Spatial and temporal variability in the response of phytoplankton and bacterioplankton to B-vitamin amendments in an upwelling system

Vanessa Joglar[1*], Antero Prieto[1], Esther Barber-Lluch[1], Marta Hernández-Ruíz[1], Emilio Fernandez[1] and Eva Teira[1]

[1] Departamento Ecoloxía e Bioloxía Animal, Universidade de Vigo, Campus Lagoas-Marcosende 36310 Vigo, Spain

*Correspondence to: Vanessa Joglar +34 986 818790 (vjoglar@uvigo.es)

Abstract. We evaluated the temporal (inter-day and inter-season) and spatial variability in microbial plankton responses to vitamins B12 and B1 supply (also in combination with inorganic nutrients) in coastal and oceanic waters of the northeast Atlantic ocean. Phytoplankton and, to a lesser extent, bacteria were strongly limited by inorganic nutrients. Inter-day variability in microbial plankton responses to B-vitamins was unimportant, suggesting that B-vitamins availability was controlled by factors operating at larger temporal scales. Phytoplankton and bacteria positively responded to B-vitamin amendments in 13% and 21%, respectively, of the 216 cases (36 experiments x 6 treatments). Negative responses represented 21% for phytoplankton and 26% for bacteria. Most positive responses were produced by treatments containing either B12 alone or B12 combined with B1 in oceanic waters, which was consistent with the significantly lower average vitamin B12 ambient concentrations compared to that in the coastal station. Growth stimulation by B1 addition was more frequent on bacteria than in phytoplankton, which is coherent with their widespread dependence on exogenous sources of this growth factor. Negative responses to B-vitamins were generalized in coastal waters in summer, and were associated to a high contribution of Flavobacteriales to the prokaryote community. This observation suggests that the external supply of B12 and/or B1 may promote negative interactions between microbial components when B-vitamin auxotrophs are abundant. The microbial response patterns to B12 and/or B1 amendments were significantly correlated with changes in the prokaryotic community composition, highlighting the pivotal role of prokaryotes in B-vitamins cycling in marine ecosystems.

1 Introduction

Phytoplankton accounts for almost half of the global net primary production (Field et al., 1998) and may eventually cause toxic episodes entailing human health problems and large economic losses (Hallegraeff, 1993; van Dolah et al., 2001). Recent emerging evidence

38 suggests the role of biologically active organic compounds, such as B-vitamins, on the
39 control of marine productivity in both coastal and oceanic waters (Panzeca et al., 2006;
40 Bertrand et al., 2007; Gobler et al., 2007; Koch et al., 2011; Browning et al., 2017, 2018).
41 B-vitamins act as cofactors for enzymatic reactions and are involved in many important
42 metabolic pathways (Madigan et al., 2005; Koch et al., 2011; Monteverde et al., 2017).
43 Vitamin B12 (B12 herein), which is exclusively synthesized by some bacteria and archaea
44 (Roth et al., 1996; Martens et al., 2002; Warren et al., 2002), acts as a cofactor of three
45 enzymes in eukaryotes (methionine synthase, methylmalonyl-coA mutase and
46 ribonucleotide reductase type II) (Helliwell et al., 2011; Bertrand and Allen, 2012). In
47 comparison, over 20 different B12-dependent enzymes are found in bacteria (Roth et al.,
48 1996), making B12 critically important also for these organisms. Vitamin B1 (B1 herein)
49 plays a pivotal role in intermediary carbon metabolism and is a cofactor for a number of
50 enzymes involved in primary carbohydrate and branched-chain amino acid metabolism
51 (Croft et al., 2006).

52 Most eukaryote phytoplankton species are auxotrophs for one or more B-vitamins,
53 consequently requiring an exogenous supply of these molecules (Carlucci and Bowes,
54 1970; Haines and Guillard, 1974; Croft et al., 2005; Tang et al., 2010; Helliwell et al.,
55 2011; Bertrand and Allen, 2012). Moreover, genomic data also indicate widespread B-
56 vitamins auxotrophy among many bacterial taxonomic groups (Sañudo-Wilhelmy et al.,
57 2014; Paerl et al., 2018), which implies that phytoplankton and bacteria may eventually
58 compete for the acquisition of these compounds (Koch et al., 2012). Auxotrophic
59 microorganisms may acquire the required vitamins from the environment or through
60 biotic interactions with prototrophic (biosynthetically competent) microorganisms
61 (Droop, 2007; Grant et al., 2014; Kazamia et al., 2012). A well-known example is the

mutualistic interaction between B12-dependent phytoplankton and bacteria (Croft et al., 2005; Amin et al., 2012; Cooper and Smith, 2015).

Even though B-vitamins appear to be important and potentially limiting factors for microbial plankton, our understanding of B-vitamins cycling in the ocean is largely limited by the complex and still evolving analytical methodology for its quantification in natural waters (Okbamichael and Sañudo-Wilhelmy, 2004, 2005; Suffridge et al., 2017). Sañudo-Wilhelmy et al. (2012) found extensive areas of coastal waters with close to undetectable B12 concentrations, suggesting that microbes might be well adapted to drive under limiting conditions for this growth factor.

The factors limiting phytoplankton and bacterial growth in marine ecosystems are known to vary over different spatial and temporal scales (Cullen et al., 1992; Arrigo, 2005; Church, 2008; Saito et al., 2008; Martínez-García et al., 2010a, 2010b; Moore et al., 2013), in accordance with the dynamic nature of microbial communities (Pinhassi et al., 2003; Pommier et al., 2007; Fuhrman et al., 2008; Carlson et al., 2009; Hernando-Morales et al., 2018; Hernández-Ruiz et al., 2018). Compared to mineral nutrient and trace elements, much less is known about B vitamin limitation and its spatial and temporal variability in marine ecosystems.

Some studies have shown enhanced phytoplankton biomass associated to B12 amendments in both temperate coastal and polar waters (Bertrand et al., 2007; Gobler et al., 2007; Koch et al., 2011; Koch et al., 2012). The simultaneous effect of vitamin B12 supply on both phytoplankton and bacteria has been barely explored (Koch et al., 2011, Barber-Lluch et al., 2019). To our knowledge, the effect of B1 amendments on marine natural microbial plankton community succession has been only assessed by Gobler et al. (2007), who suggested that high concentration of B-vitamins, associated with high bacterial abundance, caused an increase in auxotrophs, mostly dinoflagellates.

The Ría de Vigo (NW Spain) is a coastal embayment affected by intermittent upwelling of subsurface cold and inorganic nutrient-rich water from March to September and the downwelling of open ocean surface water from October to March (Fraga, 1981; Barton et al., 2015). In addition to this seasonality, fluctuations of wind patterns in the area generate upwelling and downwelling events occurring within each season (Alvarez-Salgado et al., 1993; Figueiras et al., 2002). A recent study by Barber-Lluch et al. (2019) at a shelf station off the Ría de Vigo (NW Spain) showed monthly variation in the response of phytoplankton and bacteria to nutrient and/or B12 additions in surface waters, likely related to variation in the ambient concentration of B12 and the taxonomic community composition. Unfortunately, the role of these factors on the microbial response to the amendments were not specifically assessed by these authors.

Within this context, the aim of our study was to explore spatial (horizontal and vertical) and temporal (inter-day and inter-season) variability patterns in B12 and B1 vitamin limitation in relation to the prevailing initial abiotic (e.g., nutrient and B12 concentrations) and biotic (eukaryote and prokaryote community composition) conditions in this productive ecosystem. We conducted a total of 36 microcosm bioassays in February, April, and August 2016 to evaluate the response of heterotrophic bacteria and phytoplankton biomasses to the addition of B12 and/or B1.

Considering that a large fraction of eukaryotic phytoplankton and bacterial taxa require exogenous B-vitamins and considering the different requirements and capabilities to synthetize B-vitamins by different microbial taxa, we hypothesize that microbial community composition play a relevant role in explaining B-vitamins limitation patterns in microbial plankton.

2 Methods

2.1 Experimental design

Thirty-six enrichment experiments were performed in the upwelling system near Ría de Vigo on board "B/O Ramón Margalef" in three different oceanographic cruises (ENVISION I, II & III) conducted in 2016. Two different locations of the East Atlantic Ocean, one coastal station (st3) (42º N, 8.88º W) and one oceanic station (st6) (42º N, 9.06º W) (Fig. 1), were sampled during three different seasons aimed to cover a wide range of initial hydrographic and ecological conditions. The 10-day cruises were conducted in February (ENVISION I), coinciding with the spring bloom, and April (ENVISION II) and August (ENVISION III) during the early and late summer upwelling, respectively. During each cruise, 12 enrichment experiments were carried out on board, 3 experiments in each station (3a, 3b & 3c and 6a, 6b & 6c, respectively) with water from two different depths. Water was collected using 20 l Niskin metal-free bottles. Surface and sub-surface chlorophyll maximum (SCM) samples were taken at 5 m and at the maximum fluorescence depth, between 10 m and 50 m according to the CTD data, respectively (Fig. 2). We failed to sample the SCM on two occasions, due to large vertical displacements between the downward and the upward casts. Vertical profiles of temperature, salinity and chlorophyll fluorescence were obtained using a regular stainless CTD-rosette down to 60 m in the coastal station and to 200 m in oceanic station. Samples for phytoplankton and bacterial biomasses, dissolved nutrient concentration, including vitamin B12, and microbial plankton community were collected at the beginning of each experiment. Daily upwelling index (UI) values were computed by the Instituto Español de Oceanografía (www.indicedeafloramiento. ieo.es/) in a 2º x 2º geostrophic cell centered at 42 ºN , 10 ºW, using data from atmospheric pressure at sea level, derived from the WXMAP model (Gonzalez-Nuevo et al., 2014).

Seawater samples were gently pre-filtered through a 200 µm mesh to exclude large zooplankton in order to ensure good replicability and collected into a 20 l acid-cleaned polyethylene carboy. It is important to note that incidental trace-metal contamination could have occurred during water collection. Following sample collection, 300 ml PAR and UVR transparent, sterile, and non-toxic (whirl-pak) bags were filled and nutrients were added establishing eight different enrichment treatments as follows: (1) control treatment (C): no nutrients added; (2) inorganic nutrient treatment (I): 5 µM nitrate (NO_3^-), 5 µM ammonium (NH_4^+), 5 µM silicate (SiO_4^{2-}) and 1 µM phosphate (HPO_4^{2-}); (3) vitamin B12 (Sigma, V2876) treatment: 100 pM; (4) vitamin B1 (Sigma, T4625) treatment: 600 pM); (5) Inorganic nutrients and vitamin B12 (I+B12) treatment; (6) Inorganic nutrients and vitamin B1 (I+B1) treatment; (7) vitamins B12 and B1 (B12+B1) treatment and (8) Inorganic nutrients with vitamins B12 and B1 (I+B12+B1) treatment. Inorganic nutrients were added to avoid that inorganic nutrient limitation masked the responses to B vitamins. Each treatment had 3 replicates resulting in 24 whirl-pack bags per experiment. To assess short-term effects of nutrient inputs, experimental bags were incubated on-deck during 72 h under natural light conditions. In-situ temperature and light were reproduced by submerging the bags in tanks connected to the surface-water pump system, and covered with screens simulating the light intensity at the sampling depth.

2.2 Chlorophyll-*a*

Chlorophyll-*a* (Chl-*a*) concentration was measured at time-zero and after 72 h incubation as a phytoplankton biomass proxy. 300 ml of water samples were filtered through 0.2 µm polycarbonate filters and frozen at -20ºC until further analysis. Chl-*a* was extracted with 90 % acetone and kept in darkness at 4ºC overnight. Fluorescence was determined with a TD-700 Turner Designs fluorometer calibrated with pure Chl-*a* (absorption coefficient at 665 nm = 12.6) standard solution.

160 **2.3 Flow cytometry**

161 Samples for heterotrophic bacteria abundance quantification (2 ml) were preserved with
162 1 % paraformaldehyde + 0.05 % glutaraldehyde (final concentrations). Samples were
163 incubated 20 min for the fixative to act on cells and frozen at -80ºC after 15 min.
164 immersion in liquid nitrogen. Abundance of heterotrophic bacteria was determined using
165 a FACSCalibur flow cytometer equipped with a laser emitting at 488nm. Samples were
166 stained with SYBR Green DNA fluorochrome, and bacterial abundance was detected by
167 their signature of side scatter (SSC) and green fluorescence as described by Gasol and
168 Del Giorgio, 2000. The empirical calibration between light side scatter (SSC) and cell
169 diameter described by Calvo-Díaz and Morán (2006) were used to estimate the biovolume
170 (BV) of bacterioplankton cells. BV was converted into biomass by using the allometric
171 factor of Norland (1993: fg C $cell^{-1} = 120 \times BV^{0.72}$) for the coastal experiments and using
172 the open ocean conversion factor for the oceanic experiments (fg C $cell^{-1} = 350 \times BV$).

173 **2.4 Nutrients**

174 Aliquots for inorganic nutrient determinations (ammonium, nitrite, nitrate, phosphate,
175 and silicate) were collected in first place and directly from the Niskin bottle in order to
176 avoid contamination. Polyethylene bottles 50 ml precleaned with HCl 5 % were filled
177 with the sample employing free-contamination plastic gloves and immediately frozen at
178 –20°C until analysis by standard colorimetric methods with a Bran-Luebbe segmented
179 flow analyzer (Hansen and Grasshoff 1983). The detection limit was 0.1 µmol l^{-1} for
180 nitrate, 0.02 µmol l^{-1} for nitrite and phosphate and 0.05 µmol l^{-1} for ammonium and
181 silicate. Dissolved inorganic nitrogen (DIN) concentration was calculated as the sum of
182 the ammonium, nitrite and nitrate concentrations.

183 **2.5 Vitamin B12**

184 Seawater samples for dissolved vitamin analysis were taken at surface and SCM depth in
185 the coastal and oceanic station on the first, third and fifth (or sixth) day of each cruise
186 (Table S1 in the Supplement). Samples were filtered through 0.2 µm sterivex filters and
187 frozen at -20ºC until further analysis. Samples (1 l) were preconcentrated using a solid-
188 phase extraction with a C18 resin (Bondesil C18, Agilent) at pH 6.5 and rate of 1ml/min.
189 Elution was performed with 12 ml of methanol (MeOH) LCMS grade that was removed
190 via evaporation with nitrogen in a Turbovap. Residual water behind (300-500 µl) was
191 frozen at -20ºC until further analysis using liquid chromatography coupled to mass
192 spectrometry system.

193 The concentrate was filtered again through a cellular acetate membrane 0.2 µm
194 (Phenomenex) prior to the analysis. Ultra Performance Liquid Chromatography tandem
195 Mass Spectometry 3Q (UPLC-MS/MS) methodology was adapted from Sañudo-
196 Wilhelmy et al (2012), Heal et al. (2014) and Suffridge et al (2017). Detection and
197 quantification of dissolved vitamin B12 (cyanocobalamin and hydroxocobalamin) was
198 conducted using an Agilent 1290 Infinity LC system (Agilent Technologies, Waghaeusel-
199 Wiesental, Germany), coupled to an Agilent G6460A triple quadrupole mass
200 spectrometer equipped with an Agilent Jet Stream ESI source. The LC system used a C18
201 reversed-phase column (Agilent Zorbax SB-C18 Rapid Resolution HT (2.1 × 50 mm, 1.8
202 µm) with a 100 µl sample loop. Agilent Technologies software was used for data
203 acquisition and analysis. Chromatographic separation was performed using MeOH and
204 water LCMS grade, both buffered to pH 5 with 0.5 % acetic acid, as mobile phases in a
205 15 minutes' gradient. Gradient starting at 7 % MeOH for 2 min, changing to 100 % MeOH
206 by minute 11, continuing at 100 % MeOH until 13.5 min and returning to initial
207 conditions to complete 15 min. Limits of detection (LODs) and limits of quantification
208 (LOQs) were determined using sequential dilutions of the lowest point of the calibration

curves. LODs were defined as the lowest detectable concentration of the analyte with a signal-to-noise (S/N) ratio for the qualitative transition of at least 3. In the same way, LOQs were defined as the lowest quantificable concentration with a S/N ratio of 10 for the quantitative transition. S/N ratios were calculated using the Mass Hunter Workstation software B.04.01. The LODs obtained for the two vitamin B_{12} congeners were 0.04 and 0.01 pM, while the LOQs values were 0.05 and 0.025 pM for hidroxocobalamin (OHB12). The average B12 recovery percentage after pre-concentration and extraction of B-vitamin spiked samples was 93%. B-vitamin free seawater was spiked with CNB12 and OHB12 standards for recovery percentage analysis.

2.6 Microbial plankton community

DNA samples were taken during the experimental period at surface and SCM depth in the coastal and oceanic station. In particular, sampling of the microbial plankton community was carried out on the first, second, fourth and sixth day of each cruise. Community composition was assessed by sequencing the V4 and V5 regions from 16S rRNA gene (16S rDNA) for prokaryotes and the V4 region from 18S rRNA gene (18S rDNA) for eukaryotes. Two litters of water samples were sequentially filtered through 3 µm pore size polycarbonate filters and 0.2 µm pore size sterivex filter and immediately frozen in liquid nitrogen and conserved at -80 ºC. DNA retained in the 3 µm and 0.2 µm filters was extracted by using the PowerSoil DNA isolation kit (MoBio Laboratories Inc., CA, USA) and the PowerWater DNA isolation kit (MoBio Laboratories Inc., CA, USA), respectively, according to the manufacturer's instructions. Prokaryotic DNA from 0.2 µm filters was amplified using the universal primers "515F and 926R" and eukaryotic DNA from both, 3 µm and 0.2 µm filters, using the primers "TAReuk454FWD1" and "TAReukREV3". Amplified regions were sequenced in an Illumina MiSeq platform and the sequences obtained were analyzed with software

234 package DADA2 (Callahan et al., 2016). SILVA reference database (Quast et al., 2012)
235 was used to taxonomic assignment of 16S amplicon sequence variants (ASVs) and PR2
236 (Guillou et al., 2012) and the marine protist database from the BioMarks project (Massana
237 et al., 2015) were used to taxonomic assignment of 18S ASVs. ASV table is an analogue
238 of the traditional OTU table which records the number of times each exact amplicon
239 sequence variant was observed in each sample (Callahan et al., 2016).

240 The raw ASV tables of prokaryotes and eukaryotes were subsampled to the number of
241 reads present in the sample with the lowest number of reads, which was 2080 and 1286,
242 for 16S rDNA and 18S rDNA, respectively. The abundance of ASVs was averaged for
243 coastal and oceanic samples, differentiating surface and SCM. A total of 1550 unique
244 ASVs of prokaryotes were identified. As many ASVs of eukaryotes were present in both
245 size fractions, we combined datasets derived from the 0.2 and the 3 µm filters for
246 eukaryotic community analyses. As explained in Hernández-Ruiz et al. (2018), we
247 normalized the reads from each filter size by the filter DNA yield, as recommended in
248 Dupont et al. (2015), obtaining 2293 unique ASVs. The sequence abundances of the
249 subsampled ASV tables were transformed using the centered log ratio (clr) (Fernandes et
250 al., 2014; Gloor et al., 2017). Zeros were replaced by the minimum value that is larger
251 than 0 divided by 2.

252 **2.7 Statistical analysis**

253 To compare the effect of different nutrient additions on the response variables,
254 phytoplankton and bacterial biomasses, we calculated response ratios (RR) by dividing
255 each observation (mean of triplicates) of each treatment by the respective control
256 treatment mean. A value equal to 1 implies no response, a value < 1 implies a negative
257 response and a value > 1 implies growth stimulation after nutrient addition. Secondary
258 limitation by B vitamins was calculated by dividing the mean biomass value in the

inorganic nutrients and B vitamin combined treatment by the mean biomass value in the inorganic nutrient addition treatment. In the same way, a value < 1 implies a negative effect of B vitamins and a value > 1 implies growth stimulation by B vitamin through secondary limitation.

Normal distribution was tested by a Kolmogorov-Smirnov test and variables were log transformed if necessary to attain normality. All statistical analysis were considered significant at the 0.05 significance level and p-value was standardized as proposed by Good (1982) in order to overcome the low number of replicates. Differences between station and depth (spatial variability) and among sampling months (temporal variability) in the responses to B vitamins were evaluated with factorial analysis of variance (ANOVA). Bonferroni post hoc tests analyses were conducted to test which treatments were significantly different from the control treatment in each experiment. Z-test was used to evaluate the significance of the average B vitamins response ratios for each period, sampling site and depth. The RELATE analysis implemented in PRIMER6 (Clarke and Warwick, 2001; Clarke and Gorley, 2006) was used to relate the B-vitamin response patterns (Bray-Curtis resemblance matrix built from phytoplankton and bacteria response ratios) with: (1) environmental factors (Euclidean resemblance matrix built from normalized values of ammonium, nitrite, nitrate, phosphate, silicate, B12, temperature, salinity, chlorophyll—a, bacterial biomass), (2) prokaryote community composition (Euclidean resemblance matrix built form clr-transformed sequence abundance of major taxonomic groups), or (3) eukaryote community composition (Euclidean resemblance matrix built form clr-transformed sequence abundance of major taxonomic groups). RELATE calculates the Spearman rank correlations (Rho) between two resemblance matrices, and the significance is tested by a permutation test. In order to highlight which specific taxonomic groups are associated to changes of microbial plankton

(bacterioplankton and phytoplankton) responses to vitamin B1 and B12, we conducted a distance based redundancy analysis (dbRDA) combined with a distance linear-based model (DistLM) using a step-wise procedure and adjusted r^2 as selection criteria) using the PRIMER6 software. Correlations among the prokaryotic taxa best explaining the microbial plankton responses to B-vitamins (according to the previously tests) and phytoplankton and bacterial responses to different B vitamin treatments (including primary and secondary responses) were calculated using Pearson's correlations.

3 Results

3.1 Initial conditions

Different hydrographic conditions were found during each cruise (Fig. 1 and Fig. 2). In February, heavy rainfall combined with relaxed winds (Fig. 1) caused a halocline at 10 meters depth (Fig. 2). High levels of Chl-*a* (as derived from the calibrated CTD fluorescence sensor) were observed at the coastal station, being maximum (4.97 µg l^{-1}) by the end of the cruise. At the oceanic station, Chl-*a* levels remained low (less than 3 µg l^{-1}) throughout the cruise, being slightly higher in the subsurface layer.

Strong precipitation during the April cruise (Fig. 1) caused a persistent surface halocline at the coastal station (Fig. 2). Maximum Chl-*a* concentrations ranged from 0.99 to 2.73 µg l^{-1}, declining from day 5 onwards, coinciding with an increase in water temperature associated to a downwelling situation. At the oceanic station, a persistent subsurface Chl-*a* maximum (up to 1.61 µg l^{-1}) was observed throughout the cruise.

In August, strong thermal stratification was observed at both stations (Fig. 2). At the beginning of the cruise, high Chl-*a* concentration (close to 20 µg l^{-1}) was observed in subsurface water. These high Chl-*a* levels were maintained until day 4 and then decreased, reaching minimum values by day 7, coinciding with upwelling relaxation (Fig.

1b and Fig. 2). Salinity minima during day 1 and 5 reflect precipitation events. Chl-*a* was relatively low at the oceanic station, an increased by the end of the sampling period as a consequence of an upwelling event, that brought cold and nutrient rich water to the surface, at day 5 (Fig. 2).

Abiotic and biotic conditions at the beginning of each experiment are shown in Fig. 3 and in the supplementary Table S2. Overall, the concentration of dissolved inorganic nitrogen (DIN) was higher at the coastal than at the oceanic station, where very low levels were measured in August (Fig. 3). At the coastal station, higher DIN concentrations were observed in surface compared to subsurface waters. The DIN:DIP (dissolved inorganic phosphorous) ratio was always lower in open ocean than in the coastal station and mostly below of Redfield ratio. Phosphorous limitation (DIN:DIP > 16) was frequent in coastal subsurface waters in February and April.

Phytoplankton biomass, estimated as Chl-*a* concentration greatly varied between stations and seasons but was always higher at the coastal (st3) than at the oceanic (st6) station (Fig. 3). Bacterial biomass (BB) increased from winter (February cruise) to summer (August cruise) at the two stations. In February, Chl-*a* concentrations increased by the end of the cruise at both coastal and oceanic stations, while bacterial biomass remained very low throughout this sampling period. In April, both BB and Chl-*a* were similar in the ocean and the coast, and showed reduced temporal variability, irrespective of the observed nutrient variability (Fig. 3). In August, Chl-*a* concentration was much higher at the coastal than at the oceanic station, and showed reduced temporal variability (except at the SCM in the coast) (Fig. 3). At the beginning of the sampling period, BB was higher in the ocean than in the coast, and tended to decline by the end of the cruise.

A MDS analysis revealed that microbial community composition showed a relatively reduced within period variability, with samples clustering according to the sampling

period (ANOSIM, p = 0.001) (Fig. S1 in the Supplement). Consequently, we averaged the microbial community composition for each period and sampling site. The sampling period-averaged composition of the eukaryote community showed a clear variability among sampling dates, while differences between sampling locations and depths were less pronounced (Fig. 4a). At the coastal location, *Mamiellophyceae* were relatively abundant in February and April, but their abundance sharply decreased in August. By contrast, the relative abundance of *Dinophyceae* was highest in August at both sampling locations. The contribution of diatoms (*Bacillariophyta*) was very low in summer at the oceanic station and MALV were most representative in February at both locations. Flavobacterales and Rhodobacterales were the dominant prokaryotes (Fig. 4b) in coastal waters, particularly in August, when both represented more than 80 % of sequences, while Cyanobacteria were mostly present in February and April. In oceanic waters, Flavobacterales and Cyanobacteria were the dominant prokaryotes. SAR11 clade and Archaea were most abundant in February at both sampling locations.

B12 concentration was low, ranging from 0.06 to 0.66 pM (Table S1 in the Supplement) Mean B12 concentration was significantly higher in the coast (0.30±0.13 pM) than in the ocean (0.15±0.12 pM) (t-test, p = 0.001), and showed less variability at the coastal than at the oceanic station (Fig. 4c).

3.2 Short-term phytoplankton and bacteria responses to inorganic nutrients and vitamin additions

The temporal evolution of the phytoplankton and bacterial biomass in the control treatments showed different patterns. Phytoplankton biomass remained either stable or increased after 72 h of incubation in most of the experiments conducted in February and April. However, phytoplankton biomass mostly decreased in the coastal experiments conducted in August (Fig. 5). A very similar pattern was observed for bacterial biomass,

358 although the decrease in biomass occurred both in the coastal and in the oceanic stations
359 during summer (Fig. 6).

360 The magnitude of phytoplankton and bacteria responses (i.e., the response ratios) to the
361 different addition treatments differed between sampling stations (ANOVA, $p = 0.018$)
362 and among sampling periods (ANOVA, $p = 0.014$). The most prominent responses of
363 phytoplankton, compared to the control treatment, occurred after inorganic nutrient
364 amendments, especially in surface oceanic waters (Fig. 5 and Fig. S2 in the Supplement).
365 The magnitude of the phytoplankton response to inorganic nutrients was significantly
366 higher in oceanic than in coastal waters (ANOVA, $p = 0.028$). Bacteria responded
367 comparatively less than phytoplankton to inorganic nutrients (Fig. 6) and there were no
368 significant differences between coastal and oceanic waters (ANOVA, $p = 0.203$). The
369 addition of inorganic nutrients caused significant increases in phytoplankton biomass in
370 31 out of the 36 experiments, and in 19 out of 36 experiments in bacterial biomass (Fig
371 5, Fig. 6 and Fig. S2 in the Supplement).

372 The addition of B12 stimulated phytoplankton growth in 5 out of 36 experiments (Fig. 5
373 and Fig. S3 in the Supplement) while bacteria responded positively to B12 in 6
374 experiments (Fig. 6 and Fig. S3 in the Supplement). Phytoplankton biomass increased in
375 3, and bacterial biomass in 7 out of 36 experiments after adding B1 (Fig. 5 and Fig. 6). B
376 vitamins also caused negative responses of phytoplankton (Fig. 5 and Fig. S3 in the
377 Supplement) and bacterial biomass (Fig. 6 and Fig. S3 in the Supplement). The addition
378 of vitamins induced decreases of phytoplankton biomass in 6 experiments (4 after adding
379 B12 and 2 after adding B1) and bacterial biomass in 14 experiments (6 after adding B12
380 and 8 after adding B1). Additions of inorganic nutrients combined with B-vitamins
381 caused a similar increase in phytoplankton or bacterial biomass than the inorganic
382 addition alone in most of the experiments. Secondary limitation by B1 and/or B12 was

383 occasionally observed when inorganic nutrients were limiting, leading to a higher
384 biomass increase in the treatments including both inorganic nutrients and vitamins as
385 compared to the inorganic nutrient addition alone (Fig. 5, Fig. 6 and Fig. S3 in the
386 Supplement). In the case of phytoplankton, secondary limitation by B-vitamins was found
387 in the 3b-surface, 6a-SCM and 6b-SCM experiments in February, in the 3b-surface and
388 3b-SCM experiments in April, and in the 3b-SCM, 6b-SCM and 6c-surface experiments
389 in August (Fig. 5).

390 In order to quantify the relevance of inter-day variability, we calculated the mean
391 coefficient of variation (CV) of the responses to B vitamins (i.e., excluding the responses
392 to inorganic nutrients, and normalizing the responses of the nutrient and vitamin
393 combined treatments to the corresponding response to inorganic nutrients alone) within
394 sampling periods for each sampling point (4 sites during 3 periods). The CV ranged from
395 9%, in subsurface oceanic waters in April, to 34% in surface coastal waters in April,
396 averaging 16±6 (SD) % (data not shown). Considering that short-term (within sampling
397 period) variability was overall very low, and for simplicity, we averaged the responses to
398 B vitamins in the 3 experiments conducted at each of the 12 sampling points to further
399 describe spatial and temporal patterns in the response to B vitamin amendments (Fig. 7).
400

401 **3.3 B-vitamin response patterns in relation to environmental factors and prokaryote**
402 **and eukaryote community composition**

403 When averaging the responses within each sampling point (Fig. 7), some general patterns
404 emerge. Both phytoplankton and bacteria showed more negative than positive responses
405 to B1 and/or B12 amendments. Most positive responses occurred at the oceanic station,
406 while negative responses dominated in the coast. Phytoplankton significant positive
407 responses mostly occurred in February, showing an average increase of up to 1.2-fold in

coastal subsurface waters after B12+B1 amendment (Fig. 7). The largest significant increase in phytoplankton biomass (ca. 1.4-fold) occurred in April after the combined addition of B12 and B1 in coastal surface waters. Significant positive bacterial responses mainly occurred in August, when the largest increase (ca. 1.3-fold) occurred in coastal subsurface waters after B1 amendment (Fig. 7). Most positive responses were associated with treatments containing B12 either alone or combined with B1 (Fig. 7). Phytoplankton primary B1 limitation was only found at the oceanic SCM in February (Fig. 7), while bacterial primary B1 limitation only occurred at the coastal SCM in August. In addition, bacterial secondary B1 limitation occurred in oceanic surface waters in February and August.

In order to explore the controlling factors of the observed B-vitamin response patterns, the correlation between the B-vitamin response resemblance matrix and the corresponding resemblance matrices obtained from the initial environmental factors, the initial prokaryotic community composition, or the initial eukaryotic community composition were calculated. Only the prokaryotic community composition significantly correlated with the B-vitamin responses (Spearman Rho = 0.31, p = 0.041). We then used distance-based linear modelling (DistLM) to identify the prokaryotic taxa which best explained the microbial plankton responses to B-vitamins (Fig. 8). The resulting model explained 78 % of the variation and included seven prokaryotic groups: *Planktomarina*, Actinobacteria, SAR11_clade, Cellvibrionales, Euryarchaeota, Flavobacteriales and *Synechococcus*. The sequential test identified *Planktomarina* and Actinobacteria as the taxa explaining the largest fraction of variation (ca. 24 % and 14%, respectively, data not shown). The total variation explained by the db-RDA1 and db-RDA2 was 59.4 %, both represented as x and y axis, respectively (Fig. 8). The db-RDA1 axis tended to separate coastal, where negative responses to B vitamins dominated, from oceanic samples, where

most positive responses were found (Fig. 7). The db-RDA plot showed that Cellvibrionales and *Plankomarina* highly and positively correlated with axis 1, while SAR11 and *Synechococcus* showed negative correlation with axis 1. Flavobacteriales and Actinobacteria mostly correlated with the db-RDA2 axis.

4 Discussion

Although the dependence of phytoplankton on B vitamin has been previously observed in cultures (e.g. Croft et al., 2006; Droop, 2007; Tang et al., 2010) and in natural microbial assemblages in coastal areas (e.g. Sañudo-Wilhelmy et al., 2006; Gobler et al., 2007; Koch et al., 2011, 2012, Barber-Lluch et al., 2019), this is, to the best of our knowledge, the most complete study about responses of phytoplankton and bacterial biomass to vitamin B12 and/or B1 addition. The 36 experiments developed in this study allowed a detailed evaluation of the role of vitamins B12 and B1 at different spatial and temporal scales.

Contrary to our expectations, inter-day variability of microbial responses to B vitamins and microbial plankton community composition was relatively small (Fig. 5, Fig. 6, and Fig. S1 in the supplement). The reduced short-term variability in the responses to B vitamins additions suggested that B vitamin availability might be controlled by factors operating at larger temporal scales, such as the succession of microbial communities associated to seasonal environmental variation (Hernández-Ruiz et al., 2018; Hernando-Morales et al., 2018). Considering this, and for further discussion, we averaged the responses from the three experiments conducted during each sampling period, resulting in a total of 12 experimental situations (2 stations × 2 depths × 3 periods). Overall, phytoplankton and/or bacterial growth enhancement in at least one B vitamin treatment

457 was frequent but relatively moderate in this productive ecosystem, showing 1.1 to 2.4-
458 fold increases in 75% of the experimental situations for phytoplankton and in 50% for
459 bacteria. On the other hand, negative responses to at least one B vitamin treatment
460 occurred in all but one of the experimental situations (Fig. 7). The low and constant B12
461 ambient concentration (Fig. 4) and the reduced magnitude of microbial responses suggest
462 a close balance between production and consumption of this growth factor. Different
463 patterns of response to B-vitamin amendments were observed in phytoplankton and
464 bacteria, which appear to be mostly explained by the prokaryotic community
465 composition.

466 **4.1 Positive responses to vitamin B1 and B12 amendments**

467 The experimental design allowed the detection of two categories of B vitamin dependency
468 of the microbial plankton community. A primary limitation by B vitamins occurs when
469 microorganisms respond to additions of B vitamins alone, while a secondary limitation
470 by B vitamins arises when the response to the combined addition of B vitamins and
471 inorganic nutrients is significantly higher than that to inorganic nutrients alone, as a result
472 of the ambient B-vitamin depletion associated to the plankton growth after inorganic
473 nutrient enrichment. Most positive (72% for phytoplankton and 60 % for bacteria)
474 responses occurred after single B-vitamins additions, suggesting that inorganic nutrient
475 availability enhance B-vitamin production by the prototrophic microbes. Under nutrient-
476 limiting conditions, the external supply of vitamins could reduce the energy costs
477 associated to its synthesis (Jaehme and Slotboom, 2015), stimulating the growth not only
478 of auxotrophs but also of prototrophs.

479 The significant positive effects of B12 and/or B1 addition, suggest that these compounds
480 may be eventually limiting microbial growth in marine productive ecosystems, as
481 previously observed by other authors (e.g., Panzeca et al., 2006; Sañudo-Wilhelmy et al.,

2006; Bertrand et al., 2007; Gobler et al., 2007; Koch et al 2011; 2012; Barber.-Lluch et al 2019). Most positive responses to B vitamin amendments were observed in oceanic waters, where B12 concentration was significantly lower than in coastal waters (Fig. 4c). Unfortunately we lack B1 measurements in this study, but, according to previous field studies in other oceanographic regions, a similar pattern to that observed for B12 can be expected (Cohen et al., 2017; Sañudo-Wilhelmy et al., 2012; Suffridge et al., 2018). The overall low and stable concentration of B12 at both sampling locations suggests a high turnover time of this compound in these productive, well-lit waters. Rapid cycling of B12 in surface waters may occur due to high biological uptake rates (Taylor and Sullivan, 2008; Koch et al., 2012) and/or photochemical degradation (Carlucci et al., 1969; Juzeniene and Nizauskaite, 2013; Juzeniene et al., 2015). The measured B12 concentrations were in the lower range reported for coastal sites, and similar to that found in the upwelling system off the California coast in the San Pedro Basin during winter, spring and summer (Panzeca et al., 2009).

The increase of phytoplankton biomass was mostly associated to B12 amendments, which is consistent with the known incapability of eukaryotes to synthesize this vitamin (Croft et al., 2005; Tang et al., 2010; Sañudo-Wilhelmy et al., 2014). Considering the very low concentration of B12 in the sampling area, the relatively limited phytoplankton response to B vitamins is consistent with the presence of species that may have adapted to overcome B12 limitation in the environment by using alternative enzymes. For example, changes in external B12 availability may cause shifts from vitamin B12-dependence to vitamin B12-independence in taxa possessing the vitamin B12-independent methionine synthase (MetE) gene (Bertrand et al., 2013; Helliwell et al., 2014). Other strategies used by phytoplankton to cope with low cobalamin concentration include, increased cobalamin acquisition machinery, decreased cobalamin demand, and management of reduced

507 methionine synthase activity through changes in folate and S-adenosyl methionine
508 metabolism (Bertrand et al., 2012). The available data on B12 half-saturation constants
509 for phytoplankton (0.1-10 pM) (Droop, 1968, 2007; Taylor and Sullivan, 2008; Tang et
510 al., 2010; Koch et al., 2011) are similar or higher than the B12 concentrations measured
511 here (0.3 pM in the coastal and 0.15 pM in the oceanic waters, on average), reinforcing
512 the hypothesis of a phytoplankton community adapted to B12 limiting concentrations in
513 this upwelling system.

514 The positive responses of phytoplankton in surface oceanic waters in February seemed to
515 be associated with high abundance of *Synechococcus* and SAR11 (Fig. 4a and Fig. 8).
516 *Synechococcus* produce a B12 analog known as pseudocobalamin, where the lower ligand
517 base adenine replaces 5,6-dimethylbenzimidazole (DMB) (Helliwell et al., 2016). In
518 natural conditions, pseudocobalamin is considerably less bioavailable to eukaryotic algae
519 than other cobalamin forms (Helliwell et al., 2016; Heal et al., 2017). SAR11 do not
520 require B12 and do not have pathways for its synthesis (Sañudo-Wilhelmy et al., 2014;
521 Gómez-Consarnau et al., 2018), suggesting that B12 synthesis could be limited in oceanic
522 waters in winter, due to the low abundance of potentially B12 producers.

523 Microbial responses to B vitamins in subsurface oceanic waters in February were
524 associated to high abundance of *Synechococcus* and, to some extent, of Actinobacteria
525 (Fig. 8). In these experiments, positive effects of B1 addition on phytoplankton and
526 bacteria were observed (Fig. 7). While *Synechococcus* is capable of B1 synthesis (Carini
527 et al., 2014; Sañudo-Wilhelmy et al., 2014; Gómez-Consarnau et al., 2018),
528 Actinobacteria seems to have a strong dependence on this vitamin (Gómez-Consarnau et
529 al., 2018). Among the sequenced eukaryote genomes, only Stramenopiles contain genes
530 codifying for the synthesis of thiamine monophosphate (Sañudo-Wilhelmy et al., 2014;
531 Cohen et al., 2017) . While Stramenopiles, dominated by Bacillariophyta, were ubiquitous

532 in the sampling area, their relative contribution was lower in oceanic waters (Fig. 4). The
533 simultaneous stimulation of phytoplankton and bacteria by B1 addition in subsurface
534 oceanic waters in winter suggest a strong demand for this compound under these
535 particular conditions, however what triggers the observed responses remain unclear.

536 Even though B1 caused a significant effect on phytoplankton only in subsurface waters
537 in winter, half of the positive responses of bacteria were associated to B1 supply (Fig. 7).
538 This pattern is consistent with the recently described widespread dependence of
539 bacterioplankton on external B1 supply (Paerl et al., 2018). B1 stimulated bacterial
540 growth in subsurface coastal waters and surface oceanic waters in summer (Fig. 7), when
541 the B vitamin response patterns were associated to high abundance of *Planktomarina* and
542 Actinobacteria (Fig. 8),which are expected to strongly depend on external B1 sources
543 (Giebel et al., 2013; Gómez-Consarnau et al., 2018). The generalized significant and
544 positive bacterial responses to vitamin treatments in surface oceanic waters in summer,
545 when the bacterial biomass was high and dissolved inorganic nitrogen concentration was
546 very low (Fig. 3), suggest that bacteria may have an advantage in the uptake and
547 assimilation of B vitamins under nitrogen limiting conditions.

548

549 **4.2 Negative responses to vitamin B1 and B12 amendments**

550 Similar experiments conducted in this area also reported negative responses of microbial
551 plankton to vitamin B12 additions (Barber-Lluch et al., 2019). The predominantly
552 negative bacterial responses after vitamin amendments in the coast during summer (Fig.
553 6, Fig. 7, and Fig. S3 in the Supplement), when nutrient concentrations were low (Fig. 3),
554 suggest either a strong competition between phytoplankton and bacteria or a stimulation
555 of predation. Dinoflagellates were particularly abundant in summer at both sampling sites

556 and depths. Many dinoflagellate species are auxotrophs for B1 and/or B12 (Croft et al,
557 2006; Tang et al., 2010), and also many of them are phagotrophs (Stoecker and Capuzzo,
558 1990; Smayda, 1997; Sarjeant and Taylor, 2006; Stoecker et al., 2017), thus the external
559 supply of B vitamins may have promoted their growth, ultimately leading to net decreases
560 in microbial biomass at the end of the experiments. Several studies demonstrated that
561 vitamin B12 is implicated in the occurrence of dinoflagellate blooms around the world
562 (Aldrich, 1962; Carlucci and Bowes, 1970; Takahashi and Fukazawa, 1982; Yu and
563 Rong-cheng, 2000). It has been suggested that the B12-dependent enzyme
564 methylmalonyl-CoA mutase in dinoflagellate, euglenoid, and heterokont algae allows
565 them to grow heterotrophically when B12 is available (Croft et al., 2006). Therefore, the
566 B12 enrichment could trigger such nutritional strategy, particularly in summer, when
567 mineral nutrients are less available, resulting in an increased predation pressure on
568 bacteria.

569 Strikingly, the B vitamin response patterns in surface coastal waters in summer (Fig. 7),
570 seemed to be associated with high abundance of Flavobacteriales (Fig. 8). All isolates of
571 Bacteroidetes sequenced so far are predicted to be B12 auxotrophs (Sañudo-Wilhelmy et
572 al., 2014; Gómez-Consarnau et al., 2018) and recent metatranscriptomic analyses revel
573 that B1 synthesis gene transcripts are relatively low in Flavobacteriia as a group (Gómez-
574 Consarnau et al., 2018). As both phytoplankton and bacteria are dominated by potentially
575 B12 and B1 auxotrophs (dinoflagellates and Flavobacteriales) in the coast during summer
576 (Fig. 4), the negative responses could be the result of strong competition for B vitamins.
577 However, the negative responses to B vitamins of both phytoplankton and bacteria in
578 surface coastal water in summer suggests an increase in predation over both microbial
579 groups rather than competition between them. By contrast, bacteria and phytoplankton
580 showed opposite patterns of response to B vitamins in subsurface coastal waters in

summer, which suggests competition between both microbial compartments (Fig. 7). While phytoplankton negatively responded only to single B vitamin additions, bacteria responded negatively only when both inorganic nutrients and B vitamins were added (Fig. 7). It is conceivable that phytoplankton had an advantage over bacteria when mineral nutrients were added.

In conclusion, our findings suggest that the heterogeneous responses of microbial plankton to B1 and B12 vitamins supply in this coastal upwelling system could be partially controlled by the composition of the prokaryote community, which is consistent with their major role as B12 producers and B1 consumers. The overall moderate responses in terms of biomass together with the low ambient B12 concentration, suggest that the microbial plankton in this area is well adapted to cope with B vitamin shortage and that a close balance exists between production and consumption of these important growth factors.

Author contribution.

Eva Teira designed the experiments and Vanessa Joglar carried them out with contributions from all co-authors. Vanessa Joglar analyzed the data, Vanessa and Eva Teira interpreted the results and Vanessa Joglar prepared the manuscript under Eva Teira supervision.

Competing interests. The authors declare that they have no conflict of interest.

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609

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931 **6 Tables and Figures**

932 **Figure 1:** (a) The NW Iberian margin (rectangle) and locations of the stations that were
933 sampled in the Ría de Vigo (st3) and on the shelf (st6) (diamonds), (b) distribution of
934 daily coastal upwelling index (Iw) and (c) registered precipitations during each sampling
935 period showing the initial time of each experiment (3a, 3b, 3c and 6a, 6b, 6c). ns: no
936 sampling day.

937 **Figure 2:** Vertical distribution in the coastal station of (a) fluorescence (µg l^{-1}), (b)
938 temperature (ºC) and (c) salinity (PSU) over time for February, April and August and
939 vertical distribution in the oceanic station of (d) fluorescence (µg l^{-1}), (e) temperature (ºC)
940 and (f) salinity (PSU) over time for February, April and August.

941 **Figure 3:** Initial biological conditions and abiotic factors at the coastal (st3) and oceanic
942 (st6) sampling stations. Each bar corresponds to one of the 3 experiments performed in
943 each depth and station during February, April and August. (a), Chl-*a*, total Chl-*a* (µg l^{-1});
944 (b) BB, bacterial biomass (µg C l^{-1}); (c) DIN, dissolved inorganic nitrogen (µmol N l^{-1})
945 and (d) DIN:DIP, ratio nitrogen:phosphate.

946 **Figure 4:** (a) Averaged relative contribution of reads to the major taxonomic groups of
947 eukaryotes and prokaryotes at surface and SCM in the coastal and oceanic station in
948 February, April and August. (b) Averaged B12 concentration (pM) at surface and SCM
949 in the coastal and oceanic station in February, April and August.

950 **Figure 5**: Phytoplankton biomass (estimated as Chl-a concentration) (µg l^{-1}) in the time-
951 zero of each experiment (striped bars) and in the final-time of each treatment (colored
952 bars) in the experiments conducted at surface and SCM in the coastal and oceanic station
953 in February, April and August.

954 **Figure 6**: Bacterial biomass (µgC l^{-1}) in the time-zero of each experiment (striped bars)
955 and in the final-time of each treatment (colored bars) in the experiments conducted at
956 surface and SCM in the coastal and oceanic station in February, April and August.
957 **Figure 7:** Monthly averaged response ratio (RR) of (a) total phytoplankton community
958 and of (b) bacterial community at surface and SCM in the coastal and oceanic station.
959 Horizontal line represents a response equal to 1, that means no change relative to control
960 in the pink bars (treatments with vitamins alone) and no change relative to inorganic (I)
961 treatment in the green bars (vitamins combined with I treatments). Asterisks indicate
962 phytoplankton or bacterial significant response relative to control or I (Z-test; * $p < 0.05$)
963 and a indicate response with a level of significance between 0.05 and 0.1 (Z-test; ^{a} p =
964 0.05-0.06).
965 **Figure 8:** Distance based redundancy analysis (dbRDA) of B vitamin responses by
966 microbial plankton based on Bray-Curtis similarity. Filled and open symbols represent
967 samples from coastal and oceanic station, respectively, numbers correspond to the
968 sampling station, triangles and circles represent samples from surface and SCM,
969 respectively, and colours correspond to the months: (green) February, (blue) April and
970 (pink) August. Only prokaryotic taxa that explained variability in the B vitamin responses
971 structure selected in the DistLM model (step-wise procedure with adjusted R^2 criterion)
972 were fitted to the ordination.
973