

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

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10 **Abstract.** We experimentally evaluated the temporal (inter-day and inter-season) and
11 spatial variability in microbial plankton responses to vitamin B12 and/or B1 supply
12 (solely or in combination with inorganic nutrients) in coastal and oceanic waters of the
13 northeast Atlantic Ocean. Phytoplankton and, to a lesser extent, prokaryotes were strongly
14 limited by inorganic nutrients. Inter-day variability in microbial plankton responses to B-
15 vitamins was limited compared to inter-season variability, suggesting that B-vitamins
16 availability might be partially controlled by factors operating at seasonal scale.
17 Chlorophyll-*a* (Chl-*a*) concentration and prokaryote biomass (PB) significantly increased
18 after B-vitamin amendments in 13 % and 21 %, respectively, of the 216 cases (36
19 experiments × 6 treatments). Most of these positive responses were produced by
20 treatments containing either B12 solely or B12 combined with B1 in oceanic waters,
21 which was consistent with the significantly lower average vitamin B12 ambient
22 concentrations compared to that in the coastal station. Negative responses, implying a
23 decrease in Chl-*a* or PB, represented 21 % for phytoplankton and 26 % for prokaryotes.
24 Growth stimulation by B1 addition was more frequent on prokaryotes than in
25 phytoplankton, suggesting that B1 auxotrophy in the sampling area could be more
26 widespread in prokaryotes than in phytoplankton. Negative responses to B-vitamins were
27 generalized in coastal surface waters in summer, and were associated to a high
28 contribution of Flavobacteriales to the prokaryote community. This observation suggests
29 that the external supply of B12 and/or B1 may promote negative interactions between
30 microbial components when B-vitamin auxotrophs are abundant. The microbial response
31 patterns to B12 and/or B1 amendments were significantly correlated with changes in the
32 prokaryotic community composition, highlighting the pivotal role of prokaryotes in B-
33 vitamins cycling in marine ecosystems.

34

35 **1 Introduction**

36 Phytoplankton accounts for almost half of the global net primary production (Field et al.,
37 1998) and may eventually cause toxic episodes, such as those caused by harmful algae
38 blooms of *Alexandrium* spp. or *Gymnodinium* spp., entailing human health problems and
39 large economic losses (Hallegraeff, 1993; van Dolah et al., 2001). Recent emerging
40 evidence suggests the role of biologically active organic compounds, such as B-vitamins,
41 on the control of marine productivity in both coastal and oceanic waters (Panzeca et al.,
42 2006; Bertrand et al., 2007; Gobler et al., 2007; Koch et al., 2011; Browning et al., 2017,
43 2018). B-vitamins act as cofactors for enzymatic reactions and are involved in many
44 important metabolic pathways (Madigan et al., 2005; Koch et al., 2011; Monteverde et
45 al., 2017). Vitamin B12 (B12 herein), which is exclusively synthesized by some bacteria
46 and archaea (Roth et al., 1996; Martens et al., 2002; Warren et al., 2002), acts as a cofactor
47 of three enzymes in eukaryotes (methionine synthase, methylmalonyl-coA mutase and
48 ribonucleotide reductase type II) (Helliwell et al., 2011; Bertrand and Allen, 2012). In
49 comparison, over 20 different B12-dependent enzymes are found in bacteria (Roth et al.,
50 1996), making B12 critically important also for these organisms. Vitamin B1 (B1 herein)
51 plays a pivotal role in intermediary carbon metabolism and is a cofactor for a number of
52 enzymes involved in primary carbohydrate and branched-chain amino acid metabolism
53 (Croft et al., 2006).

54 Most eukaryote phytoplankton species are auxotrophs for one or more B-vitamins,
55 consequently requiring an exogenous supply of these molecules (Bertrand and Allen,
56 2012; Carlucci and Bowes, 1970; Haines and Guillard, 1974; Helliwell et al., 2011).
57 Moreover, genomic data also indicate widespread B-vitamins auxotrophy among many
58 bacterial taxonomic groups (Sañudo-Wilhelmy et al., 2014; Paerl et al., 2018), which
59 implies that phytoplankton and bacterioplankton may eventually compete for the

60 acquisition of these compounds (Koch et al., 2012). Auxotrophic microorganisms may
61 acquire the required vitamins from the environment or through biotic interactions with
62 prototrophic (biosynthetically competent) microorganisms (Droop, 2007; Grant et al.,
63 2014; Kazamia et al., 2012). A well-known example is the mutualistic interaction
64 between B12 or B12 and B1 dependent phytoplankton and bacterioplankton (Croft et al.,
65 2005; Amin et al., 2012; Cooper and Smith, 2015; Cruz-López and Maske, 2016).

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

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

79 Some studies have shown enhanced phytoplankton biomass associated to B12
80 amendments in both temperate coastal and polar waters (Bertrand et al., 2007; Gobler et
81 al., 2007; Koch et al., 2011, 2012). The simultaneous effect of vitamin B12 supply on
82 both phytoplankton and bacteria has been barely explored (Koch et al., 2011, Barber-
83 Lluç et al., 2019). To our knowledge, the effect of B1 amendments on marine natural
84 microbial plankton community succession has been only assessed by Gobler et al. (2007),

85 who suggested that high concentration of B-vitamins, associated with high bacterial
86 abundance, caused an increase in auxotrophs, mostly dinoflagellates.

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

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

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

110

111 **2 Methods**

112 **2.1 Sampling strategy**

113 Thirty-six enrichment experiments were performed in the upwelling system near Ría de
114 Vigo on board “B/O Ramón Margalef” in three different oceanographic cruises
115 (ENVISION I, II & III) conducted in 2016. Two different locations of the East Atlantic
116 Ocean, one coastal station (C) (42° N, 8.88° W) and one oceanic station (Oc) (42° N, 9.06°
117 W) (Fig. 1a), were sampled during three different seasons aimed to cover a wide range of
118 initial hydrographic and ecological conditions. The 10-day cruises were conducted in
119 February (ENVISION I), coinciding with the spring bloom, and April (ENVISION II)
120 and August (ENVISION III) during the early and late summer upwelling, respectively.
121 During each cruise, 12 enrichment experiments were carried out on board, 3 experiments
122 in each station (C-a, C-b & C-c and Oc-a, Oc-b & Oc-c, respectively) with water from
123 two different depths. Each experiment began on the first (day 0), third (day 2) and sixth
124 (day 5) of each cruise for the coast and on the second (day 1), fourth (day 3) and seventh
125 (day 6) of each cruise for the ocean (Fig. 1b, c). Water was collected using 20 l Niskin
126 metal-free bottles. Surface (5 m) and sub-surface chlorophyll maximum (SCM) (between
127 10 m and 50 m according to the CTD data) samples were taken (Fig. 2a-f). We failed to
128 sample the SCM on two occasions (C-a in February and C-a in April), due to large vertical
129 displacements between the downward and the upward casts. Vertical profiles of
130 temperature, salinity and chlorophyll fluorescence were obtained using a regular stainless
131 CTD-rosette down to 60 m in the coastal station and to 200 m in oceanic station. Samples
132 for chlorophyll-*a* (Chl-*a*), prokaryotic biomass (PB), dissolved nutrient concentration,
133 including vitamin B12, and microbial plankton community were collected at the

134 beginning (time zero, hereafter referred to as t_0) of each enrichment experiment. Daily
135 upwelling index (UI) values were computed by the Instituto Español de Oceanografía
136 (www.indicedeafloramiento.ieo.es/) in a $2^\circ \times 2^\circ$ geostrophic cell centered at 42°N , 10°W , using data from atmospheric pressure at sea level, derived from the WXMAP model
137 $^\circ\text{W}$, using data from atmospheric pressure at sea level, derived from the WXMAP model
138 (Gonzalez-Nuevo et al., 2014). Precipitation data was obtained from the Regional
139 Weather Forecast Agency-Meteogalicia (<http://www.meteogalicia.gal>) in the
140 meteorological station Illas Cies (ID 10125).

141 **2.2. Experimental design**

142 Seawater samples were gently pre-filtered through a $200\ \mu\text{m}$ mesh to exclude large
143 zooplankton in order to ensure good replicability and collected into a 20 l acid-cleaned
144 polyethylene carboy. It is important to note that incidental trace-metal contamination
145 could have occurred during water collection. Following sample collection, 300 ml PAR
146 and UVR transparent, sterile, and non-toxic (whirl-pak) bags were filled and nutrients
147 were added establishing eight different enrichment treatments as follows: (1) control
148 treatment (C); (2) inorganic nutrient treatment (I); (3) vitamin B12 (Sigma, V2876)
149 treatment; (4) vitamin B1 (Sigma, T4625) treatment; (5) Inorganic nutrients and vitamin
150 B12 (I+B12) treatment; (6) Inorganic nutrients and vitamin B1 (I+B1) treatment; (7)
151 vitamins B12 and B1 (B12+B1) treatment and (8) Inorganic nutrients with vitamins B12
152 and B1 (I+B12+B1) treatment (see Table 1 for details). Inorganic nutrients were added to
153 avoid that inorganic nutrient limitation masked the responses to B vitamins. The nutrient
154 concentrations of the additions were the same as previously used in similar enrichment
155 experiments in the sampling area (Martinez-García et al., 2010a). The amount of B12 and
156 B1 vitamin experimentally added approximated maximum concentrations previously
157 observed in coastal areas (Okbami and Sañudo-Wilhelmy 2004, 2005, Sañudo-
158 Wilhelmy et al., 2006). Each treatment had 3 replicates resulting in 24 whirl-pak bags

159 per experiment. To assess short-term effects of nutrient inputs, experimental bags were
160 incubated on-deck during 72 h. In-situ temperature was reproduced by submerging the
161 bags in tanks filled with constantly circulating surface seawater. To simulate light
162 intensity at the SCM the incident light was attenuated by covering the tanks with mesh
163 screens.

164 **2.3 Chlorophyll-*a***

165 Chlorophyll-*a* (Chl-*a*) concentration was measured at t_0 and after 72 h incubation as a
166 phytoplankton biomass proxy. 300 ml of water samples were filtered through 0.2 μm
167 polycarbonate filters and frozen at -20°C until further analysis. Chl-*a* was extracted with
168 90 % acetone and kept in darkness at 4°C overnight. Fluorescence was determined with a
169 TD-700 Turner Designs fluorometer calibrated with pure Chl-*a* standard solution
170 (absorption coefficient at 663 nm = 87.7; Lorenzen and Newton Downs, 1986).

171 **2.4 Flow cytometry**

172 Samples for prokaryote abundance quantification (2 ml) were preserved with 1 %
173 paraformaldehyde + 0.05 % glutaraldehyde (final concentrations). Samples were
174 incubated 20 min for the fixative to act on cells, immersed in liquid nitrogen for 15 min,
175 and frozen at -80°C . Abundance of prokaryotes was determined using a FACSCalibur
176 flow cytometer equipped with a laser emitting at 488nm. Samples were stained with
177 SYBR Green DNA fluorochrome, and bacterial abundance was detected by their
178 signature of side scatter (SSC) and green fluorescence as described by Gasol and Del
179 Giorgio (2000). The empirical calibration between light side scatter (SSC) and cell
180 diameter described by Calvo-Díaz and Moran (2006) were used to estimate cell
181 biovolume (BV). BV was converted into biomass by using the allometric factor of

182 Norland (1993: $\text{fg C cell}^{-1} = 120 \times \text{BV}^{0.72}$) for the coastal experiments and using the open
183 ocean conversion factor for the oceanic experiments ($\text{fg C cell}^{-1} = 350 \times \text{BV}$).

184 **2.5 Nutrients**

185 Aliquots for inorganic nutrient determinations (ammonium, nitrite, nitrate, phosphate,
186 and silicate) were collected before all other variables and directly from the Niskin bottle
187 in order to avoid contamination. Polyethylene bottles (50 ml) precleaned with 5 % HCl
188 were filled with the sample using contamination-free plastic gloves and immediately
189 frozen at -20°C until analysis using standard colorimetric methods with a Bran-Luebbe
190 segmented flow analyzer (Hansen and Grasshoff 1983). The detection limit was $0.1 \mu\text{mol}$
191 l^{-1} for nitrate, $0.02 \mu\text{mol l}^{-1}$ for nitrite and phosphate and $0.05 \mu\text{mol l}^{-1}$ for ammonium
192 and silicate. Dissolved inorganic nitrogen (DIN) concentration was calculated as the sum
193 of the ammonium, nitrite and nitrate concentrations.

194 **2.6 Vitamin B12**

195 Seawater samples for dissolved vitamin analysis were taken at surface and SCM depth on
196 day 1, day 3 and day 5 in the coastal, and on day 1, day 3 and day 6 oceanic station of
197 each cruise (Table S1 in the Supplement). Samples were filtered through $0.2 \mu\text{m}$ sterivex
198 filters and frozen at -20°C until further analysis. Samples (1 l) were preconcentrated using
199 a solid-phase extraction with a C18 resin (Bondesil C18, Agilent) at pH 6.5 and rate of
200 1ml/min . Elution was performed with 12 ml of methanol (MeOH) LCMS grade that was
201 removed via evaporation with nitrogen in a Turbovap. Gas pressure was initially set at 5
202 PSI and was slowly increased to 15 PSI until 300-500 μl of sample remained. The
203 concentrated samples were frozen at -20°C until further analysis using liquid
204 chromatography coupled to mass spectrometry system.

205 The concentrate was filtered again through a cellular acetate membrane 0.2 μm
206 (Phenomenex) prior to the analysis. Ultra Performance Liquid Chromatography tandem
207 Mass Spectrometry 3Q (UPLC-MS/MS) methodology was adapted from Sañudo-
208 Wilhelmy et al. (2012), Heal et al. (2014) and Suffridge et al. (2017). Detection and
209 quantification of dissolved vitamin B12 (cyanocobalamin and hydroxocobalamin) was
210 conducted using an Agilent 1290 Infinity LC system (Agilent Technologies, Waghaeusel-
211 Wiesental, Germany), coupled to an Agilent G6460A triple quadrupole mass
212 spectrometer equipped with an Agilent Jet Stream ESI source. The LC system used a C18
213 reversed-phase column (Agilent Zorbax SB-C18 Rapid Resolution HT (2.1 inner
214 diameter \times 50 mm length, 1.8 μm particle size) with a 100 μl sample loop. Agilent
215 Technologies software was used for data acquisition and analysis. Chromatographic
216 separation was performed using MeOH and water LCMS grade, both buffered to pH 5
217 with 0.5 % acetic acid, as mobile phases in a 15 minutes' gradient. Gradient starting at 7
218 % MeOH for 2 min, changing to 100 % MeOH by minute 11, continuing at 100 % MeOH
219 until 13.5 min and returning to initial conditions to complete 15 min. Limits of detection
220 (LODs) and limits of quantification (LOQs) were determined using sequential dilutions
221 of the lowest point of the calibration curves. LODs were defined as the lowest detectable
222 concentration of the analyte with a signal-to-noise (S/N) ratio for the qualitative transition
223 of at least 3. In the same way, LOQs were defined as the lowest quantifiable
224 concentration with a S/N ratio of 10 for the quantitative transition. S/N ratios were
225 calculated using the Mass Hunter Workstation software B.04.01. The LODs obtained
226 were 0.04 pmol l^{-1} for hydroxocobalamin (OHB12) and 0.01 pmol l^{-1} for cyanocobalamin
227 (CNB12), while the LOQs values were 0.05 and 0.025 pmol l^{-1} for OHB12 and CNB12,
228 respectively. The average B12 recovery percentage after pre-concentration and extraction
229 of B-vitamin spiked samples was 93%. B-vitamin free seawater was spiked with CNB12

230 and OHB12 standards for recovery percentage analysis. We failed to detect B1 vitamin
231 in the pre-concentrated samples, likely due to a low ambient concentration and low pre-
232 concentration volume.

233 **2.7 Microbial plankton community**

234 DNA samples were taken during the experimental period at surface and SCM depth in
235 the coastal and oceanic station. In particular, sampling of the microbial plankton
236 community was carried out on day 0, day 1, day 3 and day 5 of each cruise. Community
237 composition was assessed by sequencing the V4 and V5 regions from 16S rRNA gene
238 (16S rDNA) for prokaryotes and the V4 region from 18S rRNA gene (18S rDNA) for
239 eukaryotes. Two liters of water samples were sequentially filtered through 3 μm pore size
240 polycarbonate filters and 0.2 μm pore size sterivex filter and immediately frozen in liquid
241 nitrogen and conserved at -80 °C. DNA retained in the 3 μm and 0.2 μm filters was
242 extracted by using the PowerSoil DNA isolation kit (MoBio Laboratories Inc., CA, USA)
243 and the PowerWater DNA isolation kit (MoBio Laboratories Inc., CA, USA),
244 respectively, according to the manufacturer's instructions. Prokaryotic DNA from 0.2 μm
245 filters was amplified using the universal primers "515F and 926R" (Parada et al., 2016)
246 and eukaryotic DNA from both, 3 μm and 0.2 μm filters, using the primers
247 "TAREuk454FWD1" and "TAREukREV3" (Logares et al., 2014). Amplified regions
248 were sequenced in an Illumina MiSeq platform and the sequences obtained were analyzed
249 with software package DADA2 (Callahan et al., 2016). SILVA reference database (Quast
250 et al., 2012) was used to taxonomic assignment of 16S amplicon sequence variants
251 (ASVs) and PR2 (Guillou et al., 2013) and the marine protist database from the BioMarks
252 project (Massana et al., 2015) were used to taxonomic assignment of 18S ASVs. The data
253 for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-
254 EBI (<https://www.ebi.ac.uk/ena>) under accession numbers PRJEB36188 (16S rDNA

255 sequences) and PRJEB36099 (18S rDNA sequences). ASV table is an analogue of the
256 traditional OTU table which records the number of times each exact amplicon sequence
257 variant was observed in each sample (Callahan et al., 2016).

258 The raw ASV tables of prokaryotes and eukaryotes were subsampled to the number of
259 reads present in the sample with the lowest number of reads, which was 2080 and 1286,
260 for 16S rDNA and 18S rDNA, respectively. The abundance of ASVs was averaged for
261 coastal and oceanic samples, differentiating surface and SCM. A total of 1550 unique
262 ASVs of prokaryotes were identified. As many ASVs of eukaryotes were present in both
263 size fractions (e.g. those having a cell size range including 3 μm), we combined datasets
264 derived from the 0.2 and the 3 μm filters for eukaryotic community analyses. As explained
265 in Hernández-Ruiz et al. (2018), we normalized the reads from each filter size by the filter
266 DNA yield, as recommended in Dupont et al. (2015), obtaining 2293 unique ASVs. The
267 sequence abundances of the subsampled ASV tables were transformed using the centered
268 log ratio (clr) (Fernandes et al., 2014; Gloor et al., 2017). Before clr transformation, zeros
269 were replaced by the minimum value that is larger than 0 divided by 2 (Aitchison, 1982;
270 Martín-Fernández et al., 2003).

271 **2.8 Statistical analysis**

272 To compare the effect of different nutrient additions on the response variables,
273 chlorophyll-*a* concentration and prokaryote biomass, we calculated response ratios (RR)
274 by dividing each observation (mean of triplicates) of each treatment by the respective
275 control treatment mean. A value equal to 1 implies no response, a value < 1 implies a
276 negative response and a value > 1 implies growth stimulation after nutrient addition.
277 Secondary limitation by B vitamins was calculated by dividing the mean value in the
278 inorganic nutrients and B vitamin combined treatment by the mean value in the inorganic

279 nutrient addition treatment. In the same way, a value < 1 implies a negative effect of B
280 vitamins and a value > 1 implies stimulation positive effect of B vitamin treatment
281 through secondary limitation.

282 Normal distribution was tested by a Kolmogorov-Smirnov test and non-normal variables
283 such as temperature, salinity, DIN, SiO_4^{2-} , and Chl-*a* and PB response ratios, were log
284 transformed to attain normality. All statistical analysis were considered significant at the
285 0.05 significance level and p-value was standardized as proposed by Good (1982) in order
286 to overcome the low number of replicates. Differences between station and depth (spatial
287 variability) and among sampling months (temporal variability) in the responses to B
288 vitamins were evaluated with factorial analysis of variance (ANOVA). Bonferroni post
289 hoc tests analyses were conducted to test which treatments were significantly different
290 from the control treatment in each experiment. Non-metric multidimensional scaling
291 (nMDS) was used to analyze the similarities between the samples based on microbial
292 assemblage structure using the PRIMER6 software (Clarke and Warwick, 2001; Clarke
293 and Gorley, 2006). The similarities were evidenced in a multidimensional space by
294 plotting more similar samples closer together. Analysis of similarity (ANOSIM) was used
295 to verify that microbial community composition from the same season and station were
296 more similar to each other than to communities from a different season and station. Z-test
297 was used to test if averaged B vitamins response ratios were significantly different from
298 1. The RELATE analysis implemented in PRIMER6 was used to relate the B-vitamin
299 response patterns (Bray-Curtis resemblance matrix built from phytoplankton and bacteria
300 response ratios) with: (1) environmental factors (Euclidean resemblance matrix built from
301 normalized values of ammonium, nitrite, nitrate, phosphate, silicate, B12, temperature,
302 salinity, Chl-*a* and prokaryote biomass), (2) prokaryote community composition
303 (Euclidean resemblance matrix built form clr-transformed sequence abundance of major

304 taxonomic groups), or (3) eukaryote community composition (Euclidean resemblance
305 matrix built from clr-transformed sequence abundance of major taxonomic groups).
306 RELATE calculates the Spearman rank correlations (Rho) between two resemblance
307 matrices, and the significance is tested by a permutation test (999 permutations). In order
308 to highlight which specific taxonomic groups are associated to changes of microbial
309 plankton (prokaryote plankton and phytoplankton) responses to vitamin B1 and B12, we
310 conducted a distance based redundancy analysis (dbRDA) combined with a distance
311 linear-based model (DistLM) using a step-wise procedure and adjusted R^2 as selection
312 criteria using the PRIMER6 software.

313

314 **3 Results**

315 **3.1 Initial conditions**

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

322 Strong precipitation during the April cruise (Fig. 1c) caused a persistent surface halocline
323 at the coastal station (Fig. 2n). Maximum Chl-*a* concentrations ranged from 0.99 to 2.73
324 $\mu\text{g l}^{-1}$, declining from day 5 onwards (Fig. 2b), coinciding with an increase in water
325 temperature associated to a downwelling situation. At the oceanic station, a persistent
326 subsurface Chl-*a* maximum (up to $1.61 \mu\text{g l}^{-1}$) was observed throughout the cruise (Fig.
327 2e).

328 In August, strong thermal stratification was observed at both stations (Fig. 2i and Fig. 2l).
329 At the beginning of the cruise, high Chl-*a* concentration (close to 20 $\mu\text{g l}^{-1}$) was observed
330 in subsurface water (Fig. 2c). Chl-*a* was relatively low at the oceanic station, and
331 increased by the end of the sampling period (Fig. 2f) as a consequence of an upwelling
332 event (Fig. 1b), that brought cold and nutrient rich water to the surface, at day 5.

333 Abiotic and biotic conditions at the beginning of each experiment are shown in Fig. 3 and
334 in the supplementary Table S2. Overall, the concentration of dissolved inorganic nitrogen
335 (DIN) was higher at the coastal than at the oceanic station, where very low levels were
336 measured in August (Fig. 3i). At the coastal station, higher DIN concentrations were
337 observed in surface compared to subsurface waters. The DIN:DIP (dissolved inorganic
338 phosphorous) ratio was always lower in open ocean than in the coastal station and mostly
339 below the Redfield ratio (16:1). Phosphorous limitation (DIN:DIP > 16) was frequent in
340 coastal surface waters in February and April (Fig. 3j and Fig. 3k).

341 On average, chl-*a* concentration varied greatly between stations and months but was
342 always higher at the coastal than at the oceanic station (Fig. 3a-c). Prokaryote biomass
343 (PB) increased from winter (February) to summer (August) at the two stations (Fig. 3d-
344 f). In February, Chl-*a* concentrations increased by the end of the cruise at both coastal
345 and oceanic stations (Fig. 3a), while PB remained very low throughout this sampling
346 period (Fig. 3d). In April, both PB and Chl-*a* were similar in the ocean and the coast, and
347 showed reduced temporal variability (Fig. 3b and Fig. 3e), irrespective of the observed
348 nutrient variability (Fig. 3h). In August, Chl-*a* concentration was much higher at the
349 coastal than at the oceanic station, and showed reduced temporal variability (except at the
350 SCM in the coast) (Fig. 3c). At the beginning of the sampling period, PB was higher in
351 the ocean than in the coast, and tended to decline by the end of the cruise (Fig. 3f).

352 A MDS analysis revealed that microbial community composition showed a relatively
353 reduced variability within period, with samples clustering according to the sampling
354 period (ANOSIM, $p = 0.001$) and station (ANOSIM, $p = 0.001$) (Fig. S1 in the
355 Supplement). Consequently, we averaged the microbial community composition for each
356 period and sampling site. The sampling period-averaged composition of the eukaryote
357 community showed a clear variability among cruises, while differences between sampling
358 locations and depths were less pronounced (Fig. 4a). At the coastal location,
359 Mamiellophyceae (*Ostreococcus* and *Micromonas*) were relatively abundant in February
360 and April, but their relative abundance sharply decreased in August. By contrast, the
361 relative abundance of Dinophyceae was highest in August at both sampling locations.
362 The contribution of diatoms (Bacillariophyta) was very low in summer at the oceanic
363 station and marine alveolates (MALV) groups (MALV-I and MALV-II) were most
364 representative in February at both locations. Flavobacteriales and Rhodobacterales were
365 the dominant prokaryotes (Fig. 4b) in coastal waters, particularly in August, when both
366 represented more than 80 % of sequences, while the Cyanobacteria *Synechococcus* were
367 mostly present in February and April. In oceanic waters, Flavobacteriales and
368 *Synechococcus* were the dominant prokaryotes. SAR11 clade and Archaea
369 (Euryarchaeota and Thaumarchaeota) were most abundant in February at both sampling
370 locations.

371 B12 concentration was low, ranging from 0.06 to 0.66 pmol l^{-1} (Table S1 in the
372 Supplement). Average B12 concentration was significantly higher in the coast (0.30 ± 0.13
373 pmol l^{-1}) than in the ocean ($0.15 \pm 0.12 \text{ pmol l}^{-1}$) (t-test, $t = 3.17$, $df = 10$, $p = 0.01$), and
374 showed less variability at the coastal than at the oceanic station (Fig. 4c).

375 **3.2 Short-term phytoplankton and prokaryote responses to inorganic nutrients and** 376 **vitamin additions**

377 The temporal development of the phytoplankton (as estimated from changes in Chl-*a*
378 concentration) and prokaryote biomass in the control treatments showed different
379 patterns. Chl-*a* remained either stable or increased after 72 h of incubation in 87.5% of
380 the experiments conducted in February and April. However, Chl-*a* mostly decreased in
381 the coastal experiments conducted in August (Fig. 5a and Fig. 5c). A very similar pattern
382 was observed for prokaryote biomass, although the decrease in biomass occurred both in
383 the coastal and in the oceanic stations during summer (Fig. 6).

384 The response ratios (RRs) of Chl-*a* and prokaryote biomass were calculated as a measure
385 of the magnitude of phytoplankton and prokaryote responses to nutrient and vitamin
386 treatments (Fig S2, S3 and S4 in the supplement). The RRs differed between sampling
387 stations (ANOVA, $F(1,502) = 18.059$, $p < 0.001$) and among sampling periods (ANOVA,
388 $F(2,501) = 6.54$, $p = 0.002$). The most prominent responses of phytoplankton, compared
389 to the control treatment, occurred after inorganic nutrient amendments, especially in
390 surface oceanic waters (Fig. 5c and Fig. S2b, f and j in the Supplement). The magnitude
391 of the phytoplankton response to inorganic nutrients was significantly higher in oceanic
392 than in coastal waters (ANOVA, $F(1,34) = 5.22$, $p = 0.028$). Prokaryotes responded less
393 than phytoplankton to inorganic nutrients and, in addition, heterotrophic prokaryote
394 responses to inorganic nutrients were similar between coastal and oceanic waters
395 (ANOVA, $F(1,34) = 1.68$, $p = 0.203$). The addition of inorganic nutrients caused
396 significant increases in Chl-*a* in 31 out of the 36 experiments (Fig. 5 and Fig S2 in the
397 supplement), while prokaryotes increased their biomass in 19 out of 36 experiments (Fig.
398 6 and Fig. S2 in the Supplement).

399 The addition of B12 stimulated phytoplankton in 5 out of 36 experiments (Fig. 5 and Fig.
400 S3 in the Supplement) and prokaryotes in 6 experiments (Fig. 6 and Fig. S4 in the
401 Supplement). Chl-*a* increased in 3, and prokaryote biomass in 7 out of 36 experiments

402 after adding B1 (Fig. 5 and Fig. 6). B vitamins also caused negative responses of
403 phytoplankton (Fig. 5 and Fig. S3 in the Supplement) and prokaryote biomass (Fig. 6 and
404 Fig. S4 in the Supplement). The addition of vitamins induced decreases of Chl-*a* in 6
405 experiments (4 after adding B12 and 2 after adding B1) and prokaryote biomass in 14
406 experiments (6 after adding B12 and 8 after adding B1). Secondary limitation by B1
407 and/or B12 was occasionally observed when inorganic nutrients were limiting, leading to
408 a higher biomass increase in the treatments including both inorganic nutrients and
409 vitamins as compared to the inorganic nutrient addition alone (Fig. 5, Fig. 6 and Fig. S3
410 and Fig. S4 in the Supplement). In the case of Chl-*a*, secondary limitation by B-vitamins
411 was found in the C-b-surface, Oc-a-SCM and Oc-b-SCM experiments in February, in the
412 C-b-surface and C-b-SCM experiments in April, and in the C-b-SCM, Oc-b-SCM and
413 Oc-c-surface experiments in August (Fig. 5).

414 In order to quantify the relevance of inter-day variability, we calculated the mean
415 coefficient of variation (CV) of the responses to B vitamins (i.e., excluding the responses
416 to inorganic nutrients, and normalizing the responses of the nutrient and vitamin
417 combined treatments to the corresponding response to inorganic nutrients alone) within
418 sampling periods for each sampling point (2 stations and 2 depths). The CV ranged from
419 9%, in subsurface oceanic waters in April, to 34% in surface coastal waters in April,
420 averaging 16 ± 6 (SD) % (data not shown). Considering that short-term (within sampling
421 period) variability was overall very low, and for simplicity, we averaged the responses to
422 B vitamins in the 3 experiments conducted at each of the 12 sampling points to further
423 describe spatial and temporal patterns in the response to B vitamin amendments (Fig. 7).

424 When averaging the responses within each sampling point (Fig. 7), some general patterns
425 emerge. Both phytoplankton and prokaryotes showed more negative than positive
426 responses to B1 and/or B12 amendments. Most positive responses occurred at the oceanic

427 station (83.3%), while negative responses dominated in the coast (61.5%). Phytoplankton
428 significant positive responses mostly occurred in February, showing an average increase
429 of up to 1.2-fold in coastal subsurface waters after B12+B1 amendment (Fig. 7a). The
430 largest significant increase in Chl-a (ca. 1.4-fold) occurred in April after the combined
431 addition of B12 and B1 in coastal surface waters. Significant positive prokaryote
432 responses mainly occurred in August, when the largest increase (ca. 1.3-fold) occurred in
433 coastal subsurface waters after B1 amendment (Fig. 7b). Most positive responses were
434 associated with treatments containing B12 either alone or combined with B1 (Fig. 7b).
435 Phytoplankton primary B1 limitation was only found at the oceanic SCM in February
436 (Fig. 7a), while prokaryote primary B1 limitation only occurred at the coastal SCM in
437 August. In addition, prokaryote secondary B1 limitation occurred in oceanic surface
438 waters in February and August.

439 **3.3 B-vitamin response patterns in relation to environmental factors and prokaryote** 440 **and eukaryote community composition**

441 In order to explore the controlling factors of the observed B-vitamin response patterns,
442 the correlation between the B-vitamin response resemblance matrix and the
443 corresponding resemblance matrices obtained from the initial environmental factors, the
444 initial prokaryotic community composition, or the initial eukaryotic community
445 composition were calculated. While eukaryotic community composition did not show a
446 significant correlation with the B-vitamin responses (Spearman Rho = 0.05, $p = 0.39$), the
447 prokaryotic community composition was significantly correlated with the B-vitamin
448 responses (Spearman Rho = 0.31, $p = 0.041$). We then used distance-based linear
449 modelling (DistLM) to identify the prokaryotic taxa which best explained the microbial
450 plankton responses to B-vitamins (Fig. 8). The resulting model explained 78% of the
451 variation and included seven prokaryotic groups: *Planktomarina* (24%), Actinobacteria

452 (14%), SAR11 (8.2%), Cellvibrionales (8.5%), Euryarchaeota (8.7%), Flavobacteriales
453 (9%) and *Synechococcus* (6.1%). The sequential test identified *Planktomarina* and
454 Actinobacteria as the taxa explaining the largest fraction of variation (ca. 24 % and 14%,
455 respectively, data not shown). The total variation explained by the db-RDA1 (34.9%) and
456 db-RDA2 (24.5%) was 59.4 %, both represented as x and y axis, respectively (Fig. 8).
457 The db-RDA1 axis separated, to some extent, coastal samples, where negative responses
458 to B vitamins dominated, from oceanic samples, where most positive responses were
459 found (Fig. 7). The db-RDA plot showed that Cellvibrionales and *Planktomarina*
460 positively correlated with axis 1, while SAR11 and *Synechococcus* showed negative
461 correlation with axis 1. Flavobacteriales and Actinobacteria mostly correlated with the
462 db-RDA2 axis.

463

464 **4 Discussion**

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

473 Considering the high short-time variability of the hydrographic conditions in the area
474 (Alvarez-Salgado et al., 1996), we expected a large inter-day variation in the responses
475 to B vitamin amendments. By contrast, inter-day variability of microbial responses to B

476 vitamins and microbial plankton community composition was relatively small (Fig. 5,
477 Fig. 6, Fig. S1 and Fig. S2 in the supplement). The reduced short-term variability in the
478 responses to B vitamins additions suggested that B vitamin availability might be
479 controlled by factors operating at larger temporal scales, such as the succession of
480 microbial communities associated to seasonal environmental variation (Hernández-Ruiz
481 et al., 2018; Hernando-Morales et al., 2018). Considering this, and for further discussion,
482 we averaged the responses from the three experiments conducted during each sampling
483 period, resulting in 12 experimental situations (2 stations \times 2 depths \times 3 periods). Overall,
484 phytoplankton and/or prokaryote growth enhancement in at least one B vitamin treatment
485 was frequent but relatively small in this productive ecosystem, showing 1.1 to 1.3-fold
486 increases in 75% of the experimental situations for phytoplankton and in 50% for
487 prokaryotes. On the other hand, negative responses to at least one B vitamin treatment
488 occurred in 83% of the experimental situations for phytoplankton and in 67% for
489 prokaryotes (Fig. 7). The low and constant B12 ambient concentration (Fig. 4c) and the
490 reduced magnitude of microbial responses suggest a close balance between production
491 and consumption of this growth factor. Different patterns of response to B-vitamin
492 amendments were observed in phytoplankton and prokaryotes (Fig. 7), which appear to
493 be mostly explained by the prokaryotic community composition (Fig. 8).

494 **4.1 Positive responses to vitamin B1 and B12 amendments**

495 The experimental design allowed the detection of two categories of B vitamin dependency
496 of the microbial plankton community. A primary limitation by B vitamins occurs when
497 microorganisms respond to additions of B vitamins alone. A secondary limitation by B
498 vitamins arises when the response to the combined addition of B vitamins and inorganic
499 nutrients is significantly higher than that to inorganic nutrients alone. Such response
500 occurs because of the ambient B-vitamin depletion associated to the plankton growth after

501 inorganic nutrient enrichment. Most positive (72% for phytoplankton and 60 % for
502 prokaryotes) responses occurred after single B-vitamins additions, suggesting that
503 inorganic nutrient availability enhance B-vitamin production by the prototrophic
504 microbes. Under nutrient-limiting conditions, the external supply of vitamins could
505 reduce the energy costs associated to its synthesis (Jaehme and Slotboom, 2015),
506 stimulating the growth not only of auxotrophs but also of prototrophs.

507 The significant positive effects of B12 and/or B1 addition, suggest that these compounds
508 may be eventually limiting microbial growth in marine productive ecosystems, as
509 previously observed by other authors (e.g., Panzeca et al., 2006; Sañudo-Wilhelmy et al.,
510 2006; Bertrand et al., 2007; Gobler et al., 2007; Koch et al., 2011; 2012; Barber.-Lluch et
511 al., 2019). Most positive responses to B vitamin amendments were observed in oceanic
512 waters, where B12 concentration was significantly lower than in coastal waters (Fig. 4c).
513 Unfortunately we lack B1 measurements in this study, but, according to previous field
514 studies in other oceanographic regions, a similar pattern to that observed for B12 can be
515 expected (Cohen et al., 2017; Sañudo-Wilhelmy et al., 2012; Suffridge et al., 2018). The
516 overall low and stable concentration of B12 at both sampling locations suggests a high
517 turnover time of this compound in these productive, well-lit waters. Rapid cycling of B12
518 in surface waters may occur due to high biological uptake rates (Taylor and Sullivan,
519 2008; Koch et al., 2012) and/or photochemical degradation (Carlucci et al., 1969;
520 Juzeniene and Nizauskaite, 2013; Juzeniene et al., 2015). The measured B12
521 concentrations were in the lower range reported for coastal sites, and similar to that found
522 in the upwelling system off the California coast in the San Pedro Basin during winter,
523 spring and summer (Panzeca et al., 2009).

524 The increase of Chl-*a* was mostly associated to B12 amendments, which is consistent
525 with the known incapability of eukaryotes to synthesize this vitamin (Croft et al., 2005;

526 Tang et al., 2010; Sañudo-Wilhelmy et al., 2014). Considering the very low concentration
527 of B12 in the sampling area, the relatively limited phytoplankton response to B vitamins
528 suggests that the existing species might have adapted to overcome B12 shortage. For
529 example, changes in external B12 availability may cause shifts from vitamin B12-
530 dependence to vitamin B12-independence in taxa possessing the vitamin B12-
531 independent methionine synthase (MetE) gene (Bertrand et al., 2013; Helliwell et al.,
532 2014). Other strategies used by phytoplankton to cope with low B12 concentration
533 include, increased cobalamin acquisition machinery, decreased cobalamin demand, and
534 management of reduced methionine synthase activity through changes in folate and S-
535 adenosyl methionine metabolism (Bertrand et al., 2012). The available data on B12 half-
536 saturation constants for phytoplankton ($0.1\text{--}10\text{ pmol l}^{-1}$) (Droop, 1968, 2007; Taylor and
537 Sullivan, 2008; Tang et al., 2010; Koch et al., 2011) are similar or higher than the B12
538 concentrations measured here (0.3 pmol l^{-1} in the coastal and 0.15 pmol l^{-1} in the oceanic
539 waters, on average), reinforcing the hypothesis of a phytoplankton community adapted to
540 B12 limiting concentrations in this upwelling system.

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

550 Microbial responses to B vitamins in subsurface oceanic waters in February were
551 associated to high abundance of *Synechococcus* and, to some extent, of Actinobacteria
552 (Fig. 8). In these experiments, positive effects of B1 addition on phytoplankton and
553 prokaryotes were observed (Fig. 7). While *Synechococcus* is capable of B1 synthesis
554 (Carini et al., 2014; Sañudo-Wilhelmy et al., 2014; Gómez-Consarnau et al., 2018),
555 Actinobacteria seems to have a strong dependence on this vitamin (Gómez-Consarnau et
556 al., 2018). Among the sequenced eukaryote genomes, only Stramenopiles contain genes
557 codifying for the synthesis of thiamine monophosphate (Sañudo-Wilhelmy et al., 2014;
558 Cohen et al., 2017). While Stramenopiles, dominated by Bacillariophyta, were ubiquitous
559 in the sampling area, their relative contribution was lower in oceanic waters (Fig. 4a).
560 The simultaneous stimulation of phytoplankton and prokaryotes by B1 addition in
561 subsurface oceanic waters in winter suggest a strong demand for this compound under
562 these particular conditions, however what triggers the observed responses remain unclear.
563 Even though B1 caused a significant effect on phytoplankton only in subsurface waters
564 in winter, half of the positive responses of prokaryotes were associated to B1 supply (Fig.
565 7b). This pattern is consistent with the recently described widespread dependence of
566 bacterioplankton on external B1 supply (Paerl et al., 2018). B1 stimulated prokaryote
567 growth in subsurface coastal waters and surface oceanic waters in summer (Fig. 7b), when
568 the B vitamin response patterns were associated to high abundance of *Planktomarina* and
569 Actinobacteria (Fig. 8), which are expected to strongly depend on external B1 sources
570 (Giebel et al., 2013; Gómez-Consarnau et al., 2018). The generalized significant and
571 positive responses of prokaryotes to vitamin treatments in surface oceanic waters in
572 summer, when the prokaryote biomass was high and dissolved inorganic nitrogen
573 concentration was very low (Fig. 3i), suggest that prokaryotes may have an advantage in
574 the uptake and assimilation of B vitamins under nitrogen limiting conditions. This is

575 consistent with the observation of small (0.7–3 μm)-plankton cells containing more B1
576 than larger cells (Fridolfsson et al., 2019). Following this, it has been speculated that
577 bacteria and small phytoplankton can transfer B1 to large cells through predation by
578 acting as an important source of this compound in the marine environment (Fridolfsson
579 et al., 2019).

580 **4.2 Negative responses to vitamin B1 and B12 amendments**

581 Similar experiments conducted in this area also reported negative responses of microbial
582 plankton to vitamin B12 additions (Barber-Lluch et al., 2019). The predominantly
583 negative prokaryote responses after vitamin amendments in the coast during summer (Fig.
584 7b), when nutrient concentrations were low (Fig. 3), suggest either a strong competition
585 between phytoplankton and prokaryotes or a stimulation of predation. Dinoflagellates
586 were particularly abundant in summer at both sampling sites and depths. Many
587 dinoflagellate species are auxotrophs for B1 and/or B12 (Croft et al, 2006; Tang et al.,
588 2010), and also many of them are phagotrophs (Stoecker and Capuzzo, 1990; Smayda,
589 1997; Sarjeant and Taylor, 2006; Stoecker et al., 2017), thus the external supply of B
590 vitamins may have promoted their growth, ultimately leading to net decreases in
591 microbial biomass at the end of the experiments. Several studies demonstrated that
592 vitamin B12 is implicated in the occurrence of dinoflagellate blooms around the world
593 (Aldrich, 1962; Carlucci and Bowes, 1970; Takahashi and Fukazawa, 1982; Yu and
594 Rong-cheng, 2000). It has been suggested that the B12-dependent enzyme
595 methylmalonyl-CoA mutase in dinoflagellate, euglenoid, and heterokont algae allows
596 them to grow heterotrophically when B12 is available (Croft et al., 2006). Therefore, the
597 B12 enrichment could trigger such nutritional strategy, particularly in summer, when
598 mineral nutrients are less available, resulting in an increased predation pressure on
599 prokaryotes.

600 The B vitamin response patterns in surface coastal waters in summer (Fig. 7), seemed to
601 be associated with high abundance of Flavobacteriales (Fig. 8). All isolates of
602 Bacteroidetes sequenced so far are predicted to be B12 auxotrophs (Sañudo-Wilhelmy et
603 al., 2014; Gómez-Consarnau et al., 2018) and recent metatranscriptomic analyses reveal
604 that B1 synthesis gene transcripts are relatively low in Flavobacteria as a group (Gómez-
605 Consarnau et al., 2018). As both phytoplankton and prokaryotes are dominated by
606 potentially B12 and B1 auxotrophs (dinoflagellates and Flavobacteriales) in the coast
607 during summer (Fig. 4b), the negative responses could be the result of strong competition
608 for B vitamins. However, the negative responses to B vitamins of both phytoplankton and
609 prokaryotes in surface coastal water in summer suggests an increase in phytoplankton and
610 prokaryote predation by mixotrophs rather than competition between them. By contrast,
611 prokaryotes and phytoplankton showed opposite patterns of response to B vitamins in
612 subsurface coastal waters in summer, which suggests competition between both microbial
613 compartments (Fig. 7). While phytoplankton negatively responded only to single B
614 vitamin additions, prokaryotes responded negatively only when both inorganic nutrients
615 and B vitamins were added (Fig. 7). It is conceivable that phytoplankton had an advantage
616 over prokaryotes when mineral nutrients were added. This hypothesis contrasts with
617 previous studies reporting that B12 and B1 vitamin uptake is dominated by picoplankton
618 (Koch et al., 2011, 2012), strongly suggesting that bacteria could outcompete larger
619 phytoplankton for vitamin uptake. By contrast, Koch et al. (2014), found that carbon-
620 specific B12 uptake by large phytoplankton was significantly lower during non-bloom
621 (low nutrient concentration) compared to bloom conditions (high nutrient concentration),
622 which suggests better competitive ability under nutrient-rich conditions.

623 **5 Conclusions**

624 In conclusion, our findings suggest that the heterogeneous responses of microbial
625 plankton to B1 and B12 vitamins supply in this coastal upwelling system could be
626 partially controlled by the composition of the prokaryote community, which is consistent
627 with their previously reported major role as B12 producers and B1 consumers. Even
628 though we lack data on B1 concentration, the overall moderate responses together with
629 the low ambient B12 concentration, suggest that the microbial plankton community in
630 this area could be well adapted to cope with B vitamin shortage and that a close balance
631 exists between production and consumption of these important growth factors.

632

633 *Author contribution.*

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

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

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647

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984

985 Table 1

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	Treatment	Nutrient included	Concentration
1.	Control (C)	No nutrient added	
2.	Inorganic nutrients (I)	NO_3^-	$5 \mu\text{mol l}^{-1}$
		NH_4^+	$5 \mu\text{mol l}^{-1}$
		HPO_4^{2-}	$1 \mu\text{mol l}^{-1}$
		SiO_4^{2-}	$5 \mu\text{mol l}^{-1}$
3.	Vitamin B12 (B12)	B12	100 pmol l^{-1}
4.	Vitamin B1 (B1)	B1	600 pmol l^{-1}
5.	B12 + B1	B12	100 pmol l^{-1}
		B1	600 pmol l^{-1}
6.	I + B12	NO_3^-	$5 \mu\text{mol l}^{-1}$
		NH_4^+	$5 \mu\text{mol l}^{-1}$
		HPO_4^{2-}	$1 \mu\text{mol l}^{-1}$
		SiO_4^{2-}	$5 \mu\text{mol l}^{-1}$
		B12	100 pmol l^{-1}
7.	I + B1	NO_3^-	$5 \mu\text{mol l}^{-1}$
		NH_4^+	$5 \mu\text{mol l}^{-1}$
		HPO_4^{2-}	$1 \mu\text{mol l}^{-1}$
		SiO_4^{2-}	$5 \mu\text{mol l}^{-1}$
		B1	600 pmol l^{-1}
8.	I + B12 + B1	NO_3^-	$5 \mu\text{mol l}^{-1}$
		NH_4^+	$5 \mu\text{mol l}^{-1}$
		HPO_4^{2-}	$1 \mu\text{mol l}^{-1}$
		SiO_4^{2-}	$5 \mu\text{mol l}^{-1}$
		B12	100 pmol l^{-1}
		B1	600 pmol l^{-1}

988

989

990 **6 Tables and Figures**

991 **Table 1:** Eight different treatments were applied consisting of: (1) control treatment (C):
992 no nutrients added; (2) inorganic (I) nutrient treatment: 5 μM nitrate (NO_3^-), 5 μM
993 ammonium (NH_4^+), 5 μM silicate (SiO_4^{2-}) and 1 μM phosphate (HPO_4^{2-}); (3) vitamin B12
994 treatment: 100 pmol l^{-1} ; (4) vitamin B1 treatment: 600 pmol l^{-1} ; (5) inorganic nutrients
995 and vitamin B12 (I+B12) treatment; (6) Inorganic nutrients and vitamin B1 (I+B1)
996 treatment; (7) vitamins B12 and B1 (B12+B1) treatment and (8) Inorganic nutrients with
997 vitamins B12 and B1 (I+B12+B1) treatment.

998

999 **Figure 1:** (a) The NW Iberian margin (rectangle) and locations of the stations that were
1000 sampled in the Ría de Vigo (C) and on the shelf (Oc) (diamonds), (b) distribution of daily
1001 coastal upwelling index (UI) and (c) registered precipitations during each sampling period
1002 showing the initial time of each experiment (C-a, C-b, C-c and Oc-a, Oc-b, Oc-c).

1003

1004 **Figure 2:** Vertical distribution over time in the coastal station of Chl-*a* ($\mu\text{g l}^{-1}$) in (a)
1005 February, (b) April and (c) August; temperature ($^\circ\text{C}$) in (g) February, (h) April and (i)
1006 August; and salinity (PSU) in (m) February, (n) April and (o) August. Vertical
1007 distribution over time in the oceanic station of Chl-*a* ($\mu\text{g l}^{-1}$) in (d) February, (e) April
1008 and (f) August; temperature ($^\circ\text{C}$) in (j) February, (k) April and (l) August; and salinity
1009 (PSU) in (p) February, (q) April and (r) August Dots show the t_0 of the experiments. Chl-
1010 *a*: Chlorophyll-*a* concentration.

1011

1012 **Figure 3:** Initial biological conditions and abiotic factors at the coastal and oceanic
1013 sampling stations. Each bar corresponds to one of the 3 experiments performed in each
1014 depth and station during February, April and August. (a, b, c), Chl-*a*, total Chl-*a* ($\mu\text{g l}^{-1}$).

1015 Note that the y-axis is broken; (d, e, f) PB, prokaryote biomass ($\mu\text{g C l}^{-1}$); (g, h, i) DIN,
1016 dissolved inorganic nitrogen ($\mu\text{mol l}^{-1}$) and (j, k, l) DIN:DIP, ratio inorganic
1017 nitrogen:phosphate. The blue line shows the Redfield ratio (16:1) and SCM refers to the
1018 sub-surface chlorophyll maximum. Chl-*a*: Chlorophyll-*a* concentration.

1019

1020 **Figure 4:** Averaged relative contribution of reads to the major taxonomic groups of (a)
1021 eukaryotes and (b) prokaryotes at surface (surf) and SCM in the coastal and oceanic
1022 station in February, April and August. (c) Averaged B12 concentration (pmol l^{-1}) at
1023 surface (surf) and SCM in the coastal and oceanic station in February, April and August.
1024 Error bars represent standard error. SCM refers to the sub-surface chlorophyll maximum.

1025

1026 **Figure 5:** Chlorophyll-*a* concentration ($\mu\text{g l}^{-1}$) in the t0 of each experiment (striped bars)
1027 and in the endpoint of each treatment (colored bars) in the experiments conducted at (a)
1028 surface and (b) SCM in the coastal and at (c) surface and (d) SCM in the oceanic station
1029 in February, April and August. Error bars represent standard error. Note that the y-axis is
1030 broken. SCM: sub-surface chlorophyll maximum.

1031

1032 **Figure 6:** Prokaryote biomass ($\mu\text{g C l}^{-1}$) in the t0 of each experiment (striped bars) and in
1033 the endpoint of each treatment (colored bars) in the experiments conducted at (a) surface
1034 and (b) SCM in the coastal and at (c) surface and (d) SCM in the oceanic station in
1035 February, April and August. Error bars represent standard error. Note that the y-axis is
1036 broken. SCM: sub-surface chlorophyll maximum.

1037

1038 **Figure 7:** Monthly averaged response ratio (RR) of (a) Chl-*a* or (b) prokaryote biomass
1039 at surface and SCM in the coastal and oceanic station. Horizontal line represents a

1040 response equal to 1, that means no change relative to control in the pink dots (treatments
1041 with vitamins alone) and no change relative to inorganic (I) treatment in the green dots
1042 (vitamins combined with I treatments). Asterisks indicate averaged RRs that were
1043 significantly different from 1 (Z-test; * $p < 0.05$) and “a” symbols indicate averaged RRs
1044 that were marginally significant (Z-test; ^a $p = 0.05-0.06$). Error bars represent standard error.
1045 SCM: sub-surface chlorophyll maximum.

1046

1047 **Figure 8:** Distance based redundancy analysis (dbRDA) of B vitamin responses by
1048 phytoplankton and prokaryotes based on Bray-Curtis similarity. Only prokaryotic taxa
1049 that explained variability in the B vitamin responses structure selected in the DistLM
1050 model (step-wise procedure with adjusted R^2 criterion) were fitted to the ordination.
1051 Filled and open symbols represent samples from coastal and oceanic station, respectively,
1052 triangles and circles represent samples from surface and SCM, respectively, and colours
1053 correspond to the months: (green) February, (blue) April and (pink) August. SCM: sub-
1054 surface chlorophyll maximum.