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

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Abstract. We evaluated the temporal (inter-day and inter-season) and spatial variability in microbial plankton responses to vitamins B12 and B1 supply in coastal and oceanic waters. Inter-day variability in microbial plankton responses to B-vitamins was not of great concern, suggesting that B-vitamins availability was controlled by factors operating at larger temporal scales, such as those driving microbial community seasonal succession. 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, which is coherent with their widespread dependence on exogenous sources for 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-vitamins 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 suggests the role of biologically active organic compounds, such as B-vitamins, on the control of marine productivity in both coastal and oceanic waters (Bertrand et al., 2007; Gobler et al., 2007; Koch et al., 2011; Panzeca et al., 2006). B-vitamins act as cofactors for enzymatic reactions and are involved in many important metabolic pathways

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

(Madigan et al., 2005; Marsh, 1999; Monteverde et al., 2017). Vitamin B12 (B12 herein), which is exclusively synthesized by prokaryotes (Roth et al, 1996; Martens et al., 2002; Warren et al., 2002), acts as a cofactor of three enzymes in eukaryotes (methionine synthase, methylmalonyl-coA mutase and ribonucleotide reductase type II) (Bertrand and Allen 2012, Helliwell et al., 2011). In comparison, over 20 different cobalamin-dependent enzymes are found in bacteria (Roth et al., 1996), making B12 critically important also for these organisms. Vitamin B1 (B1 herein) plays a pivotal role in intermediary carbon metabolism and is a cofactor for a number of enzymes involved in primary carbohydrate and branched-chain amino acid metabolism (Croft et al., 2006).

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

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

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 communities has been only assessed by Gobler et al. (2007).

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,

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

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 (seasonal and short-term) 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 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, coinciding with the spring bloom, and April and August during

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

112 the early and late summer upwelling, respectively. During each cruise, 12 enrichment
113 experiments were carried out on board, 3 experiments in each station (3a, 3b & 3c and
114 6a, 6b & 6c, respectively) with water from two different depths. Surface and sub-surface
115 chlorophyll maximum (SCM) samples were taken at 5 m and at the maximum
116 fluorescence depth, between 10 m and 50 m according to the CTD data, respectively (Fig.
117 2). We failed to sample the SCM on two occasions, due to large vertical displacements
118 between the downward and the upward casts. Vertical profiles of temperature, salinity
119 and chlorophyll fluorescence were obtained using a regular CTD-rosette down to 60 m in
120 the coastal station and to 200 m in oceanic station. Samples for phytoplankton and
121 bacterial biomasses, dissolved nutrient concentration, including vitamin B12, and
122 microbial plankton community were collected at the beginning of each experiment.

123 Seawater samples were gently pre-filtered through a 200 µm mesh to exclude large
124 zooplankton in order to ensure good replicability. Following sample collection, 300 ml
125 PAR and UVR transparent (whirl-pak) bags were filled and nutrients were added
126 establishing eight different enrichment treatments as follows: (1) control treatment (C):
127 no nutrients added; (2) inorganic nutrient treatment (I): 5 µM nitrate (NO_3^-), 5 µM
128 ammonium (NH_4^+), 5 µM silicate (SiO_4^{2-}) and 1 µM phosphate (HPO_4^{2-}); (3) vitamin B12
129 (Sigma, V2876) treatment: 100 pM; (4) vitamin B1 (Sigma, T4625) treatment: 600 pM);
130 (5) Inorganic nutrients and vitamin B12 (I+B12) treatment; (6) Inorganic nutrients and
131 vitamin B1 (I+B1) treatment; (7) vitamins B12 and B1 (B12+B1) treatment and (8)
132 Inorganic nutrients with vitamins B12 and B1 (I+B12+B1) treatment. Inorganic nutrients
133 were added to avoid that inorganic nutrient limitation masked the responses to B vitamins.
134 Each treatment had 3 replicates resulting in 24 whirl-pack bags per experiment. To assess
135 short-term effects of nutrient inputs, experimental bags were incubated on-deck during

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

72 h under natural light conditions. In-situ temperature was reproduced by submerging the bags in tanks connected to the surface-water pump system.

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 -20 ºC until further analysis. Chl-*a* was extracted with 90 % acetone and kept in darkness at 4 ºC 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.

2.3 Flow cytometry

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

2.4 Nutrients

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

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

2.5 Vitamin B12

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

Detection and quantification of dissolved vitamin B12 (cyanocobalamin and hydroxocobalamin) was conducted using an Agilent 1290 Infinity LC system (Agilent Technologies, Waghaeusel-Wiesental, Germany), coupled to an Agilent G6460A triple quadrupole mass spectrometer equipped with an Agilent Jet Stream ESI source. The LC system used a C18 reversed-phase column (Agilent Zorbax SB-C18 Rapid Resolution HT (2.1 × 50 mm, 1.8 µm) with a 100 µl sample loop. Agilent Technologies software was

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

used for data acquisition and analysis. Chromatographic separation was performed using MeOH and water LCMS grade, both buffered to pH 5 with 0.5 % acetic acid, as mobile phases in a 15 minutes' gradient. Gradient starting at 7 % MeOH for 2 min, changing to 100 % MeOH by minute 11, continuing at 100 % MeOH until 13.5 min and returning to initial conditions to complete 15 min.

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 package DADA2 (https://www.nature.com/articles/nmeth.3869). SILVA reference database (Quast et al., 2012) was used to taxonomic assignment of 16S OTUs and PR2 (Guillou et al., 2012) and the marine protist database from the BioMarks project (Massana et al., 2015) were used to taxonomic assignment of 18S OTUs.

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

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

2.7 Statistical analysis

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

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

234 significant at the 0.05 significance level and p-value was standardized as proposed by
235 Good (1982) in order to overcome the low number of replicates. Differences between
236 station and depth (spatial variability) and among sampling months (temporal variability)
237 in the responses to B vitamins were evaluated with factorial analysis of variance
238 (ANOVA). Bonferroni post hoc tests analyses were conducted to test which treatments
239 were significantly different from the control treatment in each experiment. Z-test was
240 used to evaluate the significance of the average B vitamins response ratios for each period,
241 sampling site and depth. In order to determine which factors better explain B-vitamin
242 response patterns, we calculated the correlation between the B vitamin response
243 resemblance matrix and the corresponding resemblance matrices of (a) abiotic variables,
244 (b) prokaryote community composition, and (c) eukaryotic community, using the
245 RELATE analysis implemented in PRIMER6 (Clarke and Warwick, 2001; Clarke and
246 Gorley, 2006). In order to highlight which specific taxonomic groups are associated to
247 changes of microbial plankton (bacterioplankton and phytoplankton) responses to vitamin
248 B1 and B12, we conducted a distance based redundancy analysis (dbRDA) combined
249 with a distance linear-based model (DistLM) using a step-wise procedure and adjusted r^2
250 as selection criteria) using the PRIMER6 software. Correlations among the prokaryotic
251 taxa best explaining the microbial plankton responses to B-vitamins and phytoplankton
252 and bacterial responses to different B vitamin treatments (including primary and
253 secondary responses) were calculated using Pearson's correlations.

254

255 **3 Results**

256 **3.1 Initial conditions**

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

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

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

268 In August, strong thermal stratification was observed at both stations (Fig. 2). At the
269 beginning of the cruise, high Chl-*a* concentration (close to 20 µg l^{-1}) was observed in
270 subsurface water. These high Chl-*a* levels were maintained until day 4 and then
271 decreased, reaching minimum values by day 7, coinciding with upwelling relaxation (Fig.
272 1b, Fig. 2). Salinity minima during day 1 and 5 reflect precipitation events. Chl-*a* was
273 relatively low at the oceanic station, an increased by the end of the sampling period as a
274 consequence of an upwelling event, that brought cold and nutrient rich water to the
275 surface, at day 5 (Fig. 2).

276 Abiotic and biotic conditions at the beginning of each experiment are shown in Fig. 3.
277 Overall, the concentration of dissolved inorganic nitrogen (DIN) was higher at the coastal
278 than at the oceanic station, where very low levels were measured in August (Fig. 3). At
279 the coastal station, higher DIN concentrations were observed in surface compared to
280 subsurface waters. The DIN:DIP (dissolved inorganic phosphorous) ratio was always
281 lower in open ocean than in the coastal station and mostly below of Redfield ratio.

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

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. 2 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

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

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 0.55 pmol l^{-1} (Table S1 in the Supplement) Mean B12 concentration was significantly higher in the coast (0.30±0.13 pmol l^{-1}) than in the ocean (0.15±0.12 pmol l^{-1}) (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 magnitude of phytoplankton and bacteria responses (i.e., the response ratios) to the different addition treatments differed between sampling stations (ANOVA, $p = 0.018$) and among sampling periods (ANOVA, $p = 0.014$). The most prominent responses of phytoplankton, compared to the control treatment, occurred after inorganic nutrient amendments, especially in surface oceanic waters (Fig. 1 in the Supplement). The magnitude of the phytoplankton response to inorganic nutrients was significantly higher in oceanic than in coastal waters (ANOVA, $p = 0.028$). Bacteria responded comparatively less than phytoplankton to inorganic nutrients and there were no significant differences between coastal and oceanic waters (ANOVA, $p = 0.203$). The addition of inorganic nutrients caused significant increases in phytoplankton biomass in 31 out of the 36 experiments, and in 19 out of 36 experiments in bacterial biomass (Fig. S1 in the Supplement).

The addition of B12 stimulated phytoplankton growth in 5 out of 36 experiments while bacteria responded positively to B12 in 6 experiments (Fig. 5). Phytoplankton biomass increased in 3, and bacterial biomass in 7 out of 36 experiments after adding B1. B

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

vitamins also caused negative responses of phytoplankton and bacterial biomass (Fig. 5). The addition of vitamins induced decreases of phytoplankton biomass in 6 experiments (4 after adding B12 and 2 after adding B1) and bacterial biomass in 14 experiments (6 after adding B12 and 8 after adding B1). Additions of inorganic nutrients combined with B-vitamins caused a similar increase in phytoplankton or bacterial biomass than the inorganic addition alone in most of the experiments. Secondary limitation by B1 and/or B12 was occasionally observed when inorganic nutrients were limiting, leading to a higher biomass increase in the treatments including both inorganic nutrients and vitamins as compared to the inorganic nutrient addition alone (Fig. 5).

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

3.3 B-vitamin response patterns in relation to abiotic and biotic factors

When averaging the responses within each sampling point (Fig. 6), some general patterns emerge. Both phytoplankton and bacteria showed more negative than positive responses to B1 and/or B12 amendments. Most positive responses occurred at the oceanic station, while negative responses dominated in the coast. Phytoplankton significant positive

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

357 responses mostly occurred in February, showing an average increase of up to 1.2-fold in
358 coastal subsurface waters after B12+B1 amendment (Fig. 6). The largest significant
359 increase in phytoplankton biomass (ca. 1.4-fold) occurred in April after the combined
360 addition of B12 and B1 in coastal surface waters. Significant positive bacterial responses
361 mainly occurred in August, when the largest increase (ca. 1.3-fold) occurred in coastal
362 subsurface waters after B1 amendment (Fig. 6). Most positive responses were associated
363 with treatments containing B12 either alone or combined with B1 (Fig. 6). Phytoplankton
364 primary B1 limitation was only found at the oceanic SCM in February (Fig. 6), while
365 bacterial primary B1 limitation only occurred at the coastal SCM in August. In addition,
366 bacterial secondary B1 limitation occurred in oceanic surface waters in February and
367 August.

368 In order to explore the controlling factors of the observed B-vitamin response patterns,
369 the correlation between the B-vitamin response resemblance matrix and the
370 corresponding resemblance matrices obtained from the abiotic factors, the prokaryotic
371 community composition, or the eukaryotic community composition was calculated. Only
372 the prokaryotic community composition significantly correlated with the B-vitamin
373 responses (Spearman Rho = 0.31, p = 0.041). We then used distance-based linear
374 modelling (DistLM) to identify the prokaryotic taxa which best explained the microbial
375 plankton responses to B-vitamins (Fig. 7). The resulting model explained 78 % of the
376 variation and included seven prokaryotic groups. The sequential test identified
377 *Planktomarina* as the taxon explaining the largest fraction of variation (ca. 24 %) (Fig.
378 7). The total variation explained by the db-RDA1 and db-RDA2 was 59.4 %. The db-
379 RDA1 axis tended to separate coastal, where negative responses to B vitamins dominated,
380 from oceanic samples, where most positive responses were found (Fig. 6 and 7). The db-
381 RDA plot showed that Cellvibrionales and *Plankomarina* highly and positively correlated

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

382 with axis 1, while SAR11 and *Synechococcus* showed negative correlation with axis 1.
383 Flavobacteriales and Actinobacteria mostly correlated with the db-RDA2 axis.

384 Statistically significant correlations were found between several prokaryotic taxa and
385 microbial plankton responses to B vitamins. A statistically significant negative
386 correlation was found between *Planktomarina* abundance and the phytoplankton
387 response to B12 ($r = -0.69$, $p = 0.014$) and the phytoplankton response to B1 ($r = -0.58$, p
388 $= 0.048$). Flavobacteriales abundance showed a strong significant negative correlation
389 with the secondary response of bacteria to B1 addition (i.e. response to I+B1 compared
390 to I) ($r = -0.9$, $p < 0.001$) and the phytoplankton response to B1 ($r = -0.59$, $p = 0.045$). A
391 significantly positive correlation was found between Actinobacteria and the response of
392 bacteria to B12 ($r = 0.61$, $p = 0.036$) and the secondary response of bacteria to B1 with
393 ($r2 = 0.50$, $p = 0.01$). *Synechococcus* and SAR11 also showed a significant positive
394 correlation with secondary responses of bacteria to B vitamins (Table 1).

395

396 **4 Discussion**

397 Although the dependence of phytoplankton on B vitamin has been previously observed
398 in cultures (Droop, 2007) and in natural phytoplankton assemblages in coastal areas
399 (Sañudo-Wilhelmy et al., 2006; C. J. Gobler et al., 2007; Koch et al., 2012, Barber-Lluch
400 et al., 2019), this is, to the best of our knowledge, the most complete study about responses
401 of phytoplankton and bacteria to vitamin B12 and/or B1 addition. The 36 experiments
402 developed in this study have allowed to clarify the paper of vitamins B12 and B1 at
403 different scales. On the one hand, spatial and seasonal differences were evaluated with
404 experiments in the coastal and oceanic stations during the spring bloom in February, April

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

and the upwelling in August. On the other hand, the role of B-vitamins on a very short scale (intra-day) has been studied.

Contrary to our expectations, the frequency of the experiments (every 2-3 days) conducted at different locations during contrasting hydrographic conditions revealed a reduced short-term variability of microbial plankton community composition. The slight responses to B vitamins additions suggested that B vitamin availability was 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 upon B vitamin supply was frequent but relatively moderate in this productive ecosystem, showing 1.1 to 2.4-fold increases in 75 % of the experimental situations, while negative responses to at least one B vitamin treatment occurred in all but one of the experimental situations (Fig. 6). The low and constant B12 ambient concentration and the observed microbial response patterns suggest a close balance between production and consumption of this growth factor. Different patterns of response to B-vitamin amendments were observed in phytoplankton and bacteria, which appear to be mostly explained by the prokaryotic community composition, suggesting that B vitamin bioavailability might be largely controlled by the prokaryote community

4.1 Positive responses to vitamin B1 and B12 amendments

The experimental design allowed the detection of two categories of B vitamin dependency of the microbial plankton community. A primary limitation by B vitamins occurs when microorganisms respond to additions of B vitamins alone, while a secondary limitation

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

by B vitamins arises when the response to the combined addition of B vitamins and inorganic nutrients is significantly higher than that to inorganic nutrients alone, as a result of the ambient B-vitamin depletion associated to the plankton growth after inorganic nutrient enrichment. Most positive (72 % for phytoplankton and 60 % for bacteria) responses occurred after single B-vitamins additions, suggesting that inorganic nutrient availability enhance B-vitamin production by the prototrophic microbes. Under nutrient-limiting conditions, the external supply of vitamins could reduce the energy costs associated to its synthesis (Jaehme and Slotboom, 2015), stimulating the growth not only of auxotrophs but also of prototrophs.

The significant positive effects of B12 and/or B1 addition, suggest that these compounds may be eventually limiting microbial growth in this area, as previously observed by other authors (Panzeca et al., 2006; Sañudo-Wilhelmy et al., 2006; Bertrand et al., 2007; Gobler et al., 2007; Cruz-López and Maske, 2016). 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 is consistent with the expected high turnover time of this compound in productive, well-lit waters (Bertrand et al., 2015), due to both biological uptake (Koch et al., 2012; Taylor and Sullivan, 2008) and 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).

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

455 The increase of phytoplankton biomass was mostly associated to B12 amendments, which
456 is consistent with the known incapability of eukaryotes to synthesize this vitamin (Croft
457 et al., 2005; Tang et al., 2010; Sañudo-Wilhelmy et al., 2014). Considering the very low
458 concentration of B12 in the sampling area, the limited phytoplankton response to B
459 vitamins is consistent with the presence of species that may have adapted to overcome
460 B12 limitation in the environment by using alternative enzymes. For example, changes in
461 external B12 availability may cause shifts from vitamin B12-dependence to vitamin B12-
462 independence in taxa possessing the vitamin B12-independent methionine synthase
463 (MetE) gene (Bertrand et al., 2013; Helliwell et al., 2014). Other strategies used by
464 phytoplankton to cope with low cobalamin concentration include, increased cobalamin
465 acquisition machinery, decreased cobalamin demand, and management of reduced
466 methionine synthase activity through changes in folate and S-adenosyl methionine
467 metabolism (Bertrand et al., 2012). The available data on B12 half-saturation constants
468 for phytoplankton (0.1-10 pM) (Droop, 1968, 2007; Taylor and Sullivan, 2008; Tang et
469 al., 2010; Koch et al., 2011) are similar or higher than the B12 concentrations measured
470 here (0.3 pM in the coastal and 0.15 pM in the oceanic waters, on average), reinforcing
471 the hypothesis of a phytoplankton community adapted to B12 limiting concentrations in
472 this upwelling system.

473 The positive responses of phytoplankton in surface oceanic waters in February were
474 associated with high abundance of *Synechococcus* and SAR11 (Fig. 4, 7). *Synechococcus*
475 produce a B12 analog known as pseudocobalamin, where the lower ligand base adenine
476 replaces 5,6-dimethylbenzimidazole (DMB) (Helliwell et al., 2016). In natural
477 conditions, pseudocobalamin is considerably less bioavailable to eukaryotic algae than
478 other cobalamin forms (Heal et al., 2017; Helliwell et al., 2016). SAR11 do not require
479 B12 and do not have pathways for its synthesis, suggesting that phytoplankton responds

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

to B12 when its synthesis is likely reduced, due to the low abundance of B12 producers. The higher abundance of *Synechococcus* in oceanic compared to coastal waters may explain the low concentration of B12 (Fig. 4).

There were positive effects of B1 addition on phytoplankton and bacteria in subsurface oceanic waters in winter, also associated to high abundance of Synechococcus and, to some extent, of Actinobacteria (Fig. 6 and 7). While *Synechococcus* is capable of B1 synthesis (Carini et al., 2014; Sañudo-Wilhelmy et al., 2014; Gómez-Consarnau et al., 2018), Actinobacteria has a strong dependence on this vitamin (Gómez-Consarnau et al., 2018) and both prokaryotic groups showed a strong positive correlation with secondary responses of bacteria to B1 amendments (Table 1). Among the sequenced eukaryote genomes, only Stramenopiles contain genes codifying for the synthesis of thiamine monophosphate (Cohen et al., 2017; Sañudo-Wilhelmy et al., 2014). The ubiquitous presence of Stramenopiles in the sampling area, dominated by Bacillariophyta, could explain the relatively restricted response of phytoplankton to B1. The simultaneous stimulation of phytoplankton and bacteria by B1 addition suggest a strong demand for this compound under these particular conditions, however what triggers the observed responses remain unclear.

Even though B1 caused a significant effect on phytoplankton only in subsurface waters in winter, half of the positive responses of bacteria were associated to B1 supply (Fig. 6). This pattern is consistent with the recently described widespread dependence of bacterioplankton on external B1 supply (Paerl et al., 2018). B1 stimulated bacterial growth in subsurface coastal waters and surface oceanic waters in summer, associated to high abundance of *Planktomarina* and Actinobacteria (Fig. 6 and 7), which are expected to strongly depend on external B1 sources (Giebel et al., 2013; Gómez-Consarnau et al.,

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

2018). The generalized significant and positive bacterial responses to vitamin treatments in surface oceanic waters in summer, when the bacterial biomass was high and dissolved inorganic nitrogen concentration was very low (Fig. 3) suggest that bacteria may have an advantage in the uptake and assimilation of B vitamins under nitrogen limiting conditions.

4.2 Negative responses to vitamin B1 and B12 amendments

Similar experiments conducted in this area also reported negative responses of microbial plankton to vitamin B12 additions (Barber-Lluch et al., 2019). The generalized bacterial negative responses after vitamin amendments during summer (Fig. 5 and 6), when nutrient concentrations were low (Fig. 3), suggest either a strong competition between phytoplankton and bacteria or a stimulation of grazing and/or bacterivory. Dinoflagellates were particularly abundant in summer at both sampling sites and depths. Many dinoflagellate species are auxotrophs for B1 and/or B12 (Tang et al., 2010), and also many of them are phagotrophs (Sarjeant and Taylor, 2006; Smayda, 1997; Stoecker et al., 2017; Stoecker and Capuzzo, 1990), thus the external supply of B vitamins may have promoted their growth, ultimately leading to net decreases in microbial biomass at the end of the experiments. Several studies demonstrated that vitamin B12 is implicated in the occurrence of dinoflagellate blooms around the world (Aldrich, 1962; Carlucci and Bowes, 1970; Takahashi and Fukazawa, 1982; Yu and Rong-cheng, 2000). It has been suggested that the B12-dependent enzyme methylmalonyl-CoA mutase in dinoflagellate, euglenoid, and heterokont algae allows them to grow heterotrophically when B12 is available (Croft et al., 2006). Therefore, the B12 enrichment could trigger such nutritional strategy, particularly in summer, when mineral nutrients are less available.

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

Strikingly, phytoplankton and bacteria biomass systematically decreased upon B vitamins supply in surface coastal water during summer (Fig. 6), associated to high abundance of Flavobacteriales (Fig. 7). All isolates of Bacteroidetes sequenced so far are predicted to be B12 auxotrophs (Gómez-Consarnau et al., 2018; Sañudo-Wilhelmy et al., 2014) and recent metatranscriptomic analyses revel that B1 synthesis gene transcripts are relatively low in Flavobacteria as a group (Gómez-Consarnau et al., 2018). Therefore, the systematically negative response of bacteria to B vitamins in surface coastal water in summer is most likely associated to increased predation rather than to competition with phytoplankton. By contrast, the negative responses observed in subsurface coastal waters in summer were mostly associated to high abundances of *Planktomarina* and Cellvibrionales (Fig. 7). Both bacterial groups showed a significantly negative correlation with the phytoplankton response to B1 and/or B12 (Table 1) enrichments, which suggests competition between phytoplankton and bacteria. This hypothesis is reinforced by the opposite patterns of response of these two microbial components, while phytoplankton responded negatively only to single B vitamin additions, bacteria responded negatively only when both inorganic nutrients and B vitamins were added (Fig. 6). It is conceivable that phytoplankton had an advantage over bacteria when mineral nutrients were added.

A plausible explanation for these negative responses were the stimulation of grazers or bacterivores upon vitamin B12 addition.

In conclusion, our findings indicate that the heterogeneous responses of microbial plankton to B1 and B12 vitamins supply in this coastal upwelling system is mainly driven 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

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

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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6 Tables and Figures

Table 1: Pearson correlation coefficient of phytoplankton and bacterial responses to different B vitamin treatments (including primary and secondary responses) with the seven prokaryotic taxa which best explained the microbial plankton responses to B-vitamins. Asterisks mean statistically significant Pearson correlation.

		Actinobacteria	Flavobacteriales	Synechococcus	SAR 11	*Planktomarina*	Cellvibrionales	Euryarchaeota
Bacteria	**B12**	0.609*	-0.402	0.407	0.33	-0.202	-0.147	-0.141
	B1	0.003	0.264	-0.112	-0.365	0.097	0.182	-0.211
	B12B1	0.545	-0.158	0.398	0.038	-0.207	0.103	-0.272
	IB12/I	0.566	-0.571	0.576	0.459	-0.239	-0.252	0.087
	IB1/I	0.709*	-0.900*	0.757*	0.818*	-0.487	-0.442	0.297
	IB12B1/I	0.441	-0.568	0.401	0.635*	-0.464	-0.292	0.419
Phytoplankton	**B12**	0.451	-0.43	0.527	0.536	-0.686*	-0.552	0.499
	B1	0.474	-0.587*	0.368	0.566	-0.580*	-0.600*	0.459
	B12B1	0.124	-0.078	0.26	0.233	-0.53	-0.314	0.412
	IB12/I	0.496	-0.302	0.519	0.359	-0.184	-0.287	0.058
	IB1/I	0.029	-0.027	-0.0149	-0.024	0.148	-0.0311	0.109
	IB12B1/I	0.598*	-0.422	0.381	0.347	-0.318	-0.497	0.138

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

Figure 1: (a) The NW Iberian margin (rectangle) and locations of the stations that were sampled in the Ría de Vigo and on the shelf (diamonds), (b) distribution of daily coastal upwelling index (Iw) and (c) registered precipitations during each sampling period.

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

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

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

Figure 5: Response ratio (RR) of total phytoplankton community (smooth bars) and of bacterial biomass (striped bars) at (a) surface and (b) SCM in the coastal station and at (c) surface and (d) SCM in the oceanic waters. Treatments represented are: B12; B1; B12+B1 in pink tones and I+B12/I; I+B1/I; I+B12+B1/I in green tones. Pink bars represent primary responses to B vitamins and green bars represent secondary responses to B vitamins. Horizontal line represents a response equal to 1, that means no change relative to control in the primary responses, and no change relative to inorganic treatment in the secondary responses. Asterisks indicate phytoplankton significant response (t-test;

https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

824 * $p < 0.05$) and circle indicate bacterial significant response (t-test; ^{o} $p < 0.05$). Note that
825 different scales were used.

826 **Figure 6:** Monthly averaged response ratio (RR) of (a) total phytoplankton community
827 and of (b) bacterial community at surface and SCM in the coastal and oceanic station.
828 Horizontal line represents a response equal to 1, that means no change relative to control
829 in the pink bars (treatments with vitamins alone) and no change relative to inorganic (I)
830 treatment in the green bars (vitamins combined with I treatments). Asterisks indicate
831 phytoplankton or bacterial significant response relative to control or I (Z-test; * $p < 0.05$)
832 and a indicate response with a level of significance between 0.05 and 0.1 (Z-test; ^{a} $p =$
833 0.05-0.06).

834 **Figure 7:** Distance based redundancy analysis (dbRDA) of B vitamin responses by
835 microbial plankton based on Bray-Curtis similarity. Filled and open symbols represent
836 samples from coastal and oceanic station, respectively, numbers correspond to the
837 sampling station, triangles and circles represent samples from surface and SCM,
838 respectively, and colours correspond to the months: (green) February, (blue) April and
839 (pink) August. Only prokaryotic taxa that explained variability in the B vitamin responses
840 structure selected in the DistLM model (step-wise procedure with adjusted R^2 criterion)
841 were fitted to the ordination.

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https://doi.org/10.5194/bg-2019-306
Preprint. Discussion started: 22 August 2019

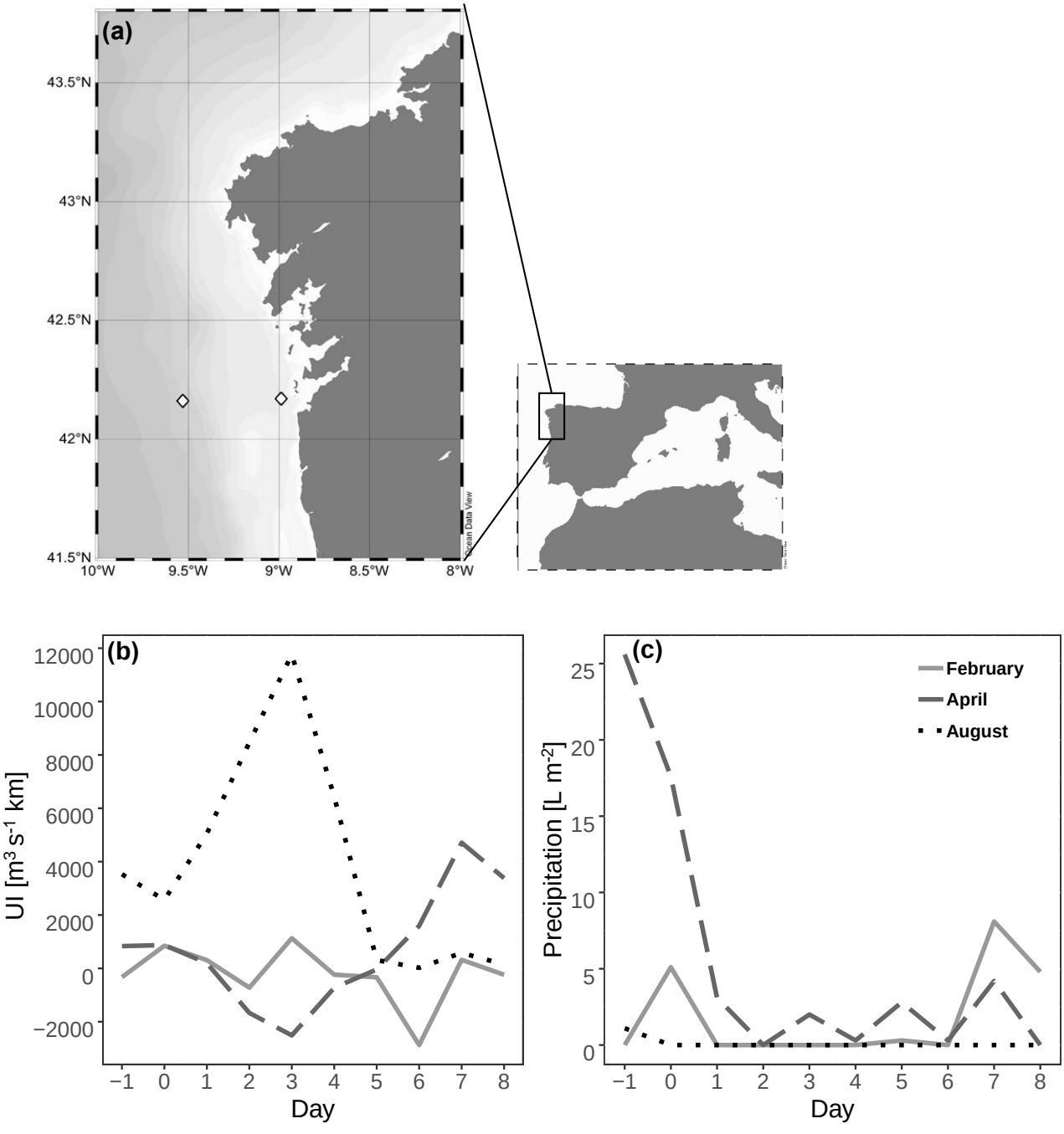

Figure 1

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Preprint. Discussion started: 22 August 2019

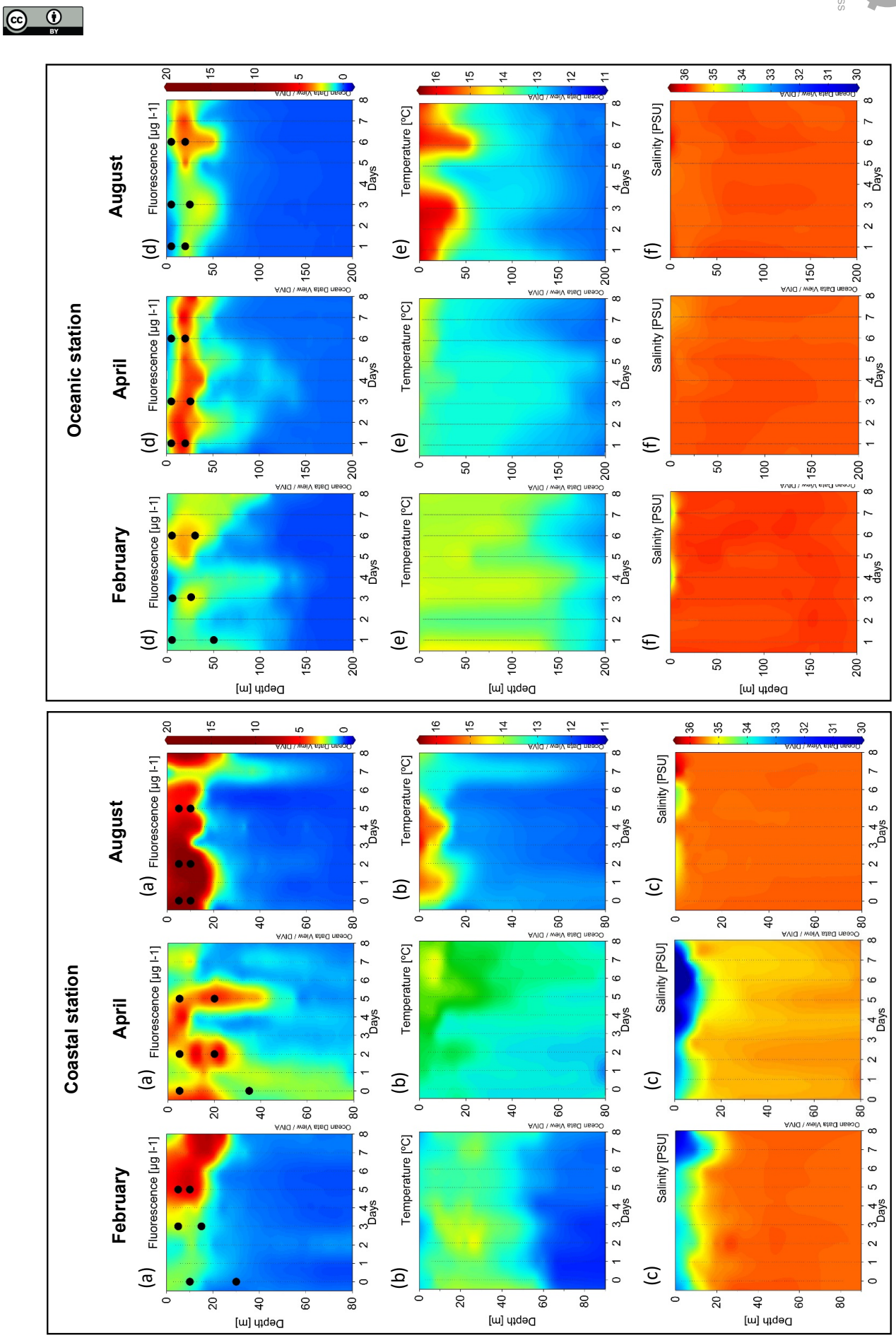

Figure 2 ∞ ω

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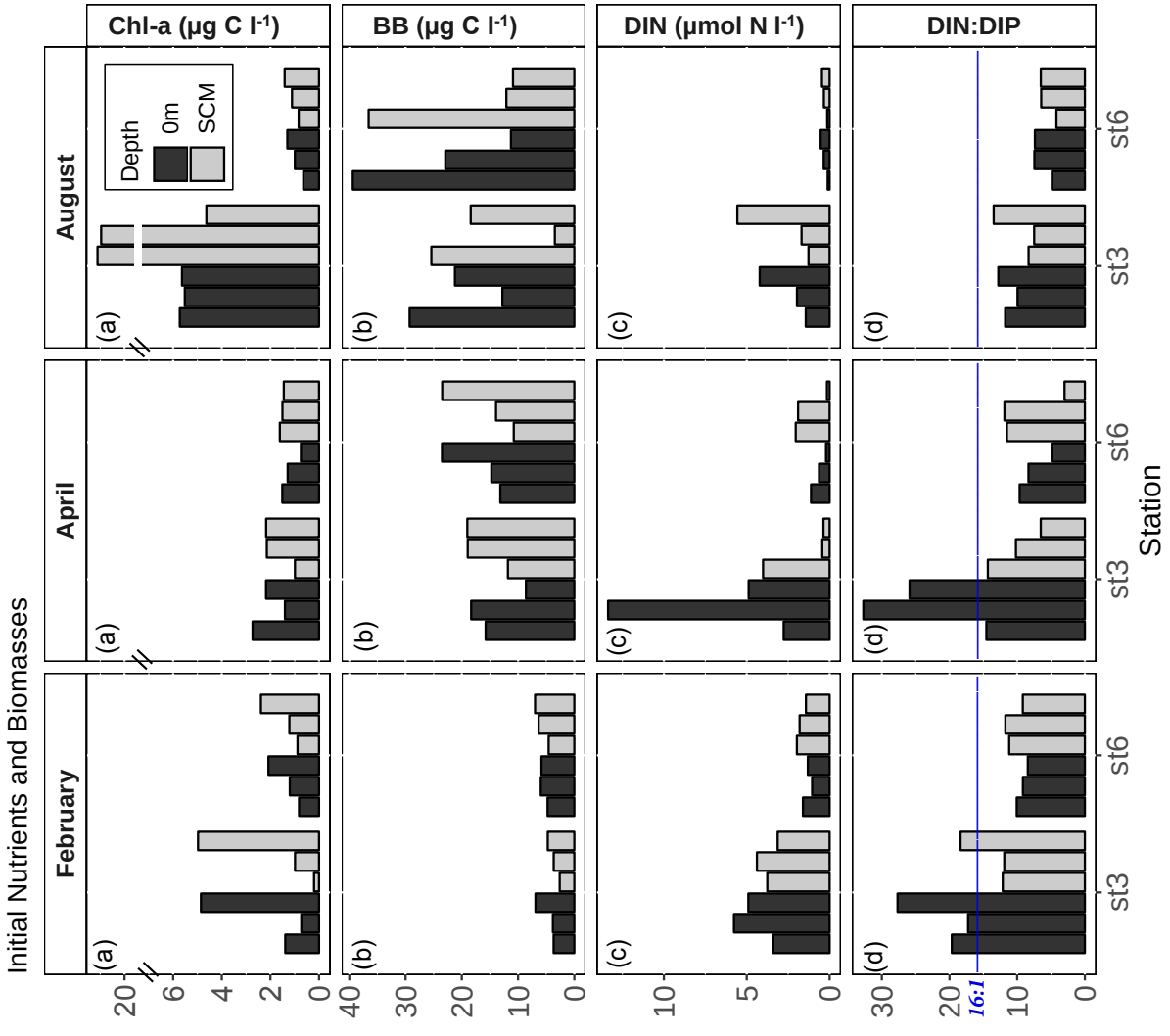

Figure 3

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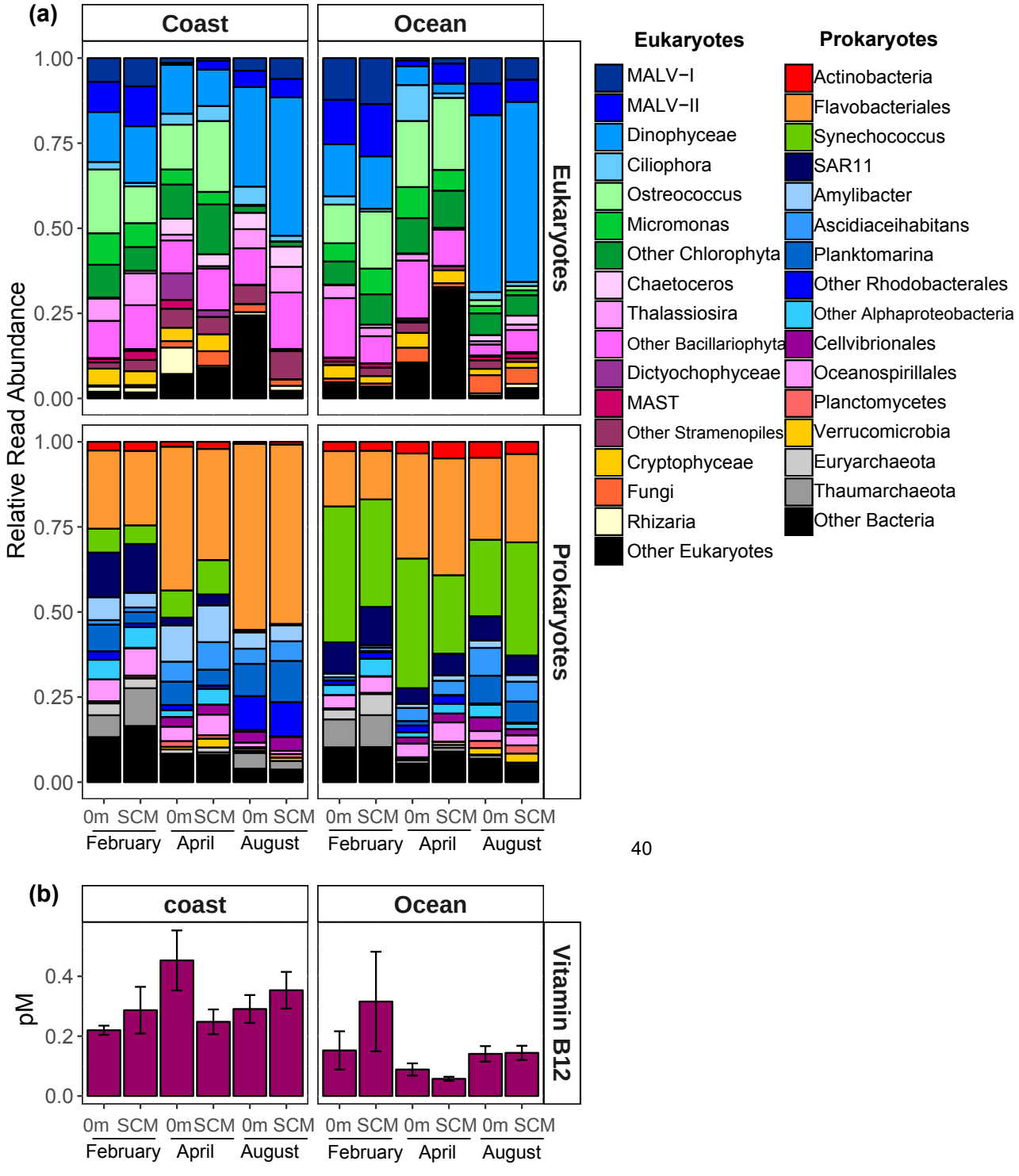

Figure 4

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Preprint. Discussion started: 22 August 2019

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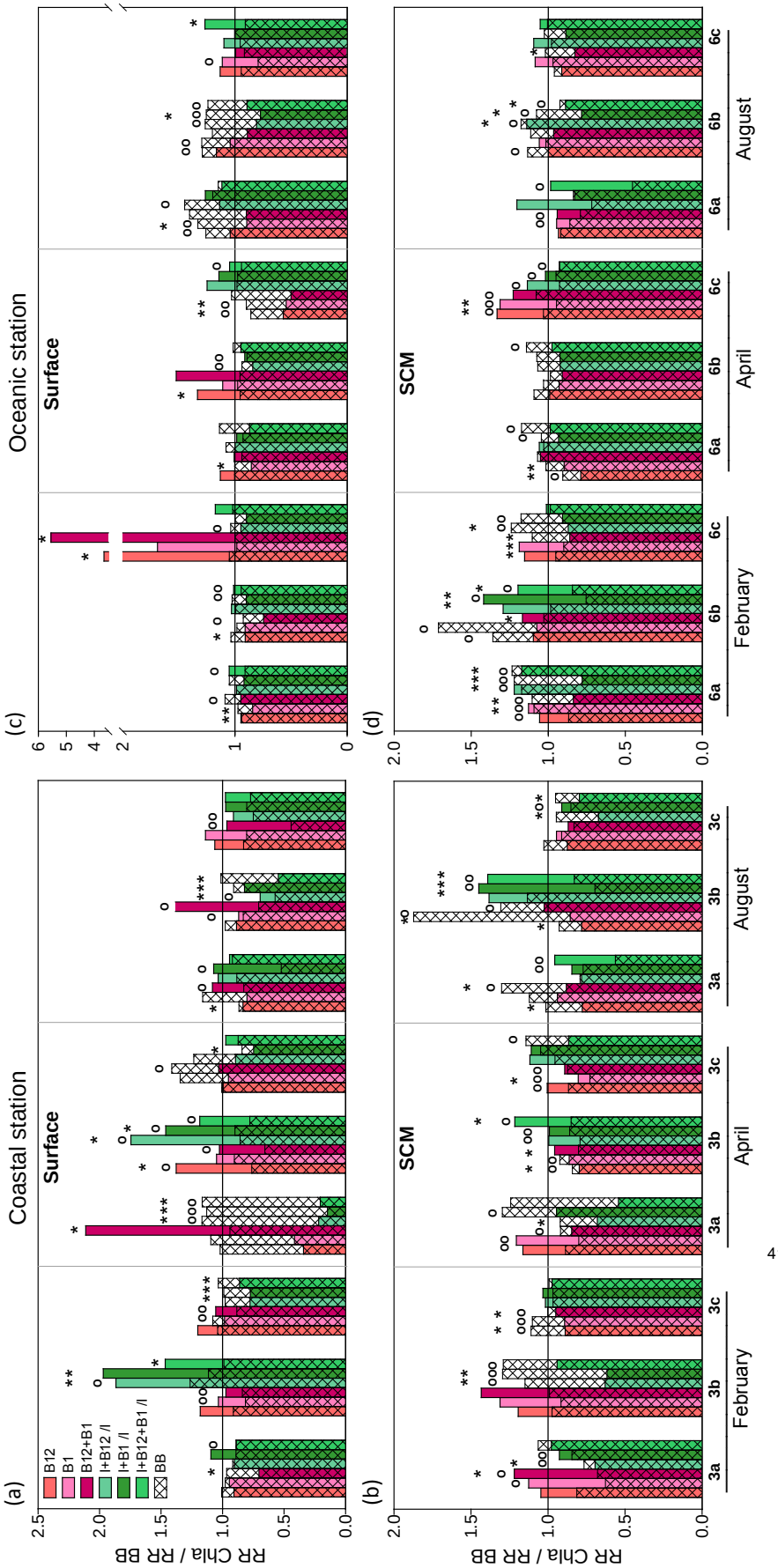

Figure 5

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Preprint. Discussion started: 22 August 2019

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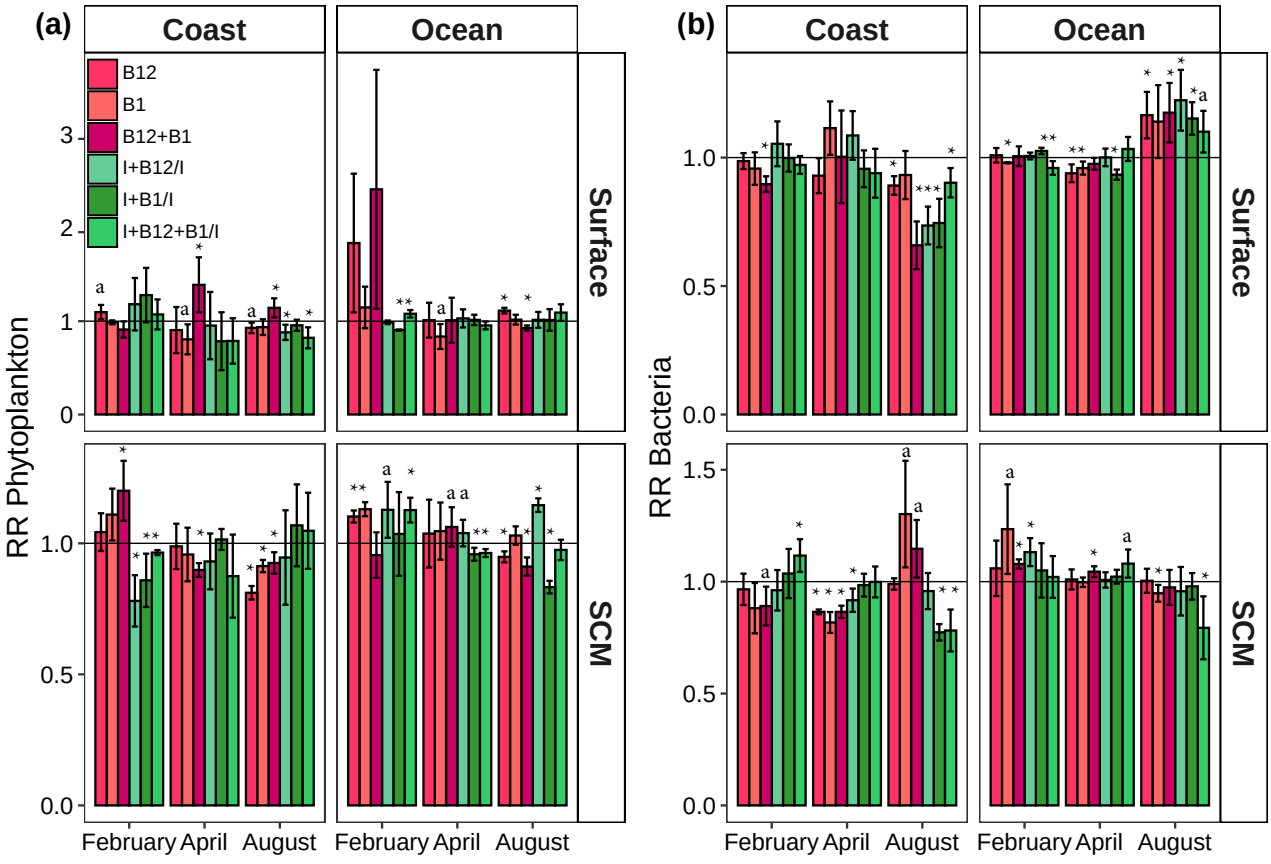

Figure 6

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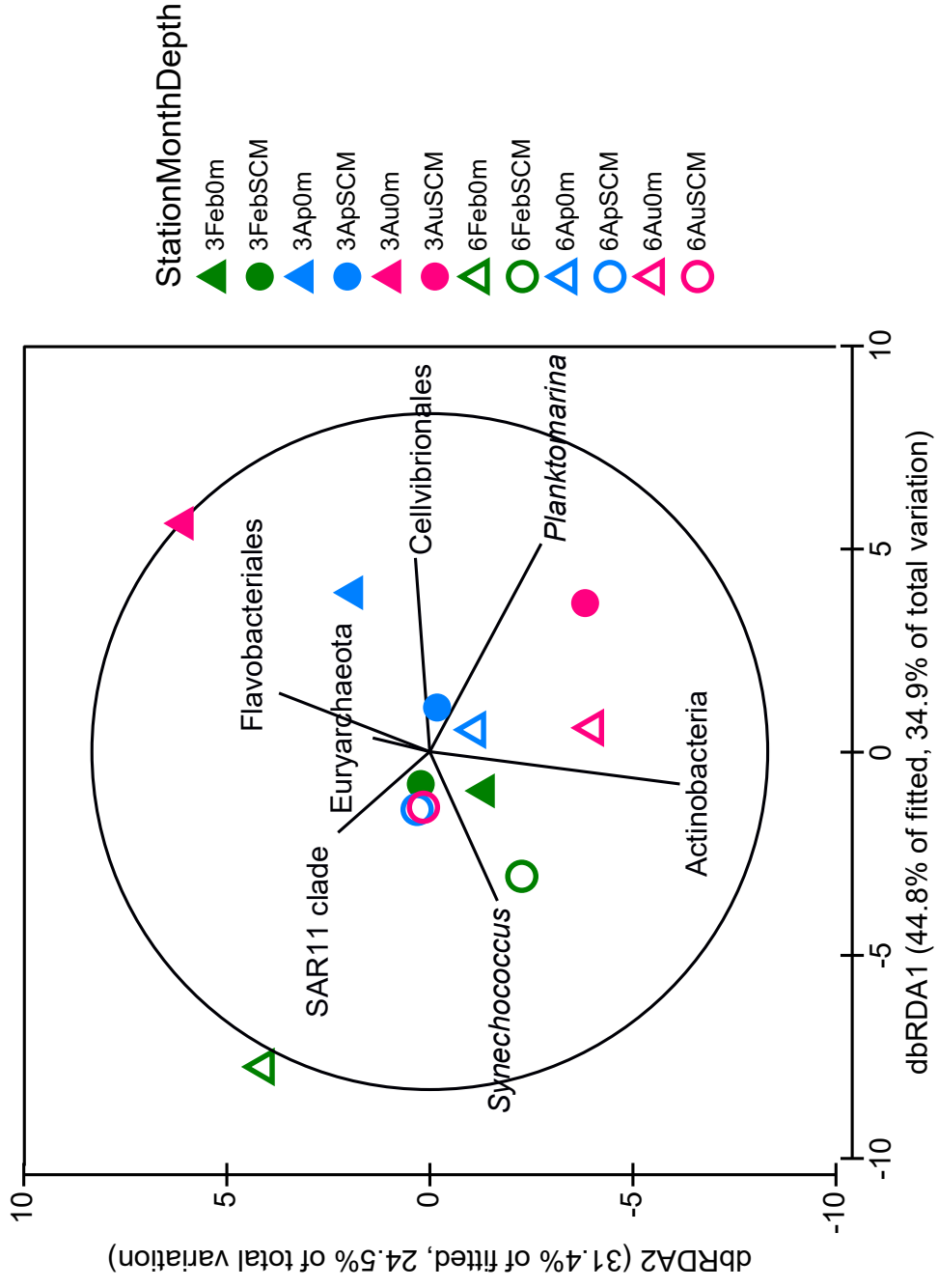

Figure 7