several studies have shown that nitrogen is the main macronutrient controlling primary production in marine coastal waters (Rabalais 2002, Howarth & Marino 2006, Moore et al. 2013). The amount of anthropogenic nitrogen reaching marine systems through atmospheric deposition (Duce et al. 2008, Jickells et al. 2017) or riverine flow (Howarth 2008) has exceeded pre-industrial inputs, inducing a global transformation of the nitrogen biogeochemical cycle (Galloway et al. 2008). Consequently, enhanced inorganic nutrient inputs into coastal systems are expected to result in higher levels of phytoplankton biomass and production (Rabalais 2002).

The NW Iberian Peninsula coastal system is characterized by the intermittent upwelling of inorganic nutrient-rich water between March and September (Fraga 1981, Figueiras et al. 2002, Barton et al. 2015) and is affected by significant allochthonous nutrient delivery through riverine discharge (Gago et al. 2005) and atmospheric deposition (Rodríguez & Macías 2006). Addition experiments conducted in

*Corresponding author: eblluch@uvigo.es

this coastal ecosystem showed that phytoplankton did not always respond after inorganic nutrient amendment, but was only stimulated when inorganic nutrients (nitrate, ammonium and phosphate) and organic substrates (amino acids and glucose) were added jointly (Martínez-García et al. 2010, 2013). These results were interpreted as indicative of phytoplankton reliance on some bacterial-derived secondary metabolites (Prieto et al. 2016), such as vitamin B_{12} (cobalamin).

Vitamin B_{12} is one of the essential growth factors commonly associated with algal–bacterial interactions (Croft et al. 2005, Amin et al. 2012, Cooper & Smith 2015) since it is exclusively synthesized by prokaryotes but required by most eukaryotic phytoplankton species (Croft et al. 2005, Tang et al. 2010, Sañudo-Wilhelmy et al. 2014). Vitamin B_{12} plays essential roles in eukaryotic cellular biochemistry, acting as a cofactor of enzymes involved in important metabolic processes such as the odd-chain fatty acid metabolism, the synthesis of methionine and the biosynthesis of deoxyribose (Matthews et al. 2003, Helliwell et al. 2011, Dowling et al. 2012, Helliwell 2017).

Vitamin B_{12} is a cobalt-containing tetrapyrrole with an α ligand of 5,6-dimethylbenzimidizole (DMB) and a β ligand of an OH-, CN-, methyl- or adenosyl-group (Warren et al. 2002). Measurements of vitamin B_{12} levels in the water column using highly sensitive chemical methodologies highlighted the scarcity of this compound in the world oceans (Okbamichael & Sañudo-Wilhelmy 2004, Sañudo-Wilhelmy et al. 2012, Bonnet et al. 2013, Heal et al. 2014, Suffridge et al. 2018). In addition, recent studies have demonstrated the existence of a vitamin B_{12} analogue, called pseudocobalamin, in which adenine replaces the DMB ligand (Helliwell et al. 2016, Heal et al. 2017). Pseudocobalamin is synthesized by marine cyanobacteria and is less bioavailable for eukaryotic phytoplankton (Heal et al. 2017).

Several studies have shown enhanced chlorophyll *a* (chl *a*) concentration associated with vitamin B_{12} amendments in diverse marine ecosystems (Panzeca et al. 2006, Sañudo-Wilhelmy et al. 2006, Bertrand et al. 2007, Gobler et al. 2007, Koch et al. 2011, 2012), suggesting an underexplored role of this growth factor in primary production. Many bacterial taxa lack a pathway for de novo vitamin B_{12} synthesis (Sañudo-Wilhelmy et al. 2014), thus potentially competing with phytoplankton for the acquisition of this growth factor from the environment. Yet, the simultaneous effect of vitamin B_{12} supply on both phytoplankton and bacteria has only been explored in 1 study (Koch et al. 2011). Considering that a large fraction of microbial taxa are vitamin B_{12} auxotrophs, it is conceivable that the response of phytoplankton to nutrient enrichment associated with increasing anthropogenic inputs could be limited by vitamin B_{12} supply.

Within this context, we conducted 10 nutrient-addition microcosm experiments to assess the role of vitamin B_{12} in the response of microbial plankton biomass to inputs of inorganic nutrients into coastal waters. We hypothesized that the response of phytoplankton to nutrient enrichment would be limited by vitamin B_{12}, which could eventually affect the ability of the autotrophic microbial community to respond to increasing nitrogen inputs derived from human activities.

2. MATERIALS AND METHODS

2.1. Sampling site

Monthly sampling was conducted at an on-shelf station off the Ría de Vigo, NW Spain (42.14° N, 8.96° W; Fig. 1), throughout 2014, except in July and August, when technical problems with the research vessel prevented sampling. Vertical profiles of temperature, salinity and *in situ* fluorescence were obtained using a conductivity-temperature-depth sensor down to 80 m. Upwelling intensity was estimated by calculating the Ekman transport from surface winds as an upwelling index (I_w). Daily values were computed by the Instituto Español de Oceanografía (www.indicedeafloramiento.ieo.es) in 2 cells of 1° × 1° centered at 42° N, 10° W, using data from atmospheric pressure at sea level, derived from the WXMAP model (Gonzalez-Nuevo et al. 2014). Solar radiation data were obtained from the Regional Weather Forecast Agency-Meteogalicia (www.meteogalicia.gal) at the nearest station (42.2° N, 8.9° W).

2.2. Experimental design

Water samples for the microcosm experiments were collected from the surface (ca. 2 m depth) and filtered through a 200 µm mesh to remove larger zooplankton. Subsequently, 12 Whirl-Pak® bags, which were transparent to photosynthetically active radiation and UV-B radiation (Davidson et al. 2007, Pakulski et al. 2007), were filled with 1 l of seawater. Ten vitamin B_{12} addition experiments, lasting 3 d, were performed during the sampling year.

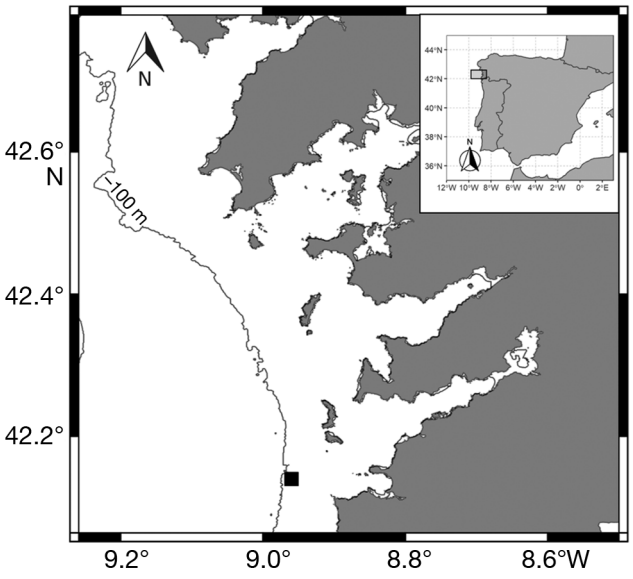

Fig. 1. Sampling site (black square) off the Ría de Vigo, Spain

At the beginning of each experiment, dissolved inorganic nutrients, phytoplankton and bacterioplankton biomasses, and primary production, bacterial production and microbial community respiration rates were measured. Size-fractionated phytoplankton biomass and primary production rates, bacterial abundance, heterotrophic bacterial production rate and size-fractionated microbial community respiration rates were quantified. In addition, inorganic nutrients were also determined.

The experimental set-up consisted of 3 treatments: (1) inorganic nutrient addition: nitrate (NO_3^-), ammonium (NH_4^+) and phosphate (PO_4^{3-}) with final concentrations of 5, 5 and 1 µM, respectively; (2) vitamin B_{12} addition (cyano-cobalamin, Sigma) with a final concentration of 500 pM; and (3) mixed addition: combination of inorganic nutrients and vitamin B_{12} previous additions (NO_3^- 5 µM, NH_4^+ 5 µM, PO_4^{3-} 1 µM and vitamin B_{12} 500 pM), added to triplicate 1 l seawater samples. A no-addition control treatment was also performed in triplicate.

The vitamin B_{12}-analogue added in our bioassays was cyanocobalamin (CN-B_{12}), an inactive form of vitamin B_{12}, which is taken up by eukaryotic phytoplankton and converted into the cofactor forms adenosylcobalamin (Ado-B_{12}) or methylcobalamin (Me-B_{12}) (Helliwell 2017). Previous studies have shown that the half-life of vitamin B_{12} in the illuminated surface ocean is about 4 d (Carlucci et al. 1969, Bertrand et al. 2012), which together with the high biological uptake rates reported (Koch et al. 2012) and the relatively high added concentration, led us to consider that there would be enough vitamin B_{12} available for consumption during the incubation before degradation.

As we lacked previous background data about vitamin B_{12} concentration at our sampling site, we added the same amount as used by Gobler et al. (2007), for comparative purposes. Although the maximum reported vitamin B_{12} concentration in marine coastal waters is 121 pM (Koch et al. 2013), toxic effects of the added vitamin B_{12} can be disregarded, as it is typically added at similar (370 pM) or much higher concentrations (370–400 nM) in phytoplankton culture media (Donald et al. 1997, Koch et al. 2013, Cruz-López & Maske 2016, Cohen et al. 2017).

The experimental units were maintained under natural temperature and solar radiation conditions (Table 1) in an outdoor large tank with continuous circulation of surface seawater during 72 h. The incu-

Table 1. Summary of initial conditions for each experiment. Sampling depth was 2 m. Data were not collected in July and August. SR: solar radiation; Sal: salinity; DIN: dissolved inorganic nitrogen

	Surface initial conditions										
Expt	Temp (°C)	SR (10 kJ m^{-2} d^{-1})	Sal	Chl *a* (µgC l^{-1})	NO_3^- (µM)	NO_2^- (µM)	NH_4^+ (µM)	DIN (µM)	HPO_4^{2-} (µM)	DIN:P	SiO_2 (µM)
Jan	12.7	205	32.2	1.40	9.56	0.85	0.94	11.35	0.33	34.39	12.47
Feb	12.9	737	31.7	1.75	9.58	0.29	0.40	10.27	0.27	38.03	13.73
Mar	13.8	1460	29.3	1.82	4.23	0.11	0.20	4.53	0.19	23.84	6.72
Apr	13.6	2260	33.7	0.56	3.63	0.28	2.11	6.01	0.84	7.15	5.92
May	14.3	2938	35.4	5.33	0.57	0.01	0.04	0.62	0.08	7.75	2.23
Jun	17.6	2292	35.3	0.41	0.39	0.01	0.07	0.47	0.03	15.66	1.25
Sep	20.2	1940	35.2	4.10	0.66	0.10	0.58	1.34	0.12	11.16	3.81
Oct	19.4	949	33.9	2.07	3.41	0.24	0.69	4.34	0.25	17.36	6.23
Nov	16.5	373	33.0	1.21	4.56	0.58	0.28	5.42	0.23	23.56	8.93
Dec	13.3	330	35.3	1.80	5.80	0.34	0.24	6.38	0.41	15.56	5.58

bation time was fixed based on the experimental constraints described in the meta-analysis study of Downing et al. (1999) and on the vitamin B_{12} consumption rates reported by Koch et al. (2013). In addition, previous similar nutrient amendment bioassays carried out in the area demonstrated that 72 h incubations were appropriate to detect responses in both phytoplankton and bacteria, avoiding long incubation times in relatively reduced volumes (see e.g. Martínez-García et al. 2010). In order to follow the changes in the microbial community structure, size-fractionated phytoplankton biomass and bacterial abundance were monitored every 24 h. By the end of the experiment, the volume of culture left in the incubation bags was approximately 80% of the initial volume.

2.3. Size-fractionated phytoplankton biomass (chl *a*)

Phytoplankton biomass was estimated from chl *a* concentration; 100 ml seawater samples were sequentially filtered through 20, 3 and 0.2 µm Whatman polycarbonate (PC) filters. We considered cells retained by the 20 µm filters as belonging to the microplankton fraction, whereas those retained by the 3 and 0.2 µm pore-size filters constituted the nanoplankton and the picoplankton, respectively. After filtration, filters were frozen at −20°C, for at least 12 h, for cell lysis. Pigment extraction was carried out using 90% acetone, and samples were kept in darkness at 4°C overnight. Samples were then analysed using a TD-700 Turner Designs fluorometer calibrated with pure chl *a* standard (Parsons et al. 1984).

2.4. Size-fractionated particulate primary production

Rates of size-fractionated photosynthetic carbon incorporation were assessed using ^{14}C-sodium bicarbonate according to Marañón et al. (2001). Surface seawater samples were transferred to 70 ml acid-cleaned polycarbonate bottles and spiked with 10 µCi (370 kBq) radiolabelled $NaH^{14}CO_3$ (specific activity: 50 mCi mmol^{-1}; Perkin Elmer). Incubation lasted 3 h under *in situ* irradiance and temperature conditions. For each experiment, 1 dark and 3 light bottles were incubated.

At the end of the incubation, samples were sequentially filtered through 20, 3 and 0.2 µm pore-size Whatman PC filters under low-vacuum pressure. Filters were exposed to hydrochloric acid fumes for 12 h, removing any non-incorporated ^{14}C. After removal of inorganic ^{14}C, filters were transferred to scintillation vials, to which 4 ml of scintillation cocktail were added. Radioactivity in each sample was measured with a WALLAC mod. 1409-012 scintillation counter. Daily production rates were estimated by taking into account the variable daylight period (Marañón et al. 2001, Teira et al. 2001).

2.5. Heterotrophic bacterial abundance and biomass

Samples for bacterial abundance determination (1.8 ml) were preserved with 1% paraformaldehyde + 0.05% glutaraldehyde and frozen at −80°C after liquid nitrogen immersion. Analysis of the samples was performed using a Becton Dickinson FACSCalibur flow cytometer equipped with a laser emitting at 488 nm (Gasol & Del Giorgio 2000). Prior to analysis, bacteria were stained with SybrGreen DNA fluorochrome. Total bacterial abundance was determined by their green fluorescence after SybrGreen staining and side scatter (SSC) signals. The empirical calibration between light SSC and cell diameter described by Calvo-Díaz & Morán (2006) was used to estimate biovolume of heterotrophic bacterioplankton. Biovolume was then converted to biomass by using the allometric relationship described by Norland et al. (1993).

2.6. Heterotrophic bacterial production

For each experiment, initial heterotrophic bacterial production was estimated as the incorporation rates of tritiated leucine (L-[4,5-^{3}H]) (Kirchman et al. 1985) modified as described by Smith & Azam (1992). Triplicates of 1 ml seawater samples, plus 2 controls, killed with cold trichloroacetic acid 50% (TCA), were placed into Eppendorf® tubes and inoculated with 40 nM (112 Ci mmol^{-1}) ^{3}H-Leu. Samples were incubated under *in situ* irradiance and temperature conditions. Incubation ended with the addition of 100 µl of cold TCA 50% after 1.5 h. The vials were centrifuged at 10 000 × *g* (10 min at 18°C), washed with 1 ml of cold 5% TCA and centrifuged again (10 000 × *g* for 10 min at 18°C). The samples were counted on a WALLAC mod. 1409-012 scintillation counter after addition of 1 ml of scintillation cocktail.

2.7. Size-fractionated microbial plankton community respiration

In vivo electron transport system (ETS) activity was used as an estimator of the initial community respiration rate. Size-fractionated *in vivo* ETS activity rates were measured using the *in vivo* 2-para(iodophenyl)-3(nitro-phenyl)-5(phenyl)tetrazolium chloride (INT salt) reduction method (Martínez-García et al. 2009). Four dark bottles were filled with a variable volume (100–200 ml, depending on the expected microbial biomass) of the seawater used in the addition experiment. One flask was immediately fixed by adding formaldehyde (2% w/v final concentration) and used as a killed-control. Incubation, performed under *in situ* temperature conditions, lasted 1.5 h and was stopped using formaldehyde. After incubation, samples were sequentially filtered through Whatman PC filters (20, 3, 0.2 µm pore size). In order to transform ETS activity into carbon respiration (R) rates, 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.

2.8. Nutrients

Aliquots for determination of inorganic nutrients (ammonium, nitrate, nitrite, phosphate and silicate) were collected monthly in polyethylene bottles and frozen at −20°C until analysis by standard colorimetric methods with a Bran-Luebbe segmented flow analyser (Hansen & Grasshoff 1983). Dissolved inorganic nitrogen (DIN) was estimated from the sum of nitrate, nitrite and ammonium concentrations.

2.9. Data analysis

To study the effect of different addition treatments on microbial standing stocks, response ratios were calculated as an effect-size metric (Downing et al. 1999, Hedges et al. 1999, Elser et al. 2007). Response ratios were calculated for the addition treatment by dividing the mean value of phytoplankton or bacterial biomass at the end of the experiment (i.e. at 72 h) by the corresponding mean biomass value in the control. A value of 1 implies no response, whereas a value <1 implies a negative response and a value >1 implies growth stimulation after nutrient addition. We conducted *t*-tests to test for response ratios significantly different from 1 in each set of experiments. The p-values were standardized as proposed by Good (1982) in order to overcome the low number of replicates. Pearson correlation coefficients were calculated to estimate the degree to which individual variables were correlated with each other.

3. RESULTS

3.1. Hydrographic conditions

In general, mostly positive upwelling index values were observed from April to September (upwelling season), and negative values from January to March and from October to December (downwelling season) (Fig. 2a). Vertical profiles of temperature (Fig. 2b) and salinity (Fig. 2c) illustrate 4 hydrographic sampling periods. During the downwelling season, winter mixing (January, February and December) and autumn-mixing (October and November) periods were characterized by vertical thermal homogeneity of the water column that was cooler in winter (13–14°C) and warmer in autumn (16–18°C, Fig. 2b). These 2 mixing periods were associated with slight haline stratification, which was stronger in winter than in autumn (Fig. 2c). During the upwelling season we sampled the early spring period, when transition from mixing to stratification occurs (March and April), and the thermal stratification period (May to September) when the thermal gradient promotes the development of a marked thermocline (Fig. 2b).

3.2. Nutrients

DIN surface concentration decreased from a maximum value in January (11.35 µM), to the minimum value reached in late spring (June; <0.5 µM). Concentrations increased thereafter during autumn (Table 1). DIN concentration was significantly and negatively correlated with salinity ($r = -0.86$, $p < 0.01$) and upwelling index ($r = -0.64$, $p = 0.06$). DIN vertical profiles showed maximum values in the surface during winter and autumn (Fig. 2d). In the summer stratification period, when surface DIN was depleted, maximum values were measured at the bottom of the water column. Surface phosphate (HPO_4^{2-}) and silicate (SiO_2) concentrations displayed a temporal variability very similar to that of DIN (Table 1). Maximum surface phosphate concentration occurred in April (0.84 µM, Table 1). Surface waters showed high DIN:P ratios during winter and early spring. The late spring–summer period was

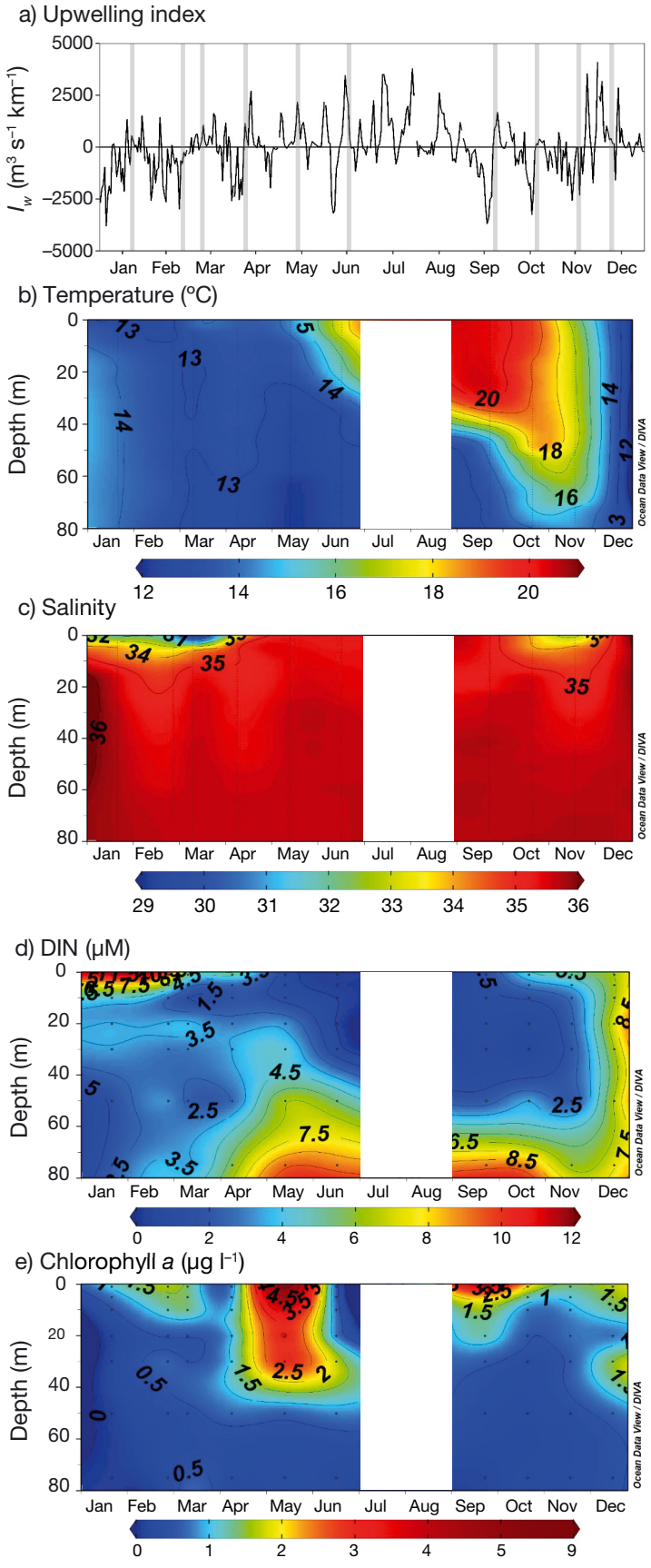

Fig. 2. (a) Upwelling index (I_w), with grey shaded areas indicating the 3 d period before each sampling. Vertical distribution of (b) temperature, (c) salinity, (d) dissolved inorganic nitrogen (DIN) and (e) chlorophyll *a* concentration over the annual cycle. Data were not collected in July and August

characterized by low DIN:P ratios and remained close to the Redfield ratio from September to December, except in November (Table 1).

3.3. Chl *a*, production and microbial community structure

The size structure of the autotrophic microbial community changed over the study period (Fig. 3a). Seasonal changes in total phytoplankton chl *a* biomass were mainly associated with changes in the larger size fraction (>20 µm). Total phytoplankton biomass showed the highest values in May (5.33 µg chl *a* l^{-1}) and September when phytoplankton was dominated by the microplankton fraction (Figs. 2e & 3a). The lowest values were measured in June (0.41 µg chl *a* l^{-1}), when picoplankton was the most abundant size fraction and DIN concentrations were the lowest registered throughout the study period. Nanoplankton was the dominant fraction in winter and autumn when the phytoplankton biomass remained fairly constant. Daily total primary production rates ranged from 147.7 µgC l^{-1} d^{-1} in May, to a minimum value of 8.6 µgC l^{-1} d^{-1} in June (Fig. 3b). The observed seasonal trend as well as the relative contribution of the different phytoplankton size fractions to total primary production paralleled that found in chl *a* concentration in all experiments except in September, November and December when the phytoplankton size fraction dominated in terms of biomass but not in primary production. Community respiration rates also exhibited a noticeable variability throughout the year, ranging from 8.3 to 58.1 µgC l^{-1} d^{-1} in December and May, respectively (Fig. 3c). Bacterial biomass ranged from 2.7 µgC l^{-1} in January to 21.4 µgC l^{-1} in October, coinciding with the minimum and maximum bacterial production rates of 2.5 and 7.4 µgC l^{-1} d^{-1}, respectively (Fig. 3e,f). Ratios of bacterial

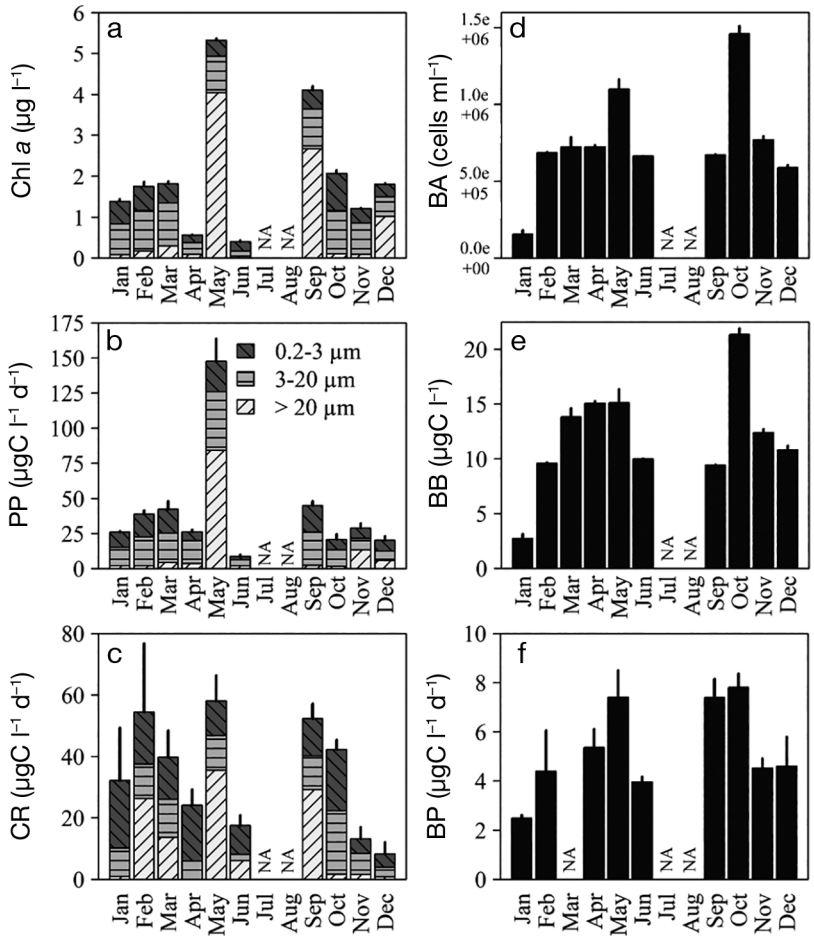

Fig. 3. Initial biological conditions at the sampling stations. (a) Size-fractionated chl *a*. (b) Size-fractionated primary production (PP). (c) Size-fractionated community respiration (CR) estimated from *in vivo* electron transport system (ETS) activity. (d) Heterotrophic bacterial abundance (BA). (e) Heterotrophic bacterial biomass (BB). (f) Bacterial production (BP). NA: data not available

biomass production to primary production can be assumed as a proxy of the relative magnitude of the dissolved organic matter flux between phytoplankton and bacteria. Values of this ratio ranged from 5 to 20% except in June (45%) and October (37%), when primary production was low and was dominated by small phytoplankton (Fig. 3b,f). A minimum value was reached in May (5%) during the bloom of >20 µm phytoplankton cells.

3.4. Autotrophic responses to inorganic nutrients and vitamin B_{12}

The temporal evolution of the autotrophic biomass in the control treatments showed different patterns, either remaining rather stable (June), increasing (January to April and October to December) or showing a slight decrease (May and September) (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m626p029_supp.pdf). Inorganic nutrients frequently stimulated total phytoplankton biomass (80% of the experiments; Fig. 4a). Generally, inorganic nutrients limited the micro- and pico-phytoplankton size classes during the stratification period and, to a lesser extent, during warmer autumn mixing periods (Fig. 4c,e). The nanophytoplankton size fraction responded in 90% of the experiments (Fig. 4d). The magnitude of the response to inorganic nutrients progressively increased from winter to a maximum value reached in late spring (May), thereafter gradually decreasing until the end of the year (Fig. 4d).

In 3 out of 10 incubation experiments (March, April and October) the combined addition of vitamin B_{12} and inorganic nutrients resulted in a significant increase in phytoplankton biomass above that measured in the inorganic nutrient treatment ($p < 0.01$) (Fig. 4a). Given that in March and October the responses to inorganic nutrients alone were also significant ($p < 0.05$), this response pattern indicates a secondary vitamin B_{12} limitation (Moore et al. 2013). By contrast, in April, the addition of vitamin B_{12} alone also caused a significant effect, suggesting single vitamin B_{12} limitation. All phytoplankton size classes showed a vitamin B_{12} single or secondary limitation in at least 1 experiment (Fig. 4c–e). Microphytoplankton biomass (>20 µm) increased by 40% after the addition of vitamin B_{12} alone in March (Fig. 4c), causing a sharp change in phytoplankton size structure, as the other size fractions did not significantly respond to the enrichment. Nevertheless, this single vitamin B_{12} limitation of microphytoplankton was not reflected in total phytoplankton biomass (Fig. 4a). Picophytoplankton, on the other hand, was co-limited by inorganic nutrients and vitamin B_{12} in March (Fig. 4e), as a significant response only occurs when both inorganic nutrients and vitamin B_{12} are jointly added (Arrigo 2005, Saito et al. 2008, Moore et al. 2013). During the April and December experiments, when vitamin B_{12} and inorganic nutrients

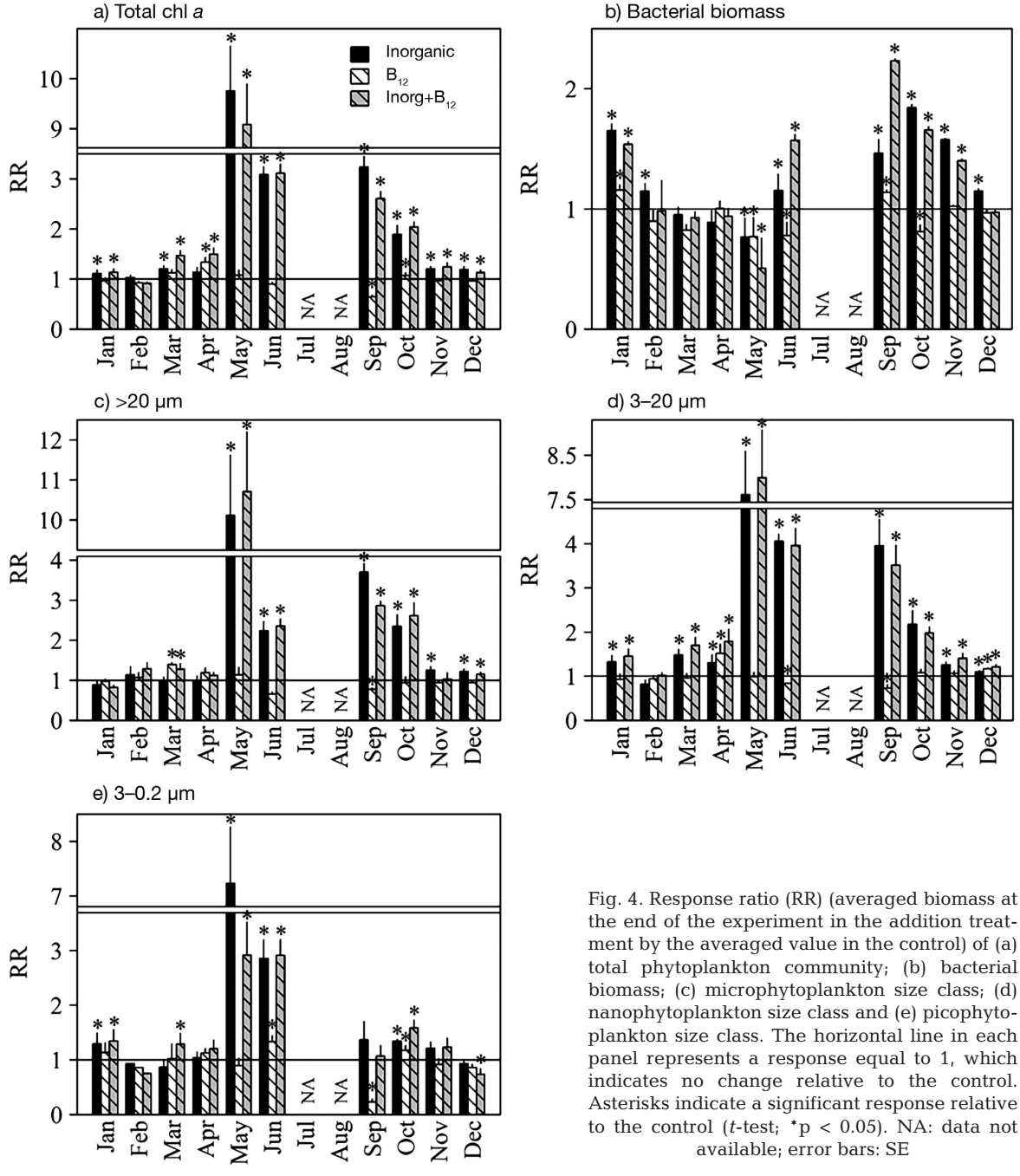

Fig. 4. Response ratio (RR) (averaged biomass at the end of the experiment in the addition treatment by the averaged value in the control) of (a) total phytoplankton community; (b) bacterial biomass; (c) microphytoplankton size class; (d) nanophytoplankton size class and (e) picophytoplankton size class. The horizontal line in each panel represents a response equal to 1, which indicates no change relative to the control. Asterisks indicate a significant response relative to the control (*t*-test; *p < 0.05). NA: data not available; error bars: SE

were added together, nanophytoplankton biomass was enhanced above each individual treatment (Fig. 4d). In those experiments, nanophytoplankton was primarily limited by B_{12}, as the responses to individual vitamin additions were higher (50 and 17%, respectively) than for inorganic nutrients (30 and 10%, respectively) with respect to the control. Nanophytoplankton secondary limitation by vitamin B_{12} was noticeable in January, April and December (Fig. 4d), although this response pattern was not reflected in the total phytoplankton biomass in January and December since its contribution to the entire community was very low by the end of the incubation in all treatments (data not shown). A similar pattern of interaction among vitamin and inorganic nutrients was observed in the pico-phytoplankton size class during the October experiment (Fig. 4e) when inorganic nutrients were the primary limiting factors of

phytoplankton growth and the vitamin B_{12} secondary limitation was noticeable both in picophytoplankton and total phytoplankton biomass (Fig. 4a).

Finally, the addition of vitamin B_{12} depressed phytoplankton biomass in 1 out of the 10 experiments, resulting in a significant decrease in the biomass of all phytoplankton size classes (Fig. 4a,c–e).

3.5. Heterotrophic bacterial responses to inorganic nutrients and vitamin B_{12}

Bacterial biomass continuously increased until 72 h in all treatments in January, February and April, whereas this variable reached maximum values at 48 h and declined afterwards in March, May, September and October (Fig. S2). In June, November and December, bacterial biomass remained more or less constant (with small variations) after the maximum values measured at 48 h when inorganic nutrients were added alone or in combination with vitamin B_{12}, whereas it decreased in the control and vitamin B_{12} addition treatments (Fig. S2). The bacterial response ratios were markedly lower than those calculated for the autotrophic community (Fig. 4b), showing a clear temporal variability. Significant responses of bacterial biomass to inorganic nutrient additions were observed in 70% of the experiments. The magnitude of these response ratios decreased from January to May, when the response ratio was significantly lower than 1 (Fig. 4b; Fig. S2e). This ratio then increased progressively from June to October, when a ca. 2-fold increase was observed, and then decreased towards the end of the year (Fig. 4b). As previously reported for phytoplankton, bacterial biomass increased after the addition of vitamin B_{12}, alone or in combination with inorganic nutrients, in 3 out of 10 experiments. Vitamin B_{12} alone promoted the increase in bacterial biomass by 15% in January and September and resulted in a 20% decrease in June and October (Fig. 4b). However, the combined addition of inorganic nutrients and vitamin B_{12} resulted in a significant increase in bacterial biomass, greater than that observed after the addition of inorganic nutrients alone (ca. 35% in June and 50% in September; Fig. 4b). The biomass increase in the combined treatment compared to the control was above 2-fold in September (Fig. 4b).

4. DISCUSSION

Monthly amendment experiments conducted over an annual cycle revealed that phytoplankton and bacterial inorganic nutritional constraints and growth limitation by vitamin B_{12} vary seasonally in this coastal upwelling ecosystem (Fig. 5). Inorganic nutrients severely limited phytoplankton growth in late spring and summer and, to a much lesser extent, controlled phytoplankton and bacterial biomass yields in autumn and winter. This finding is consistent with the temporal variability in inorganic nutrient concentrations observed during the sampling period (Fig. 2d, Table 1). The addition of vitamin B_{12}, either alone (single limitation) or combined with inorganic nutrients (secondary limitation), stimulated the production of phytoplankton biomass in early spring (March and April) and autumn (October) and bacterial biomass in winter (January) and summer (June and September) (Fig. 5). The phytoplankton response pattern to vitamin B_{12} supply likely reflects temporal changes in its availability and demand, and

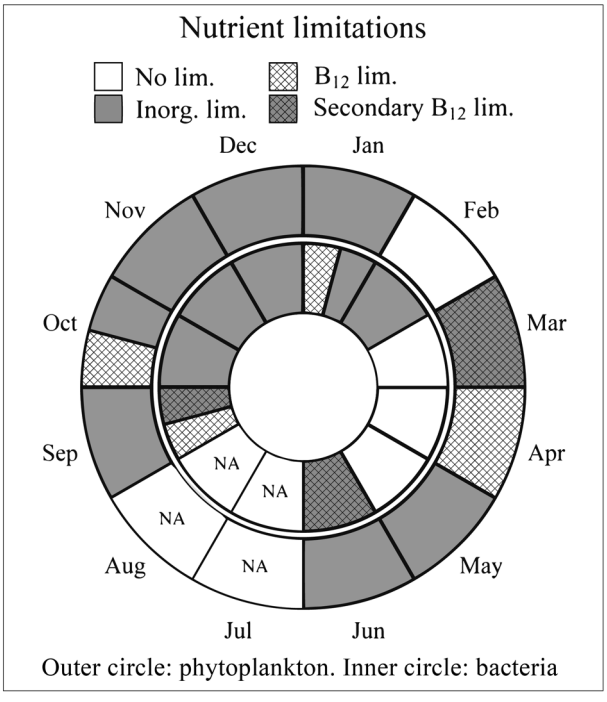

Fig. 5. Nutritional limitations over an annual cycle for phytoplankton (outer circle) and heterotrophic bacteria (inner circle). White: no growth limitation for any of the nutrients added in this study. Grey: single limitation by inorganic nutrients (N and P). White with pattern: single limitation by vitamin B_{12}. Grey with pattern: secondary vitamin B_{12} limitation, meaning the response to the combined treatment is higher than the response when inorganic nutrients are added alone. Months that are divided into 2 sections indicate that 2 types of nutrient limitation were observed (e.g. 2 single limitations by individual nutrients, or single limitation by individual nutrients combined with a secondary vitamin B_{12} limitation). Note that the secondary B_{12} limitation occurs only when inorganic nutrients are also limiting. NA: data not available

stresses the potential of this growth factor to eventually restrict inorganic nutrient drawdown in this productive ecosystem.

4.1. Microbial responses to nutrients and vitamin B_{12} in the winter-mixing period

The bacterial response to inorganic nutrient additions observed in January and February (Figs. 4b & 5) is likely related to the very high DIN:P ratios (Table 1), which suggest P limitation. The intense precipitation measured during this period as indicated by the low surface seawater salinities (Fig. 2c), and the associated inputs of matter from continental sources, would explain the elevated DIN:P ratio and the observed relative P deficiency (Seitzinger et al. 2010). Previous studies suggest that riverine inflow is not a relevant source for vitamin B_{12} in marine coastal ecosystems (Gobler et al. 2007, Tovar-Sánchez et al. 2016). In December, when DIN was still high and the DIN:P ratio was close to the Redfield ratio, both phytoplankton and bacteria responded to inorganic nutrients, likely due to nutrient depletion at the end of the incubation. Previous studies have measured active B_{12} uptake by heterotrophic bacteria (Koch et al. 2012), suggesting that these microorganisms can take advantage of the presence of this compound in the environment. The positive response of heterotrophic bacteria to vitamin B_{12} addition observed in January, when bacterial production was the lowest measured in this study (Fig. 3f), suggests that under growth-limiting conditions, the external supply of vitamin B_{12} could reduce the energy costs associated with its synthesis (Jaehme & Slotboom 2015).

4.2. Microbial responses to nutrients and vitamin B_{12} in early spring

During the early spring period, when inorganic nutrient concentrations were relatively high and bacterial abundance relatively low (Table 1, Fig. 3d), the significant secondary limitation of phytoplankton by vitamin B_{12} (i.e. the response to the combined treatment was higher than the response to inorganic nutrients alone) observed in March ($p = 0.016$) was followed by a single vitamin B_{12} limitation of phytoplankton in April ($p = 0.03$, Figs. 4a & 5). The phytoplankton response observed in March suggests an increase in vitamin B_{12} demand in the presence of extra inorganic nutrients that cannot be sustained by the bacterial community present. A similar interaction between nitrate and vitamin B_{12} amendments was observed by Gobler et al. (2007) in a coastal ecosystem, which they attributed to the increasing demand of vitamin B_{12} by phytoplankton when DIN levels are high. On the other hand, the single vitamin B_{12} limitation observed in April, when inorganic nutrients were high, suggests an insufficient stock of this organic micronutrient as the productive season progressed. Consequently, the probable progressive exhaustion of vitamin B_{12} at the beginning of the productive season and its subsequent effect on the phytoplankton community might have potential implications for the total amount of organic matter photosynthetically produced during the spring phytoplankton bloom.

Enhanced phytoplankton biomass associated with vitamin B_{12} additions is consistent with the results obtained in other coastal zones and reinforces the idea that increasing bioavailability of vitamin B_{12} may favour phytoplankton when inorganic nutrients are present (Sañudo-Wilhelmy et al. 2006, Gobler et al. 2007).

The response pattern described thus far suggests the presence of vitamin B_{12} auxotrophs and moderate to low vitamin B_{12} availability at the beginning of the productive season. The phytoplankton responses to vitamin B_{12} amendment could also reflect a methionine synthase shift, from vitamin B_{12}-independent to vitamin B_{12}-dependent, in taxa possessing the vitamin B_{12}-independent methionine synthase (MetE) gene (Bertrand et al. 2013, Helliwell et al. 2014). Vitamin B_{12} concentrations typically range from subpicomolar quantities to up to 121 pM, ca. 4-fold below our experimental addition (Panzeca et al. 2009, Suárez-Suárez et al. 2011, Sañudo-Wilhelmy et al. 2012, Bonnet et al. 2013, Koch et al. 2013). In our sampling area, vitamin B_{12} concentrations recently measured by our research group in shelf waters near the station sampled here, showed temporal variability from winter to summer, exhibiting minimum, subpicomolar values in spring and maximum values (up to 2.7 pM) in winter (authors' unpublished data), which is consistent with the responses to vitamin B_{12} in early spring. Previous studies in temperate coastal areas also found temporal variability in vitamin B_{12} concentration, reporting low levels of vitamin B_{12} following phytoplankton blooms (Gobler et al. 2007, Koch et al. 2012, 2013). Temporal variability in vitamin B_{12} concentration directly correlates with bacterial abundance (Bertrand et al. 2007). The moderate bacterial abundance registered in early spring (Fig. 3d) reinforces the hypothesis of low vitamin concentrations during this period. Recent studies describing

the existence of pseudocobalamin, a vitamin B_{12}-analogue produced by cyanobacteria where the DMB group is replaced by adenine (Helliwell et al. 2016, Heal et al. 2017), challenge the connection between bacterial abundance and the occurrence of vitamin B_{12}. However, cyanobacteria are not very abundant in our sampling area, contributing <5% to total chl *a* (Rodríguez et al. 2006), which suggests that pseudocobalamin concentrations are not likely to be relevant.

4.3. Microbial responses to nutrients and vitamin B_{12} in the thermal stratification period

During the stratification period (May to September), inorganic nutrient concentrations reached their minimum. Under a situation of nutrient depletion, the nutritional constraints on phytoplankton shifted from vitamin B_{12} limitation to inorganic nutrient limitation (Figs. 4a & 5). In the algal bloom sampled in May, large phytoplankton cells probably outcompeted bacteria in the uptake of inorganic nutrients, resulting in the overall negative responses observed for bacteria (Fig. 4b). Inorganic nutrient limitation of phytoplankton biomass has been previously observed in this coastal region, when increasing thermal stratification results in a rapid exhaustion of nutrients in the surface layer (Casas et al. 1999, Martínez-García et al. 2010, 2015). The lack of phytoplankton response in the presence of inorganic nutrients when vitamin B_{12} was supplied suggests that ambient vitamin B_{12} concentration was sufficient to cover phytoplankton demands during the experimental incubation. In this regard, Koch et al. (2012) found high concentrations of vitamin B_{12} during summer in a temperate coastal site, likely associated with organic matter decomposition (Gobler et al. 2007).

During this thermal stratification period, bacteria showed an apparent secondary limitation by vitamin B_{12} in June and September (Figs. 4b & 5) suggesting that the external supply of vitamin B_{12} could be stimulating vitamin B_{12} auxotrophic bacteria (Sañudo-Wilhelmy et al. 2014) or promoting vitamin-producing bacteria under nutrient-limiting conditions (Koch et al. 2012, Jaehme & Slotboom 2015). When inorganic nutrient concentrations are low (Table 1), competitive interactions between phytoplankton and bacteria may explain the opposite responses observed (Fig. 4a,b). Since inorganic nutrients are the primary limiting nutrients of both microbial compartments in this period, vitamin B_{12} enrichment alone would not result in higher growth rates but could change the competitive balance between phytoplankton and heterotrophic bacteria. A negative response was observed in bacterial biomass (in May and June) and in chl *a* (in September) after the addition of vitamin B_{12}. A plausible explanation for these negative responses may be the stimulation of grazers or bacterivores, such as heterotrophic/mixotrophic dinoflagellates or mixotrophic cryptophyceans, upon vitamin B_{12} addition alone. Nevertheless, the contrasting responses observed in phytoplankton and bacteria after the vitamin B_{12}-only addition in September suggest a competitive interaction between both compartments. Bacteria might be taking advantage of the presence of this micronutrient outcompeting phytoplankton for the uptake of the limiting inorganic nutrients. Moreover, these contrasting responses reveal a direct effect of the vitamin B_{12} on bacteria rather than the effect of other compounds produced by vitamin B_{12}-stimulated phytoplankton as suggested in previous studies (Koch et al. 2011).

4.4. Microbial responses to nutrients and vitamin B_{12} in the autumn mixing period

The hydrographic context of the 2 experiments performed in autumn (October and November) was characterized by a warm mixed water column (Fig. 2b) with intermediate DIN and phosphate concentrations (Table 1), moderate values of chl *a*, low rates of primary production and maximum bacterial abundance and production in October (Fig. 3a,b,d,f). Both microbial compartments responded to inorganic nutrient additions (Fig. 5), although a slight temporal lag occurs between the phytoplankton and bacterial responses (Figs. S1.2 & S2). This is probably related to the stimulation of bacterioplankton biomass by the concomitant increase of phytoplankton-derived dissolved organic carbon, in agreement with the previously reported primary organic carbon limitation of heterotrophic bacteria in this area (Martínez-García et al. 2010, Teira et al. 2016). The magnitude of the vitamin B_{12} secondary limitation of phytoplankton in October was relatively low (ca. 8% increase in the combined treatment compared to the inorganic nutrient treatment) when compared to spring, and was driven by picoeukaryotes (Figs. 4e & 5). Experimental and genomic data suggest that some eukaryotic picophytoplankton species might be vitamin B_{12} auxotrophs (Helliwell et al. 2011, Koch et al. 2011, 2012, Sañudo-Wilhelmy et al. 2014), including the genus *Ostreococcus* (Helliwell 2017), which dominates eukaryotic picophytoplankton at the sampling

site (Hernández-Ruiz et al. 2018). The fact that only picophytoplankton responded to vitamin B_{12} additions might be related to the presence of vitamin B_{12}-binding proteins exuded by diatoms and other algae, which can make the ambient vitamin inaccessible to other species (Droop 1968, Gobler et al. 2007). These vitamin B_{12}-binding proteins have been hypothesized to participate in vitamin B_{12} transport to the cell (Davies & Leftley 1985), possibly interacting with the cobalamin acquisition protein 1 (CBA1), which has been found exclusively in the stramenopile lineage (Bertrand et al. 2012). The current limited knowledge of the mechanisms and specificity of cobalamin transport systems in phytoplankton strongly constrains our ability to interpret the response of primary producers to vitamin B_{12} availability (Helliwell 2017).

4.5. Concluding remarks

The temporal sequence of microbial plankton nutrient limitations illustrated in Fig. 5 is in agreement with the seasonal cycle of inorganic nutrients in the study area (Nogueira et al. 1997) and with the vitamin B_{12} seasonal cycle described in the literature in other coastal ecosystems (Koch et al. 2012). Our results suggest that at the very first stage of the productive season off the NW Iberian Peninsula, the concentration of vitamin B_{12} might become limiting for phytoplankton growth, and, consequently, an eventual external input of anthropogenic inorganic nutrients during this period might not result in enhanced primary production rates. As autotrophic activity increases, inorganic nutrients reach minimum values in summer and dissolved organic matter also increases, promoting bacterial production by the end of the productive period. Levels of dissolved vitamin B_{12} are thus expected to increase in summer and autumn due to the activity of the prokaryotic organisms and be sufficient to maintain the phytoplankton community. Similar patterns are expected in other productive coastal areas where a time lag between phytoplankton and bacterial production typically occurs.

In conclusion, we have shown that the response of phytoplankton biomass to inorganic nutrient loads may be dependent on the presence of vitamin B_{12}. Our findings are relevant in the context of future global change scenarios, where reactive nitrogen is expected to increase in coastal regions. The impact of nutrient enrichment on ecosystem productivity must therefore be evaluated by adopting a multivariate approach, including the availability of growth factors, such as B vitamins.

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