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Biological Oceanography Group

Koji Suzuki

Associate Editor

Biogeosciences

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Dear Koji

Please find attached a revised version of manuscript entitled “Spatial and temporal variability in the response of phytoplankton and bacterioplankton to B-vitamin amendments in an upwelling system”. The manuscript was co-authored by myself, Antero Prieto, Esther Barber-Lluch, Marta Hernández-Ruíz, Emilio Fernandez and Eva Teira.

We would like to acknowledge the insightful and constructive comments of the reviewers, which clearly helped us to improve the overall merit of the manuscript. We have taken into account all the suggestions raised resulting in a higher quality work. We attach a detailed response to all the comments made by the reviewers. In the individual responses to referee comments (RCs), the suggestions and comments of the individual reviewers are in plain font and our responses are in italics and blue font. The major changes are summarized below:

- Both referees agreed that the calculated response ratios could be useful in the discussion and that the actual chlorophyll-a and bacterial biomass data at the beginning and after the 72 h incubation in all the treatments should be shown. As we agree that this information is of great value, we have prepared two new figures (Fig. 5 and 6) where we plot all the requested information. In the revised version, the former figure 5, showing the response ratios has been included as supplementary information.
- Another suggestion by both referees was to provide details about the methodology related to B12 measurement. This section has been expanded by referring to previous work and indicating the particular conditions and instruments used in our laboratory.
- We have fully reviewed the statistical analyses. We now better explain the RELATE analyses. We have also reconsidered the usefulness of the Pearson's correlation analyses between the individual responses to vitamins and prokaryotic taxa (summarized in former table 1). We believe that this analyses could be redundant and its conclusions somewhat speculative. Based on this, we have removed this analysis, eliminated the former table 1,

and fully reviewed the discussion, eliminating speculative statements, and toning down some of our conclusions.

- The genomic data of this study will be publicly available at the European Nucleotide Archive (ENA) at EMBL-EBI (https://www.ebi.ac.uk/ena) as soon as possible.

Looking forward to hearing from you,

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#Referee1 comments

We very much appreciate the useful and constructive comments made by the reviewer, which surely contribute to improve the manuscript quality. We have considered all the suggestions and made the requested modifications, as detailed below.

My main initial request is to include figures for the actual bacterial and phytoplankton biomass changes in the experiments, rather than simply ratios, including the values for the initial conditions. I believe this should be in the main manuscript, not just the Supporting Information. These data can be displayed as a mean with error bars representing the spread across the three treatment replicates. I believe this will give a better indication of how the community responded in the experiments. The ratio figures can be included too for discussion/interpretation purposes. Please also label the treatments below each bar in each case – I found treatment identification a little difficult in the current figures.

We have now included in the main manuscript the requested figure (new figure 5 and 6). We present the response of phytoplankton and that of bacteria separately. The former figure 5 with the response ratios is now included as supplementary information.

Secondly I think the manuscript should also note how trace metal contamination could have biased the results. This is currently not discussed at all, but could have had an important influence. For instance, if contaminating iron had been inadvertently included in the treatments. Contamination would likely originate from the metal CTD-rosette, the rosette bottles, during bottle sampling, from the incubation bags, from the nutrient additions etc. Where certain procedures were carried out to reduce this, these should be described. This is significant, as this microbes in this region could be experiencing primary iron limitation – see Blain et al. (2004).

Blain, S., Guieu, C., Claustre, H., Leblanc, K., Moutin, T., Quéguiner, B., Ras, J. and Sarthou, G., 2004. Availability of iron and major nutrients for phytoplankton in the northeast Atlantic Ocean. Limnology and Oceanography, 49(6), pp.2095-2104.

We did not use trace metal clean techniques for sampling. We used standard stainless CTD-rosette and Niskin metal-free bottles. We did not have a trace metal clean lab on board, so, even though samples were carefully manipulated contamination by trace metals could have eventually occurred. It is important to note that the water for the

experiments was pooled into a 20 l acid-cleaned carboy before filling the bags (L134-L136), thus all the bags would have the same incidental input of trace metals. We are aware that microbes could be limited by other trace elements or nutrients not considered in our treatments, such as iron or other B vitamins. For this reason, we based the discussion on the response ratios at the end of the experiments.

Specific comments

In the abstract I would recommend making reference to the study region (i.e. 'North east Atlantic', or 'off the northwest coast of Spain')

The study area has been specified in the abstract (L15)

Figure 1b and c: please indicate when experiments were sampled for (i.e. which day? day 0?)

Sampling day for each experiment has been indicated in the graphs (Fig. 1b and Fig. 1c)

I would recommend noting the microbial responses to major nutrient supply, in addition to B12/B1, in the abstract.

The response to inorganic nutrient additions has been considered in the abstract (L13-L14)

Line 15–16: rephrase 'was not of great concern'

This has been rewritten (L15)

I would recommend stating the number of the 36 experiments where bacteria/ phytoplankton responded positively/negatively to vitamin supply in the abstract.

This information has been included in the abstract (L16-L19)

Line 21 'Growth stimulation by B1 addition was more frequent on bacteria' – relative to phytoplankton?

This has been clarified (L22-L23)

Lines 35–36 and elsewhere: I would recommend seeing the more recent studies of Browning et al., 2017 and Browning et al., 2018, which also perform trace-metal-clean B12 addition bioassay experiments in upwelling/coastal/offshore regions.

Browning, T.J., Achterberg, E.P., Rapp, I., Engel, A., Bertrand, E.M., Tagliabue, A. and Moore, C.M., 2017. Nutrient co-limitation at the boundary of an oceanic gyre. Nature, 551(7679), p.242.

Browning, T.J., Rapp, I., Schlosser, C., Gledhill, M., Achterberg, E.P., Bracher, A. and Le Moigne, F.A., 2018. Influence of iron, cobalt, and vitamin B12 supply on phytoplankton

growth in the tropical East Pacific during the 2015 El Niño. Geophysical Research Letters, 45(12), pp.6150-6159.

Both studies have been cited in the revised version (L38)

Line 39: synthesized by prokaryotes and archaea?

Archaea is included within the prokaryote organisms. This has been clarified in the manuscript (L41)

Line 42: Have not defined 'cobalamin' (In general I recommend choosing B12 or cobalamin and sticking to it throughout)

This has been corrected (L45)

Line 79: Perhaps mention here succinctly what Gobler et al. (2007) found?

This has been explained in the revised text (L83-L84)

Line 79: the reference Barber-Lluch et al. (2019) does not appear in the reference list â˘A.

This citation has been included in the reference list (L620-L622)

Lines 114–115: How was this water sampled? From the regular stainless CTD? If so, trace element contamination should be acknowledged. Also see general comment.

This has been acknowledged in the revised manuscript (L120; L134-L136)

Line 125: Was there any treatment of the whirl-pak bags (e.g. acid and deionized water rinses) to remove contamination? Also see general comment.

*We used these bags mainly because they are sterile, non-toxic and transparent to the whole solar spectrum, thus avoiding UVR absorption of most other materials, and have been frequently used for experimentation with plankton communities (*Gonzalez et al., 1990; Davidson and van der Heijden, 2000; Pakulski et al., 2007; Teixeira et al., 2018). *The bags were not additionally treated as were used only once.*

Line 127 and on: What were the chemical stocks of the nutrients (e.g. brand and purity). Again, if these nutrients were not pre-treated to remove trace element contamination, this should be acknowledged. Also see general comment.

All the reagents were from Sigma of highest purity. Stocks were prepared with autoclaved Milli-Q water. No additional treatment for trace element contamination was applied.

Line 137: Were the tanks screened, or open to the air?

Tanks were screened to attenuate light intensity (L148-L150)

Line 147: Was any time given for the fixative to act on cells before flash freezing in liquid nitrogen?

Samples were incubated 20 min for fixative to act (L160-L161)

Section 2.5: If known, what was the recovery percent of the B12 preconcentration/ extraction? (i.e. via use of a standard)

Average B12 recovery percentage was 93% (L213-L214)

Line 271: How was the upwelling index calculated (cannot see this in methods).

Upwelling index was calculated by calculating the Ekman transport from surface winds at fix-station (st3) located at 42º N and 8.88º W. This information has been included in the revised text (L129-L132)

Figure 5: It is not clear that the value being displayed is the RR Chla OR RR BB and not the ratio of these.

This has been clarified (Fig. S3 in the supplement)

As the Figure 5 has signs indicating statistical significance, the error in the spread across the treatment replicates must have been prorogated somehow? Can this error be included as error bars in the figure?

The error bars representing the standard error of the three replicates have been included in the new figure 5 (now Figure 5 and 6).

337–338: Specifically which experiments showed serial limitation by B vitamins?

This has been specified in the revised manuscript (L387-L390)

Line 402: 'clarify the paper of vitamins'?

This fragments has been corrected for clarity (L444)

Lines 417–419: Please distinguish between the phytoplankton/bacteria responses in this value of 75%.

Taking into account the responses of phytoplankton and bacteria separately, the percentages were 75% for phytoplankton and 50% for bacteria (L457-L548)

Line 425: No full stop (perhaps also rephrase to 'community assemblage'?)

This sentence was removed.

Lines 491–495: This doesn't quite make sense – in the first sentence it states that phytoplankton responses to B1 supply were restricted, and in the second the stimulation of phytoplankton is discussed.

Phytoplankton responses to B1 are overall restricted. The second sentence refers to the particular simultaneous stimulation of phytoplankton and bacteria by B1 addition found in subsurface oceanic waters in February (L532-L533)

I would advise including a table summarizing initial conditions (i.e., nutrient concentrations, temperature, chlorophyll-a, initial bacteria and so on).

We have added a supplementary table including detailed information about initial conditions (Table S2 in the supplement)

In addition to the modifications suggested by the reviewer, we have made the following changes. The named OTUs (operation taxonomic units) has been replaced by ASV (amplicon sequence variant) due to the sequence analysis method used DADA2 (section 2.6).

We have eliminated the Pearson correlation between response ratios and the clr (centered-log-ratio) abundance of taxonomic groups (reported in former table 1) as was redundant with the dbRDA. Regarding the RELATE analysis to explore the relationship between the responses to B vitamin treatments (response ratios of phytoplankton and bacteria) and (1) the environmental variables (including nutrients, temperature, salinity, B12, chla and BB), (2) the prokaryotic, or (3) eukaryotic community structure, we believe that nicely shows that the responses are only significantly related to the prokaryotic community structure.

#Referee 2 comments

We are very grateful for the reviewer's comments, which have contributed to improve the manuscript.

The role that the availability of B-vitamins,specifically vitamin B12 and B1,play in shaping the marine microbial community is very relevant. The authors of this manuscript conducted an extensive experimental campaign with the goal of providing some insight to these processes. Unfortunately, their findings are poorly communicated and overstated in this manuscript. Most of the discussion is highly speculative and is insufficiently referenced.

We have made our best to refer adequately all the relevant studies and eliminating some speculative statements.

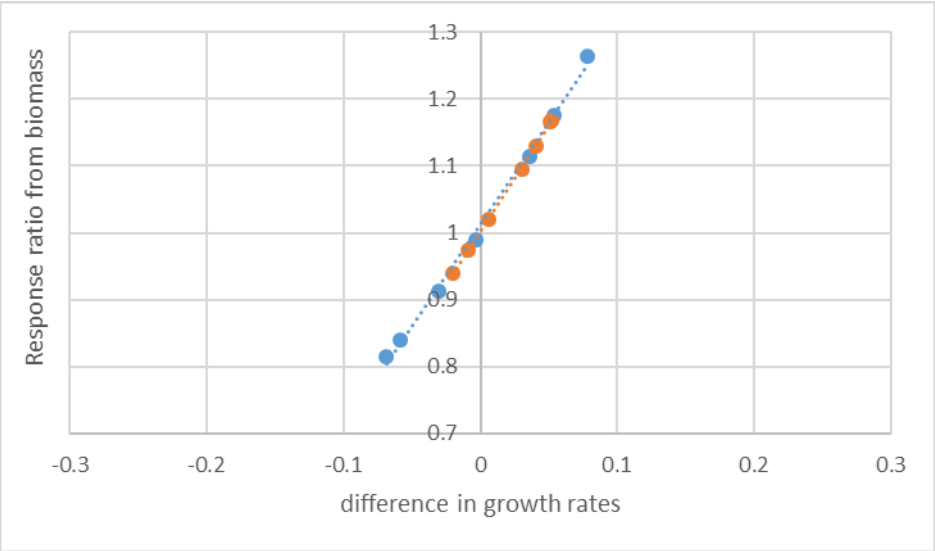
The authors have gone “all-in” on the poorly justified concept of “response ratio”. I feel like this calculated metric is overly general and prevents an in-depth analysis of the actual data which likely contains subtle variations that could either support or undermine the authors primary conclusions. I don’t understand why the authors chose to use response ratios rather more traditional ecological and physiological metrics. While response ratios could be a useful part of the discussion, they should be just that, a part of the discussion. Additionally, the authors ignore the rates of community growth and dynamics and only assess the response at the end time point relative to the initial point. While it is not possible at this point to change the experimental design, the authors need to change their interpretation of the data to acknowledge the limits of their data.

We are aware that sampling only at one endpoint (after 72 h incubation in our case) does not allow to discuss in detail the dynamics during each experiment, however we were particularly interested in extensively exploring the temporal and spatial variability of the response to vitamin enrichment. The experimental design, involved 36 experiments, with 8 triplicate treatments (24 experimental units per experiment). Even sampling only at the

beginning and at the end we collected 972 samples for chlorophyll-a, and 972 for bacterial biomass. Initial and endpoint sampling is a common practice in enrichment microcosm experiments (e. g. Mills et al., 2004; Moore et al., 2006; Gobler et al., 2007; Bonnet et al., 2008; Koch et al., 2011), and allows the estimation of net growth rates using the following formula: ln (endpoint biomass/initial biomass)/incubation time. A previous work by Barber-Lluch et al (2019) in the same sampling area allowed us to conclude that sampling at 72 h was adequate to explore the effect of vitamins on both phytoplankton and bacterial biomass. As we agree with the referee that the dynamics of phytoplankton and bacteria during the experiments are of interest, and following also the advice of referee 1, we now include in the manuscript two new figures where the initial and endpoint value of chlorophyll-a and bacterial biomass is represented. The response ratio figure is now included in the supplementary information. We accordingly now describe the dynamics of both planktonic components in the different experiments. We nevertheless decided to keep the response ratio as a measure of the magnitude of the effect (see below), which is very useful for the sake of comparison.

We used here the response ratio as the quotient between the measured quantity of a response variable in experimental and control experimental units. Previous studies dealing with the effects of nutrients additions on microbial communities have noted the importance of expressing the change in the treatment relative to the control (Downing et al., 1999; Hedges et al., 1999; Elser et al., 2007, among others). We find that this variable is particularly adequate as a measure of the experimental effect because it quantifies the proportionate change that results from an experimental manipulation. This metric is widely use in marine ecology, and particularly in nutrient amendment experiments (e.g. Martínez-García et al., 2010; Teira et al., 2013; Barber-Lluch et al., 2019). The use of the response ratio calculated from endpoint biomass data provides the same information as the comparison of growth rates between treatments. Below we plot, as an example, the relationship between the response ratio from biomass (endpoint biomass in treatment divided by endpoint biomass in control) and the difference between growth rates (growth

rate in treatment minus growth rate in the control) using data from two of our experiments (represented with different colours). It can be appreciated that the information provided by the response ratios follows exactly the same pattern as that provided by comparing growth rates. Moreover, the range of variation is higher for the response ratio, which allows to statistically detecting more subtle changes.



It is unfortunate that the only measures of biomass performed by these authors during their experiments were bacterial abundance and chlorophyll A. These are very broad, unspecific measures of community structure, that can be impacted by a myriad of environmental factors. The authors make some substantial claims about the roles that B-vitamin additions are playing on the microbial community; however, I wonder if they really have enough resolution in their measurements to make these claims. The author's use of "response rate" to obscures the fact that they are only measuring bacterial abundance and chlorophyll concentration. There are so many variables that impact these measures, it's not clear to me that the authors are actually looking at responses from B-vitamins.

B vitamins are essential growth factors for all microorganisms; therefore, the ultimate effect of a vitamin deficiency will be an impairment of growth, which is typically evaluated

from changes in biomass. It is true that we only measure the effect on bulk phytoplankton and bacteria, and thus we have toned down all the conclusions about the effect on microbial community structure. We do not think that is unfortunate to have chosen phytoplankton and bacterial biomass as response variable, considering that most previous studies evaluating the role of B vitamins were based on biomass measurements (Sañudo-Wilhelmy et al., 2006; Gobler et al., 2007; Koch et al., 2011, 2012; Browning et al., 2018; Barber-Lluch et al., 2019). We are aware of many variables that could affect bacterial and phytoplankton biomass, for this reason, we compared the response of B vitamin treatments with their corresponding controls. B12, B1 and B12+B1 treatments were compared to the unamended control, while I+B12, I+B1, I+B12+B1 were compared with the I treatment.

I have some substantial concerns about the conclusions the authors make about community diversity and B-vitamins. Their exact statistical methods need to be better explained. Additionally, the authors need to fully explain the limits of their statistical methods, and not overstate or be overly speculative about the observed correlations between abiotic/biotic factors, B-vitamins, and the amplicon data. The manuscript needs substantial copy editing/English language editing. All sections need to be streamlined. The interpretation of results tends to be far too speculative. The authors need to only make claims that their data can support.

We agree that some statistical methods needed further clarification and we recognize that some analyses were somehow redundant and have been excluded from this revised version. Specifically, we have eliminated the Pearson correlation between response ratios and the clr (centered-log-ratio) abundance of taxonomic groups (reported in former table 1) as was redundant with the dbRDA. Regarding the RELATE analysis to explore the relationship between the responses to B vitamin treatments (response ratios of phytoplankton and bacteria) and (1) the environmental variables (including nutrients, temperature, salinity, B12, chla and BB), (2) the prokaryotic, or (3) eukaryotic community structure, we believe that nicely shows that the responses are only significantly related to the prokaryotic community structure. We have clarified how we constructed the resemblance matrices (L273-L283).

It is important to note, that as we are aware of the statistical limitations when working with relative abundance of sequences, prior to statistical analyses, ASV abundances were transformed using the centered log ratio (Fernandes et al., 2014; Gloor et al., 2017).

The B12 analytical method appears to be derived from previously published methods. Specifically, those published by Heal et al. 2014, Sañudo et al. 2012, and Suffridge et al. 2017. It is troubling to me that the authors do not cite any of these papers in the methods section, despite the fact that the described method is a nearly an exact match of those described in the above papers. Additionally, SPE extraction efficiency and limits of detection need to be included.

We now provided all the requested details about the vitamin B12 quantification method and included all the references (Section 2.5, L191-L194, L205-L215).

How were the whirl-pak bags prepared? Were they prepared to be trace clean? Were they sterile? What sort of plastic are they made out of? Trace metal or trace organic (B-vitamin) contamination is a real concern in experiments like these, especially when the authors want to make conclusions about the impact of a trace-component. Many plastics contain trace contamination from the factory, and if the bags were not properly prepared, this variability could interfere with all results.

We did not use strict trace metal clean techniques for sampling. It is important to note that the water for the experiments was pooled into a 20 l acid-cleaned carboy before filling the bags, thus all the bags would have the same incidental input of trace metals. We are aware that microbes could be limited by other trace elements or nutrients not considered in our treatments, such as iron or other B vitamins. For this reason, we based the discussion on the response ratios at the end of the experiments.

The whirl-pak® bags are made of low density polyethylene, are sterile, non-toxic and transparent to the whole solar spectrum, thus avoiding UVR absorption of most other materials, and have been frequently used for experimentation with plankton communities

(Gonzalez et al., 1990; Davidson and van der Heijden, 2000; Pakulski et al., 2007; Teixeira et al., 2018). The bags were not additionally treated as were used only once.

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

1 Introduction

Phytoplankton accounts for almost half of the global net primary production (Field et al., 1998) and may eventually cause toxic episodes entailing human health problems and large economic losses (Hallegraeff, 1993; van Dolah et al., 2001). Recent emerging evidence suggests the role of biologically active organic compounds, such as B-vitamins, on the control of marine productivity in both coastal and oceanic waters (Panzeca et al., 2006; Bertrand et al., 2007; Gobler et al., 2007; Koch et al., 2011; Browning et al., 2017, 2018). B-vitamins act as cofactors for enzymatic reactions and are involved in many important metabolic pathways (Madigan et al., 2005; Koch et al., 2011; Monteverde et al., 2017). Vitamin B12 (B12 herein), which is exclusively synthesized by some bacteria and archaea

(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) (Helliwell et al., 2011; Bertrand and Allen, 2012). In comparison, over 20 different B12-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; Grant et al., 2014; Kazamia et al., 2012). A well-known example is the mutualistic interaction between B12-dependent phytoplankton and bacteria (Croft et al., 2005; Amin et al., 2012; Cooper and Smith, 2015).

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

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

75 elements, much less is known about B vitamin limitation and its spatial and temporal
76 variability in marine ecosystems.

77 Some studies have shown enhanced phytoplankton biomass associated to B12
78 amendments in both temperate coastal and polar waters (Bertrand et al., 2007; Gobler et
79 al., 2007; Koch et al., 2011; Koch et al., 2012). The simultaneous effect of vitamin B12
80 supply on both phytoplankton and bacteria has been barely explored (Koch et al., 2011,
81 Barber-Lluch et al., 2019). To our knowledge, the effect of B1 amendments on marine
82 natural microbial plankton community succession has been only assessed by Gobler et al.
83 (2007), who suggested that high concentration of B-vitamins, associated with high
84 bacterial abundance, caused an increase in auxotrophs, mostly dinoflagellates.

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

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

103 Considering that a large fraction of eukaryotic phytoplankton and bacterial taxa require
104 exogenous B-vitamins and considering the different requirements and capabilities to
105 synthetize B-vitamins by different microbial taxa, we hypothesize that microbial

community composition play a relevant role in explaining B-vitamins limitation patterns in microbial plankton.

2 Methods

2.1 Experimental design

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

Seawater samples were gently pre-filtered through a 200 µm mesh to exclude large zooplankton in order to ensure good replicability and collected into a 20 l acid-cleaned polyethylene carboy. It is important to note that incidental trace-metal contamination could have occurred during water collection. Following sample collection, 300 ml PAR

and UVR transparent, sterile, and non-toxic (whirl-pak) bags were filled and nutrients were added establishing eight different enrichment treatments as follows: (1) control treatment (C): no nutrients added; (2) inorganic nutrient treatment (I): 5 µM nitrate (NO_3^-), 5 µM ammonium (NH_4^+), 5 µM silicate (SiO_4^{2-}) and 1 µM phosphate (HPO_4^{2-}); (3) vitamin B12 (Sigma, V2876) treatment: 100 pM; (4) vitamin B1 (Sigma, T4625) treatment: 600 pM); (5) Inorganic nutrients and vitamin B12 (I+B12) treatment; (6) Inorganic nutrients and vitamin B1 (I+B1) treatment; (7) vitamins B12 and B1 (B12+B1) treatment and (8) Inorganic nutrients with vitamins B12 and B1 (I+B12+B1) treatment. Inorganic nutrients were added to avoid that inorganic nutrient limitation masked the responses to B vitamins. Each treatment had 3 replicates resulting in 24 whirl-pack bags per experiment. To assess short-term effects of nutrient inputs, experimental bags were incubated on-deck during 72 h under natural light conditions. In-situ temperature and light were reproduced by submerging the bags in tanks connected to the surface-water pump system, and covered with screens simulating the light intensity at the sampling depth.

2.2 Chlorophyll-*a*

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

2.3 Flow cytometry

Samples for heterotrophic bacteria abundance quantification (2 ml) were preserved with 1 % paraformaldehyde + 0.05 % glutaraldehyde (final concentrations). Samples were incubated 20 min for the fixative to act on cells 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

169 factor of Norland (1993: fg C cell^{-1} = 120 × BV$^{0.72}$) for the coastal experiments and using
170 the open ocean conversion factor for the oceanic experiments (fg C cell^{-1} = 350 × BV).

171 **2.4 Nutrients**

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

181 **2.5 Vitamin B12**

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

191 The concentrate was filtered again through a cellular acetate membrane 0.2 µm
192 (Phenomenex) prior to the analysis. Ultra Performance Liquid Chromatography tandem
193 Mass Spectometry 3Q (UPLC-MS/MS) methodology was adapted from Sañudo-
194 Wilhelmy et al (2012), Heal et al. (2014) and Suffridge et al (2017). Detection and
195 quantification of dissolved vitamin B12 (cyanocobalamin and hydroxocobalamin) was
196 conducted using an Agilent 1290 Infinity LC system (Agilent Technologies, Waghaeusel-
197 Wiesental, Germany), coupled to an Agilent G6460A triple quadrupole mass
198 spectrometer equipped with an Agilent Jet Stream ESI source. The LC system used a C18
199 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 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. Limits of detection (LODs) and limits of quantification (LOQs) were determined using sequential dilutions of the lowest point of the calibration curves. LODs were defined as the lowest detectable concentration of the analyte with a signal-to-noise (S/N) ratio for the qualitative transition of at least 3. In the same way, LOQs were defined as the lowest quantificable concentration with a S/N ratio of 10 for the quantitative transition. S/N ratios were calculated using the Mass Hunter Workstation software B.04.01. The LODs obtained for the two vitamin B_{12} congeners were 0.04 and 0.01 pM, while the LOQs values were 0.05 and 0.025 pM for hidroxocobalamin (OHB12). The average B12 recovery percentage after pre-concentration and extraction of B-vitamin spiked samples was 93%. B-vitamin free seawater was spiked with CNB12 and OHB12 standards for recovery percentage analysis.

2.6 Microbial plankton community

DNA samples were taken during the experimental period at surface and SCM depth in the coastal and oceanic station. In particular, sampling of the microbial plankton community was carried out on the first, second, fourth and sixth day of each cruise. Community composition was assessed by sequencing the V4 and V5 regions from 16S rRNA gene (16S rDNA) for prokaryotes and the V4 region from 18S rRNA gene (18S rDNA) for eukaryotes. Two litters of water samples were sequentially filtered through 3 µm pore size polycarbonate filters and 0.2 µm pore size sterivex filter and immediately frozen in liquid nitrogen and conserved at -80 ºC. DNA retained in the 3 µm and 0.2 µm filters was extracted by using the PowerSoil DNA isolation kit (MoBio Laboratories Inc., CA, USA) and the PowerWater DNA isolation kit (MoBio Laboratories Inc., CA, USA), respectively, according to the manufacturer's instructions. Prokaryotic DNA from 0.2 µm filters was amplified using the universal primers "515F and 926R" and eukaryotic DNA from both, 3 µm and 0.2 µm filters, using the primers "TAReuk454FWD1" and "TAReukREV3". Amplified regions were sequenced in an Illumina MiSeq platform and the sequences obtained were analyzed with software package DADA2 (Callahan et al., 2016). SILVA reference database (Quast et al., 2012)

was used to taxonomic assignment of 16S amplicon sequence variants (ASVs) 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 ASVs. The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI (https://www.ebi.ac.uk/ena) under accession numbers XXXXXX (16S rDNA sequences) and YYYYYY (18S rDNA sequences). ASV table is an analogue of the traditional OTU table which records the number of times each exact amplicon sequence variant was observed in each sample (Callahan et al., 2016).

The raw ASV 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 ASVs was averaged for coastal and oceanic samples, differentiating surface and SCM. A total of 1550 unique ASVs of prokaryotes were identified. As many ASVs 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 ASVs. The sequence abundances of the subsampled ASV 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 significant at the 0.05 significance level and p-value was standardized as proposed by Good (1982) in order to overcome the low number of replicates. Differences between station and depth (spatial variability) and among sampling months (temporal variability) in the responses to B vitamins were evaluated with factorial analysis of variance (ANOVA). Bonferroni post hoc tests analyses were conducted to test which treatments were significantly different from the control treatment in each experiment. Z-test was used to evaluate the significance of the average B vitamins response ratios for each period, sampling site and depth. The RELATE analysis implemented in PRIMER6 (Clarke and Warwick, 2001; Clarke and Gorley, 2006) was used to relate the B-vitamin response patterns (Bray-Curtis resemblance matrix built from phytoplankton and bacteria response ratios) with: (1) environmental factors (Euclidean resemblance matrix built from normalized values of ammonium, nitrite, nitrate, phosphate, silicate, B12, temperature, salinity, chlorophyll—a, bacterial biomass), (2) prokaryote community composition (Euclidean resemblance matrix built form clr-transformed sequence abundance of major taxonomic groups), or (3) eukaryote community composition (Euclidean resemblance matrix built form clr-transformed sequence abundance of major taxonomic groups). RELATE calculates the Spearman rank correlations (Rho) between two resemblance matrices, and the significance is tested by a permutation test. In order to highlight which specific taxonomic groups are associated to changes of microbial plankton (bacterioplankton and phytoplankton) responses to vitamin B1 and B12, we conducted a distance based redundancy analysis (dbRDA) combined with a distance linear-based model (DistLM) using a step-wise procedure and adjusted r^2 as selection criteria) using the PRIMER6 software. Correlations among the prokaryotic taxa best explaining the microbial plankton responses to B-vitamins (according to the previously tests) and phytoplankton and bacterial responses to different B vitamin treatments (including primary and secondary responses) were calculated using Pearson's correlations.

3 Results

3.1 Initial conditions

Different hydrographic conditions were found during each cruise (Fig. 1 and Fig. 2). In February, heavy rainfall combined with relaxed winds (Fig. 1) caused a halocline at 10

meters depth (Fig. 2). High levels of Chl-*a* (as derived from the calibrated CTD fluorescence sensor) were observed at the coastal station, being maximum (4.97 µg l^{-1}) by the end of the cruise. At the oceanic station, Chl-*a* levels remained low (less than 3 µg l^{-1}) throughout the cruise, being slightly higher in the subsurface layer.

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

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

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

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

328 observed nutrient variability (Fig. 3). In August, Chl-*a* concentration was much higher at
329 the coastal than at the oceanic station, and showed reduced temporal variability (except
330 at the SCM in the coast) (Fig. 3). At the beginning of the sampling period, BB was higher
331 in the ocean than in the coast, and tended to decline by the end of the cruise.

332 A MDS analysis revealed that microbial community composition showed a relatively
333 reduced within period variability, with samples clustering according to the sampling
334 period (ANOSIM, $p = 0.001$) (Fig. S1 in the Supplement). Consequently, we averaged
335 the microbial community composition for each period and sampling site. The sampling
336 period-averaged composition of the eukaryote community showed a clear variability
337 among sampling dates, while differences between sampling locations and depths were
338 less pronounced (Fig. 4a). At the coastal location, *Mamiellophyceae* were relatively
339 abundant in February and April, but their abundance sharply decreased in August. By
340 contrast, the relative abundance of *Dinophyceae* was highest in August at both sampling
341 locations. The contribution of diatoms (*Bacillariophyta*) was very low in summer at the
342 oceanic station and MALV were most representative in February at both locations.
343 Flavobacterales and Rhodobacterales were the dominant prokaryotes (Fig. 4b) in coastal
344 waters, particularly in August, when both represented more than 80 % of sequences, while
345 Cyanobacteria were mostly present in February and April. In oceanic waters,
346 Flavobacterales and Cyanobacteria were the dominant prokaryotes. SAR11 clade and
347 Archaea were most abundant in February at both sampling locations.

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

352 **3.2 Short-term phytoplankton and bacteria responses to inorganic nutrients and**
353 **vitamin additions**

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

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

361 The magnitude of phytoplankton and bacteria responses (i.e., the response ratios) to the
362 different addition treatments differed between sampling stations (ANOVA, $p = 0.018$)
363 and among sampling periods (ANOVA, $p = 0.014$). The most prominent responses of
364 phytoplankton, compared to the control treatment, occurred after inorganic nutrient
365 amendments, especially in surface oceanic waters (Fig. 5 and Fig. S2 in the Supplement).
366 The magnitude of the phytoplankton response to inorganic nutrients was significantly
367 higher in oceanic than in coastal waters (ANOVA, $p = 0.028$). Bacteria responded
368 comparatively less than phytoplankton to inorganic nutrients (Fig. 6) and there were no
369 significant differences between coastal and oceanic waters (ANOVA, $p = 0.203$). The
370 addition of inorganic nutrients caused significant increases in phytoplankton biomass in
371 31 out of the 36 experiments, and in 19 out of 36 experiments in bacterial biomass (Fig
372 5, Fig. 6 and Fig. S2 in the Supplement).

373 The addition of B12 stimulated phytoplankton growth in 5 out of 36 experiments (Fig. 5
374 and Fig. S3 in the Supplement) while bacteria responded positively to B12 in 6
375 experiments (Fig. 6 and Fig. S3 in the Supplement). Phytoplankton biomass increased in
376 3, and bacterial biomass in 7 out of 36 experiments after adding B1 (Fig. 5 and Fig. 6). B
377 vitamins also caused negative responses of phytoplankton (Fig. 5 and Fig. S3 in the
378 Supplement) and bacterial biomass (Fig. 6 and Fig. S3 in the Supplement). The addition
379 of vitamins induced decreases of phytoplankton biomass in 6 experiments (4 after adding
380 B12 and 2 after adding B1) and bacterial biomass in 14 experiments (6 after adding B12
381 and 8 after adding B1). Additions of inorganic nutrients combined with B-vitamins
382 caused a similar increase in phytoplankton or bacterial biomass than the inorganic
383 addition alone in most of the experiments. Secondary limitation by B1 and/or B12 was
384 occasionally observed when inorganic nutrients were limiting, leading to a higher
385 biomass increase in the treatments including both inorganic nutrients and vitamins as
386 compared to the inorganic nutrient addition alone (Fig. 5, Fig. 6 and Fig. S3 in the
387 Supplement). In the case of phytoplankton, secondary limitation by B-vitamins was found
388 in the 3b-surface, 6a-SCM and 6b-SCM experiments in February, in the 3b-surface and
389 3b-SCM experiments in April, and in the 3b-SCM, 6b-SCM and 6c-surface experiments
390 in August (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. 7).

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

When averaging the responses within each sampling point (Fig. 7), 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 responses mostly occurred in February, showing an average increase of up to 1.2-fold in coastal subsurface waters after B12+B1 amendment (Fig. 7). The largest significant increase in phytoplankton biomass (ca. 1.4-fold) occurred in April after the combined addition of B12 and B1 in coastal surface waters. Significant positive bacterial responses mainly occurred in August, when the largest increase (ca. 1.3-fold) occurred in coastal subsurface waters after B1 amendment (Fig. 7). Most positive responses were associated with treatments containing B12 either alone or combined with B1 (Fig. 7). Phytoplankton primary B1 limitation was only found at the oceanic SCM in February (Fig. 7), while bacterial primary B1 limitation only occurred at the coastal SCM in August. In addition, bacterial secondary B1 limitation occurred in oceanic surface waters in February and August.

In order to explore the controlling factors of the observed B-vitamin response patterns, the correlation between the B-vitamin response resemblance matrix and the corresponding resemblance matrices obtained from the initial environmental factors, the initial prokaryotic community composition, or the initial eukaryotic community composition were calculated. Only the prokaryotic community composition significantly

correlated with the B-vitamin responses (Spearman Rho = 0.31, p = 0.041). We then used distance-based linear modelling (DistLM) to identify the prokaryotic taxa which best explained the microbial plankton responses to B-vitamins (Fig. 8). The resulting model explained 78 % of the variation and included seven prokaryotic groups: *Planktomarina*, Actinobacteria, SAR11_clade, Cellvibrionales, Euryarchaeota, Flavobacteriales and *Synechococcus*. The sequential test identified *Planktomarina* and Actinobacteria as the taxa explaining the largest fraction of variation (ca. 24 % and 14%, respectively, data not shown). The total variation explained by the db-RDA1 and db-RDA2 was 59.4 %, both represented as x and y axis, respectively (Fig. 8). The db-RDA1 axis tended to separate coastal, where negative responses to B vitamins dominated, from oceanic samples, where most positive responses were found (Fig. 7). The db-RDA plot showed that Cellvibrionales and *Plankomarina* highly and positively correlated with axis 1, while SAR11 and *Synechococcus* showed negative correlation with axis 1. Flavobacteriales and Actinobacteria mostly correlated with the db-RDA2 axis.

4 Discussion

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

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

phytoplankton and/or bacterial growth enhancement in at least one B vitamin treatment was frequent but relatively moderate in this productive ecosystem, showing 1.1 to 2.4-fold increases in 75% of the experimental situations for phytoplankton and in 50% for bacteria. On the other hand, negative responses to at least one B vitamin treatment occurred in all but one of the experimental situations (Fig. 7). The low and constant B12 ambient concentration (Fig. 4) and the reduced magnitude of microbial responses 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.

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 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 marine productive ecosystems, as previously observed by other authors (e.g., Panzeca et al., 2006; Sañudo-Wilhelmy et al., 2006; Bertrand et al., 2007; Gobler et al., 2007; Koch et al 2011; 2012; Barber.-Lluch et al 2019). Most positive responses to B vitamin amendments were observed in oceanic waters, where B12 concentration was significantly lower than in coastal waters (Fig. 4c). Unfortunately we lack B1 measurements in this study, but, according to previous field studies in other oceanographic regions, a similar pattern to that observed for B12 can be expected (Cohen et al., 2017; Sañudo-Wilhelmy et al., 2012; Suffridge et al., 2018). The

487 overall low and stable concentration of B12 at both sampling locations suggests a high
488 turnover time of this compound in these productive, well-lit waters. Rapid cycling of B12
489 in surface waters may occur due to high biological uptake rates (Taylor and Sullivan,
490 2008; Koch et al., 2012) and/or photochemical degradation (Carlucci et al., 1969;
491 Juzeniene and Nizauskaite, 2013; Juzeniene et al., 2015). The measured B12
492 concentrations were in the lower range reported for coastal sites, and similar to that found
493 in the upwelling system off the California coast in the San Pedro Basin during winter,
494 spring and summer (Panzeca et al., 2009).

495 The increase of phytoplankton biomass was mostly associated to B12 amendments, which
496 is consistent with the known incapability of eukaryotes to synthesize this vitamin (Croft
497 et al., 2005; Tang et al., 2010; Sañudo-Wilhelmy et al., 2014). Considering the very low
498 concentration of B12 in the sampling area, the relatively limited phytoplankton response
499 to B vitamins is consistent with the presence of species that may have adapted to
500 overcome B12 limitation in the environment by using alternative enzymes. For example,
501 changes in external B12 availability may cause shifts from vitamin B12-dependence to
502 vitamin B12-independence in taxa possessing the vitamin B12-independent methionine
503 synthase (MetE) gene (Bertrand et al., 2013; Helliwell et al., 2014). Other strategies used
504 by phytoplankton to cope with low cobalamin concentration include, increased cobalamin
505 acquisition machinery, decreased cobalamin demand, and management of reduced
506 methionine synthase activity through changes in folate and S-adenosyl methionine
507 metabolism (Bertrand et al., 2012). The available data on B12 half-saturation constants
508 for phytoplankton (0.1-10 pM) (Droop, 1968, 2007; Taylor and Sullivan, 2008; Tang et
509 al., 2010; Koch et al., 2011) are similar or higher than the B12 concentrations measured
510 here (0.3 pM in the coastal and 0.15 pM in the oceanic waters, on average), reinforcing
511 the hypothesis of a phytoplankton community adapted to B12 limiting concentrations in
512 this upwelling system.

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

Gómez-Consarnau et al., 2018), suggesting that B12 synthesis could be limited in oceanic waters in winter, due to the low abundance of potentially B12 producers.

Microbial responses to B vitamins in subsurface oceanic waters in February were associated to high abundance of *Synechococcus* and, to some extent, of Actinobacteria (Fig. 8). In these experiments, positive effects of B1 addition on phytoplankton and bacteria were observed (Fig. 7). While *Synechococcus* is capable of B1 synthesis (Carini et al., 2014; Sañudo-Wilhelmy et al., 2014; Gómez-Consarnau et al., 2018), Actinobacteria seems to have a strong dependence on this vitamin (Gómez-Consarnau et al., 2018). Among the sequenced eukaryote genomes, only Stramenopiles contain genes codifying for the synthesis of thiamine monophosphate (Sañudo-Wilhelmy et al., 2014; Cohen et al., 2017) . While Stramenopiles, dominated by Bacillariophyta, were ubiquitous in the sampling area, their relative contribution was lower in oceanic waters (Fig. 4). The simultaneous stimulation of phytoplankton and bacteria by B1 addition in subsurface oceanic waters in winter 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. 7). 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 (Fig. 7), when the B vitamin response patterns were associated to high abundance of *Planktomarina* and Actinobacteria (Fig. 8),which are expected to strongly depend on external B1 sources (Giebel et al., 2013; Gómez-Consarnau et al., 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 predominantly negative bacterial responses after vitamin amendments in the coast during summer (Fig. 6, Fig. 7, and Fig. S3 in the Supplement), when nutrient concentrations were low (Fig. 3),

552 suggest either a strong competition between phytoplankton and bacteria or a stimulation
553 of predation. Dinoflagellates were particularly abundant in summer at both sampling sites
554 and depths. Many dinoflagellate species are auxotrophs for B1 and/or B12 (Croft et al,
555 2006; Tang et al., 2010), and also many of them are phagotrophs (Stoecker and Capuzzo,
556 1990; Smayda, 1997; Sarjeant and Taylor, 2006; Stoecker et al., 2017), thus the external
557 supply of B vitamins may have promoted their growth, ultimately leading to net decreases
558 in microbial biomass at the end of the experiments. Several studies demonstrated that
559 vitamin B12 is implicated in the occurrence of dinoflagellate blooms around the world
560 (Aldrich, 1962; Carlucci and Bowes, 1970; Takahashi and Fukazawa, 1982; Yu and
561 Rong-cheng, 2000). It has been suggested that the B12-dependent enzyme
562 methylmalonyl-CoA mutase in dinoflagellate, euglenoid, and heterokont algae allows
563 them to grow heterotrophically when B12 is available (Croft et al., 2006). Therefore, the
564 B12 enrichment could trigger such nutritional strategy, particularly in summer, when
565 mineral nutrients are less available, resulting in an increased predation pressure on
566 bacteria.

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

584 ## 5 Conclusions

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

Author contribution.

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

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

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927 **7 Figures**

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

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

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

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

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

950 **Figure 6**: Bacterial biomass (µgC l^{-1}) in the time-zero of each experiment (striped bars)
951 and in the final-time of each treatment (colored bars) in the experiments conducted at
952 surface and SCM in the coastal and oceanic station in February, April and August.

953 **Figure 7:** Monthly averaged response ratio (RR) of (a) total phytoplankton community
954 and of (b) bacterial community at surface and SCM in the coastal and oceanic station.
955 Horizontal line represents a response equal to 1, that means no change relative to control
956 in the pink bars (treatments with vitamins alone) and no change relative to inorganic (I)
957 treatment in the green bars (vitamins combined with I treatments). Asterisks indicate
958 phytoplankton or bacterial significant response relative to control or I (Z-test; * $p < 0.05$)
959 and a indicate response with a level of significance between 0.05 and 0.1 (Z-test; ^{a} p =
960 0.05-0.06).

961 **Figure 8:** Distance based redundancy analysis (dbRDA) of B vitamin responses by
962 microbial plankton based on Bray-Curtis similarity. Filled and open symbols represent
963 samples from coastal and oceanic station, respectively, numbers correspond to the
964 sampling station, triangles and circles represent samples from surface and SCM,
965 respectively, and colours correspond to the months: (green) February, (blue) April and
966 (pink) August. Only prokaryotic taxa that explained variability in the B vitamin responses
967 structure selected in the DistLM model (step-wise procedure with adjusted R^2 criterion)
968 were fitted to the ordination.
969

fig. 01

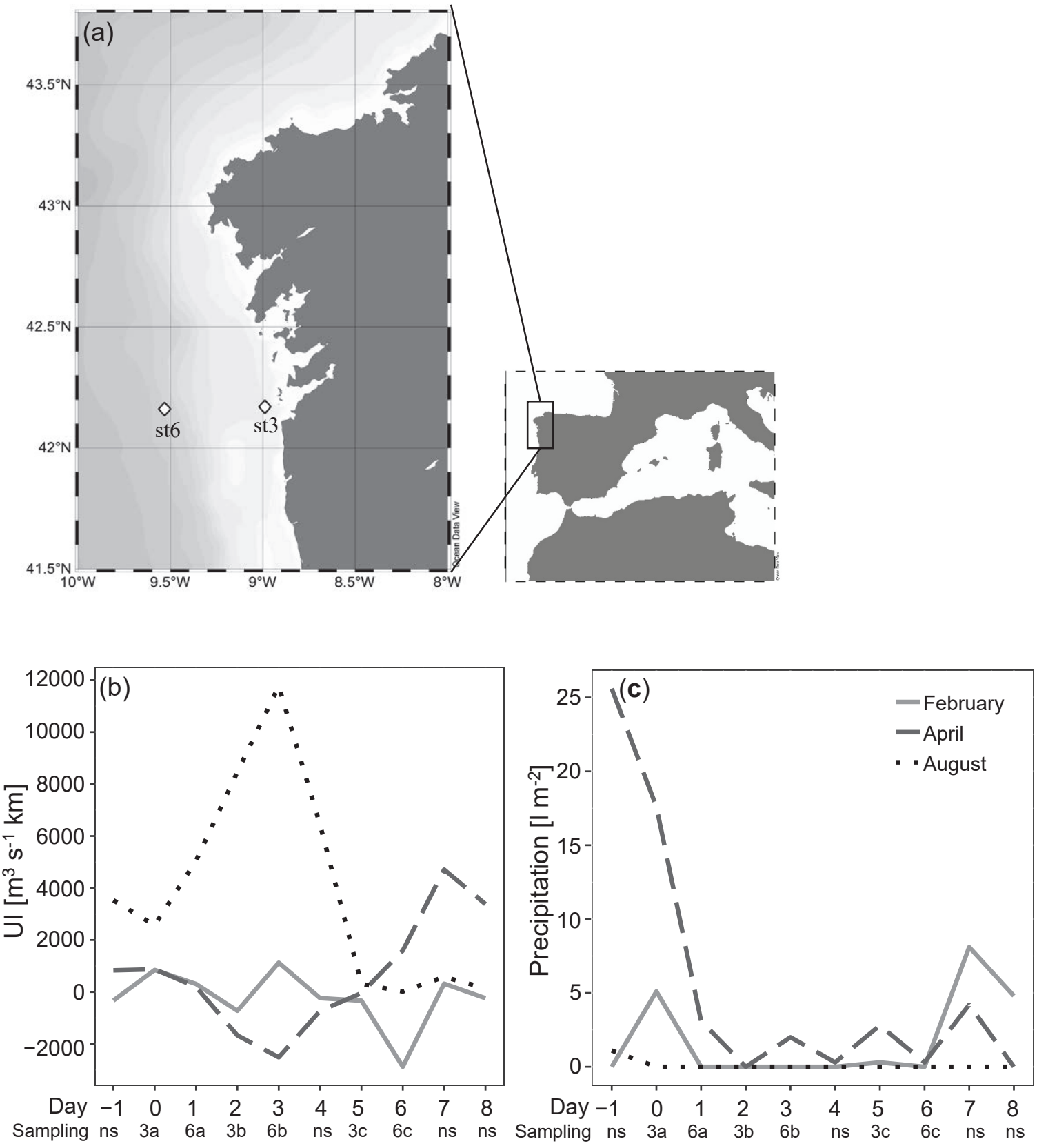
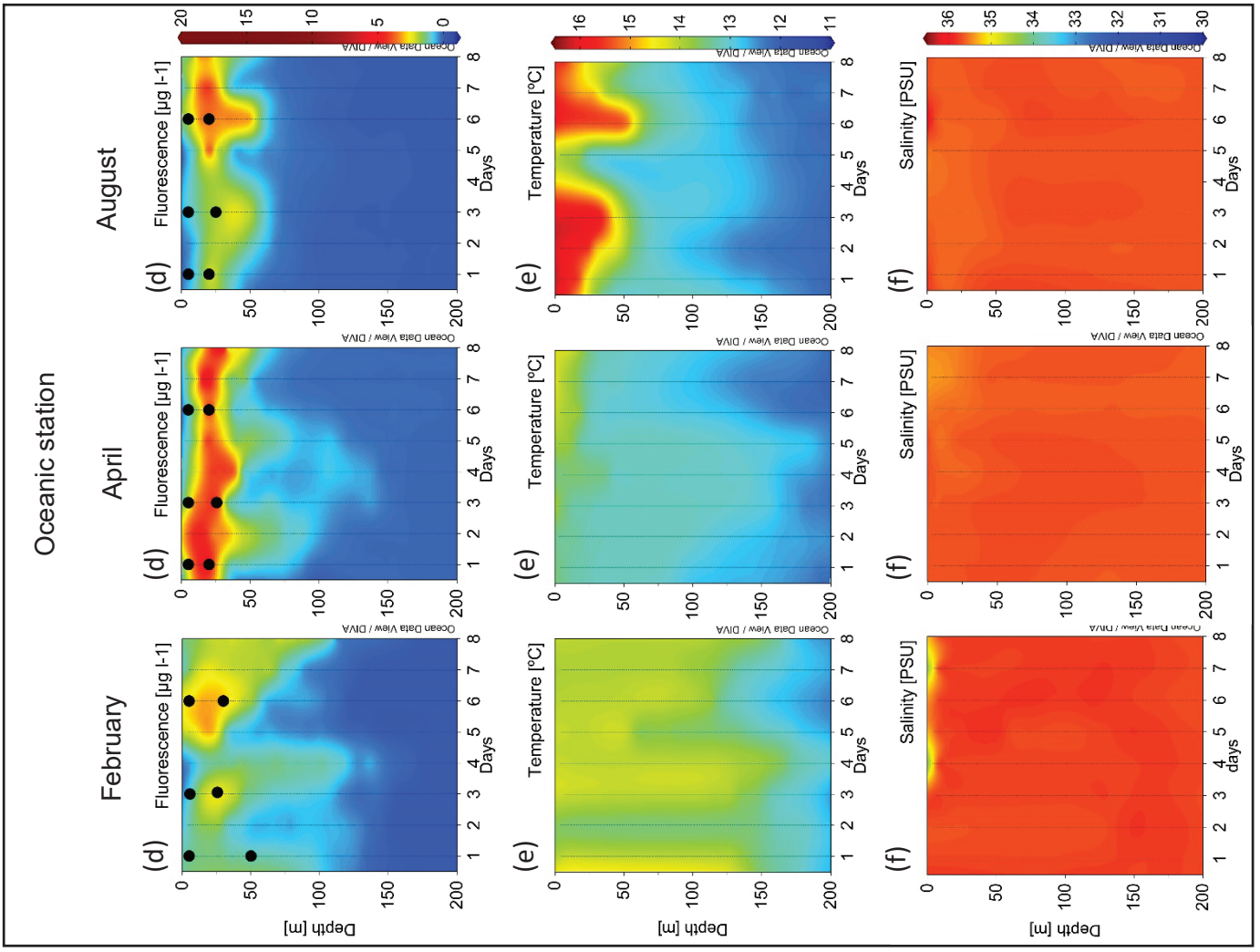

(a)
43.5°N
43°N
42.5°N
42°N
41.5°N
10°W
9.5°W
9°W
8.5°W
8°W
st6
st3
Ocean Data View
(b)
UI [m3 s-1 km]
12000
10000
8000
6000
4000
2000
0
−2000
Day −1 0 1 2 3 4 5 6 7 8
Sampling ns 3a 6a 3b 6b ns 3c 6c ns ns
(c)
Precipitation [l m-2]
25
20
15
10
5
0
February
April
August
Day −1 0 1 2 3 4 5 6 7 8
Sampling ns 3a 6a 3b 6b ns 3c 6c ns ns

fig. 02


Oceanic station
February
April
August
(d)
(e)
(f)
Fluorescence [µg l-1]
Temperature [°C]
Salinity [PSU]
Depth [m]
Days
Ocean Data View / DIVA

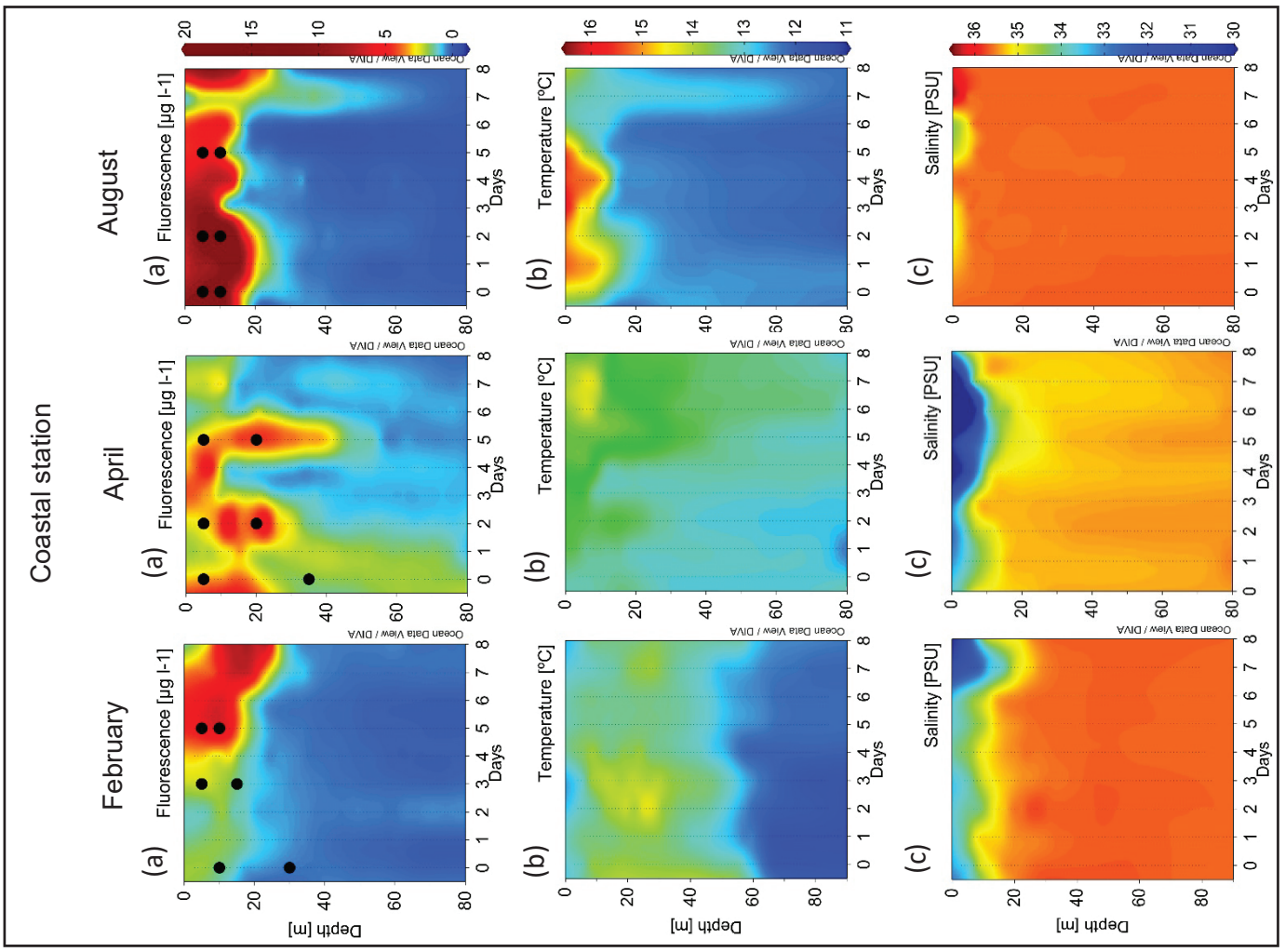

Coastal station
February
April
August
(a)
(b)
(c)
Fluorescence [µg l-1]
Temperature [°C]
Salinity [PSU]
Depth [m]
Days
Ocean Data View / DIVA

fig. 03

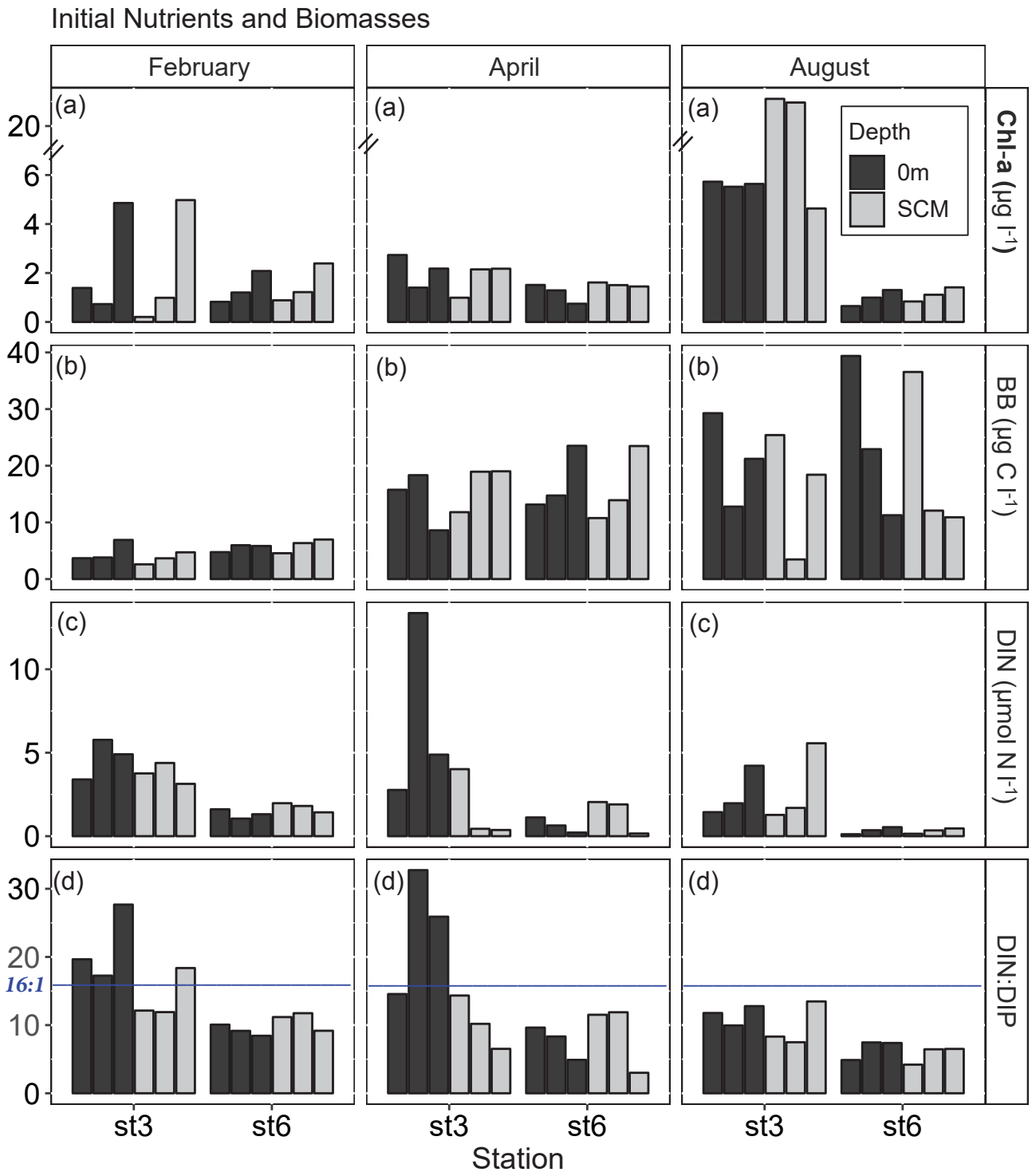

Initial Nutrients and Biomasses
February
April
August
(a)
(b)
(c)
(d)
Depth
0m
SCM
Chl-a (µg l-1)
BB (µg C l-1)
DIN (µmol N l-1)
DIN:DIP
16:1
st3
st6
Station

fig. 04

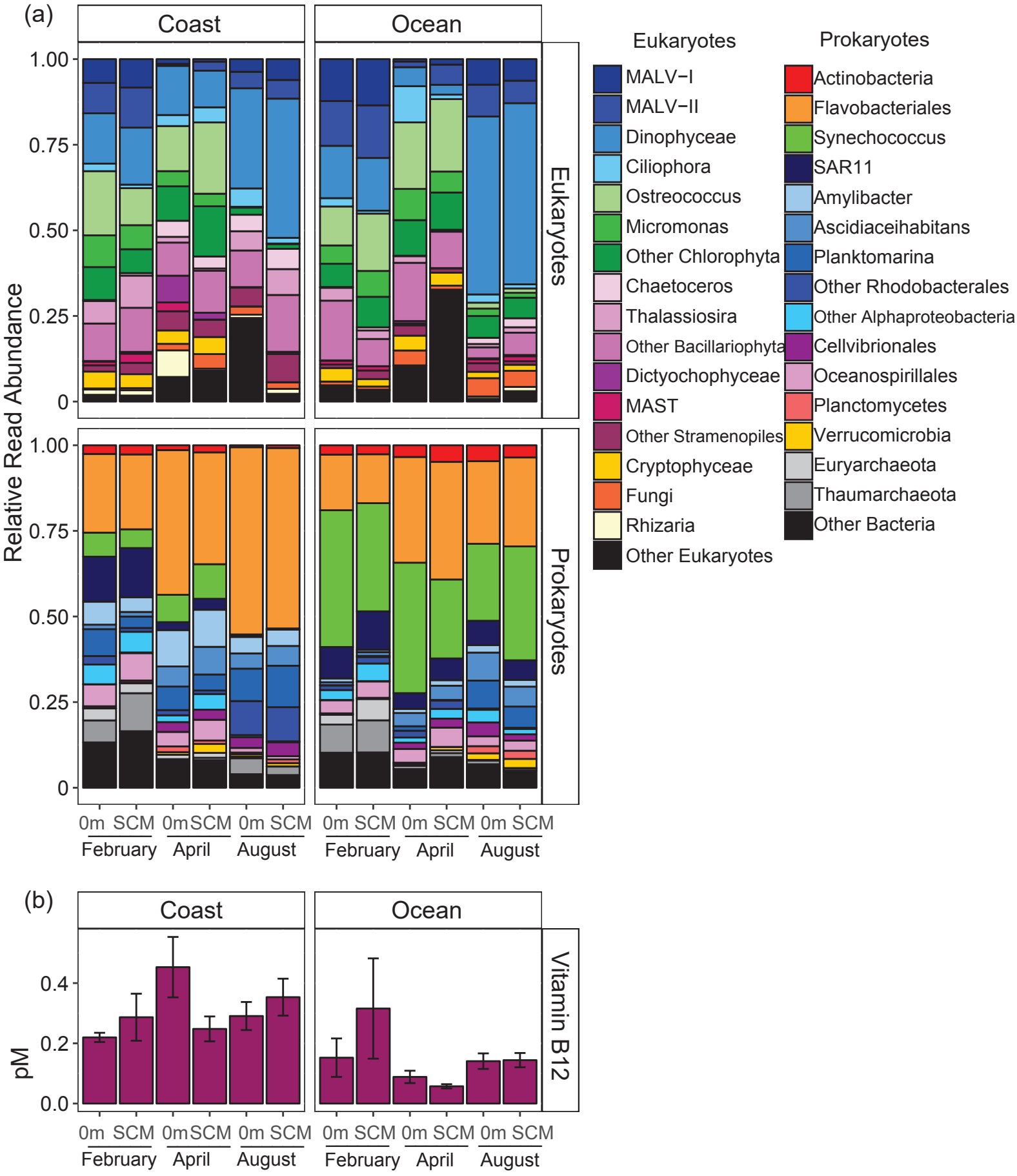

(a)
Coast
Ocean
Eukaryotes
Prokaryotes
Relative Read Abundance
1.00
0.75
0.50
0.25
0
0m SCM 0m SCM 0m SCM 0m SCM 0m SCM 0m SCM
February April August February April August
Eukaryotes
MALV−I
MALV−II
Dinophyceae
Ciliophora
Ostreococcus
Micromonas
Other Chlorophyta
Chaetoceros
Thalassiosira
Other Bacillariophyta
Dictyochophyceae
MAST
Other Stramenopiles
Cryptophyceae
Fungi
Rhizaria
Other Eukaryotes
Prokaryotes
Actinobacteria
Flavobacteriales
Synechococcus
SAR11
Amylibacter
Ascidiaceihabitans
Planktomarina
Other Rhodobacterales
Other Alphaproteobacteria
Cellvibrionales
Oceanospirillales
Planctomycetes
Verrucomicrobia
Euryarchaeota
Thaumarchaeota
Other Bacteria
(b)
Coast
Ocean
Vitamin B12
pM
0.4
0.2
0.0
0m SCM 0m SCM 0m SCM 0m SCM 0m SCM 0m SCM
February April August February April August

fig. 05

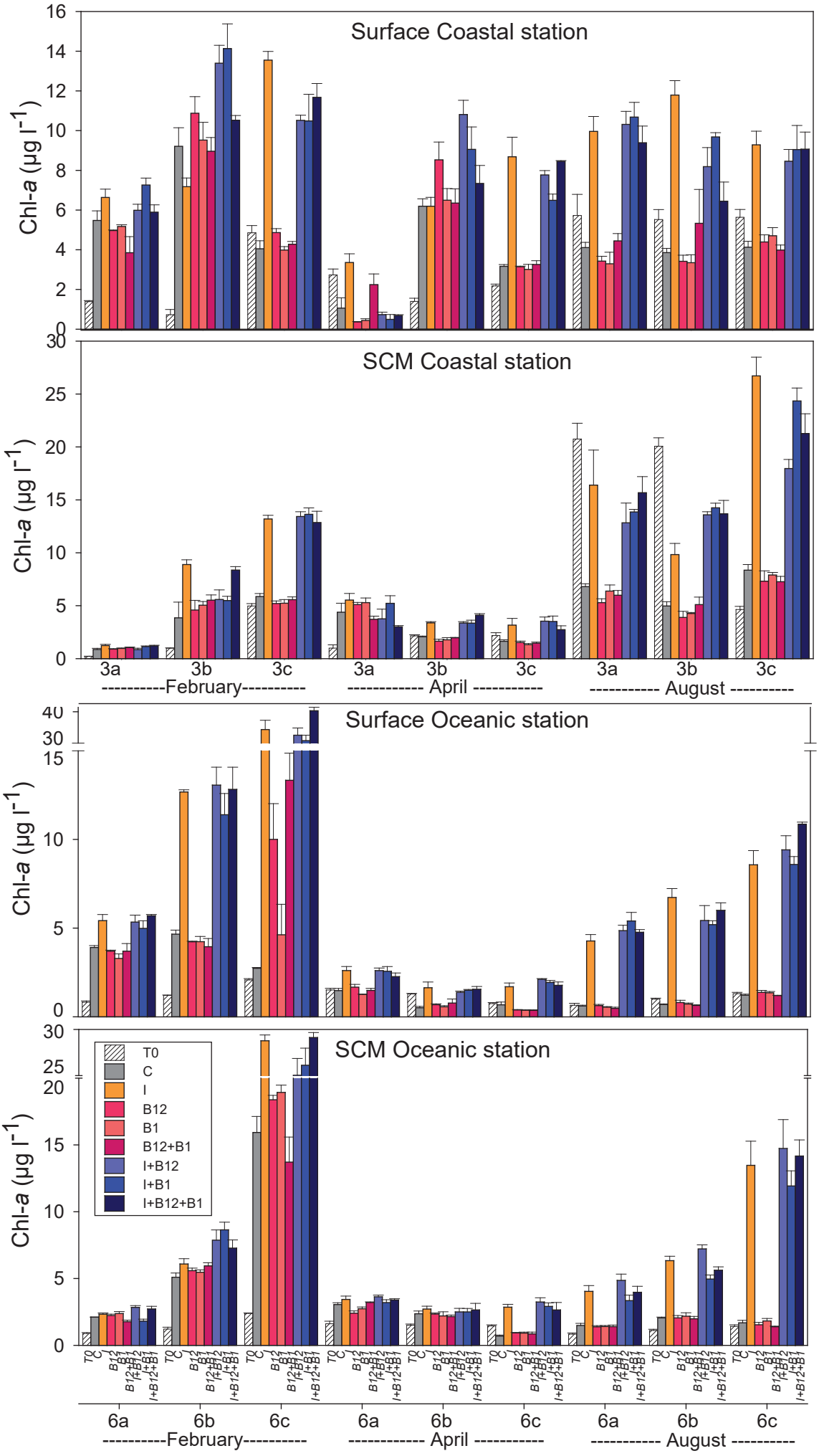

Surface Coastal station
SCM Coastal station
Surface Oceanic station
SCM Oceanic station
Chl-a (µg l-1)
T0
C
I
B12
B1
B12+B1
I+B12
I+B1
I+B12+B1
3a
3b
3c
6a
6b
6c
----------February----------
---------- April ----------
---------- August ----------

fig. 06

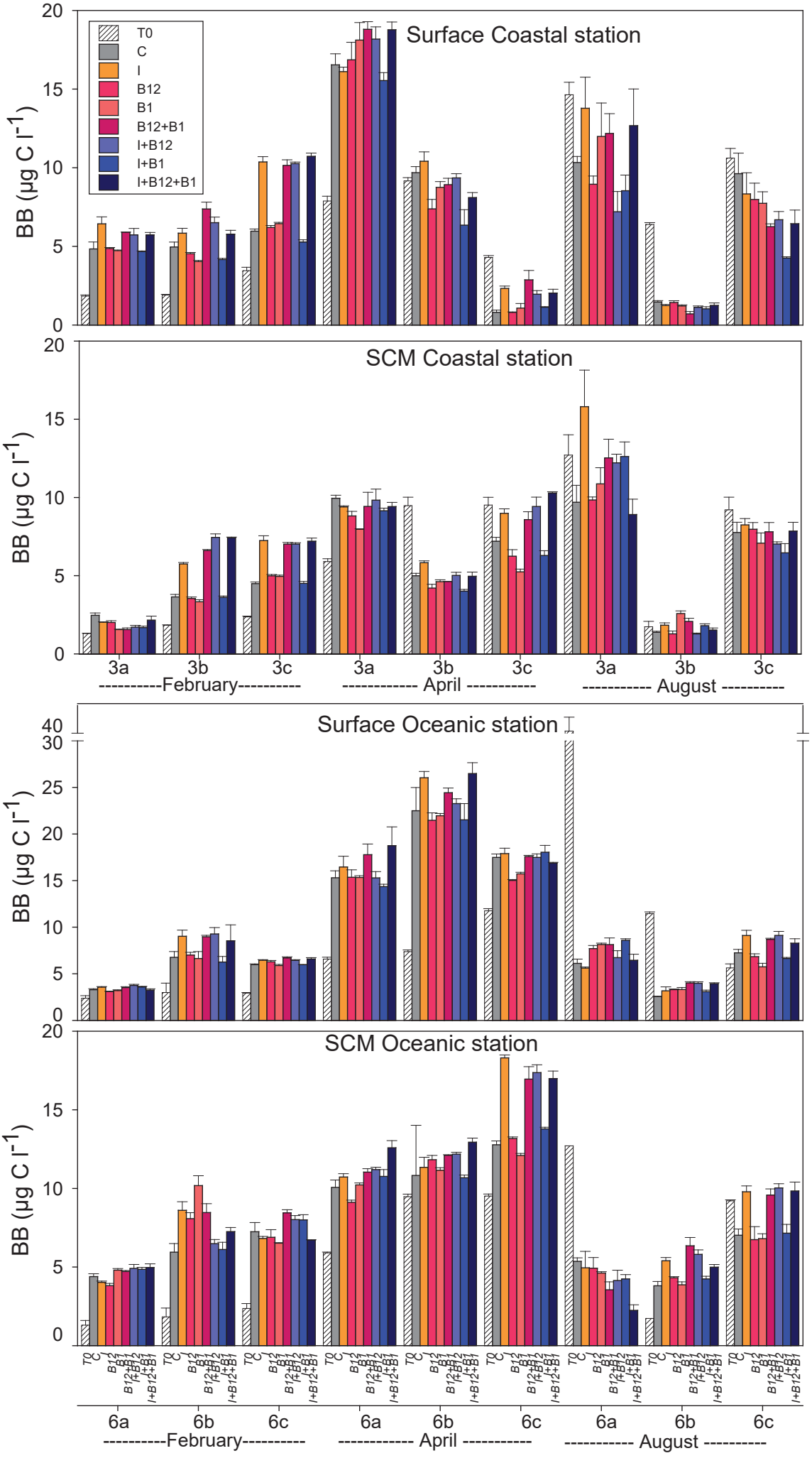

Surface Coastal station
SCM Coastal station
Surface Oceanic station
SCM Oceanic station
T0
C
I
B12
B1
B12+B1
I+B12
I+B1
I+B12+B1
BB (µg C l-1)
3a
3b
3c
6a
6b
6c
February
April
August

fig. 07

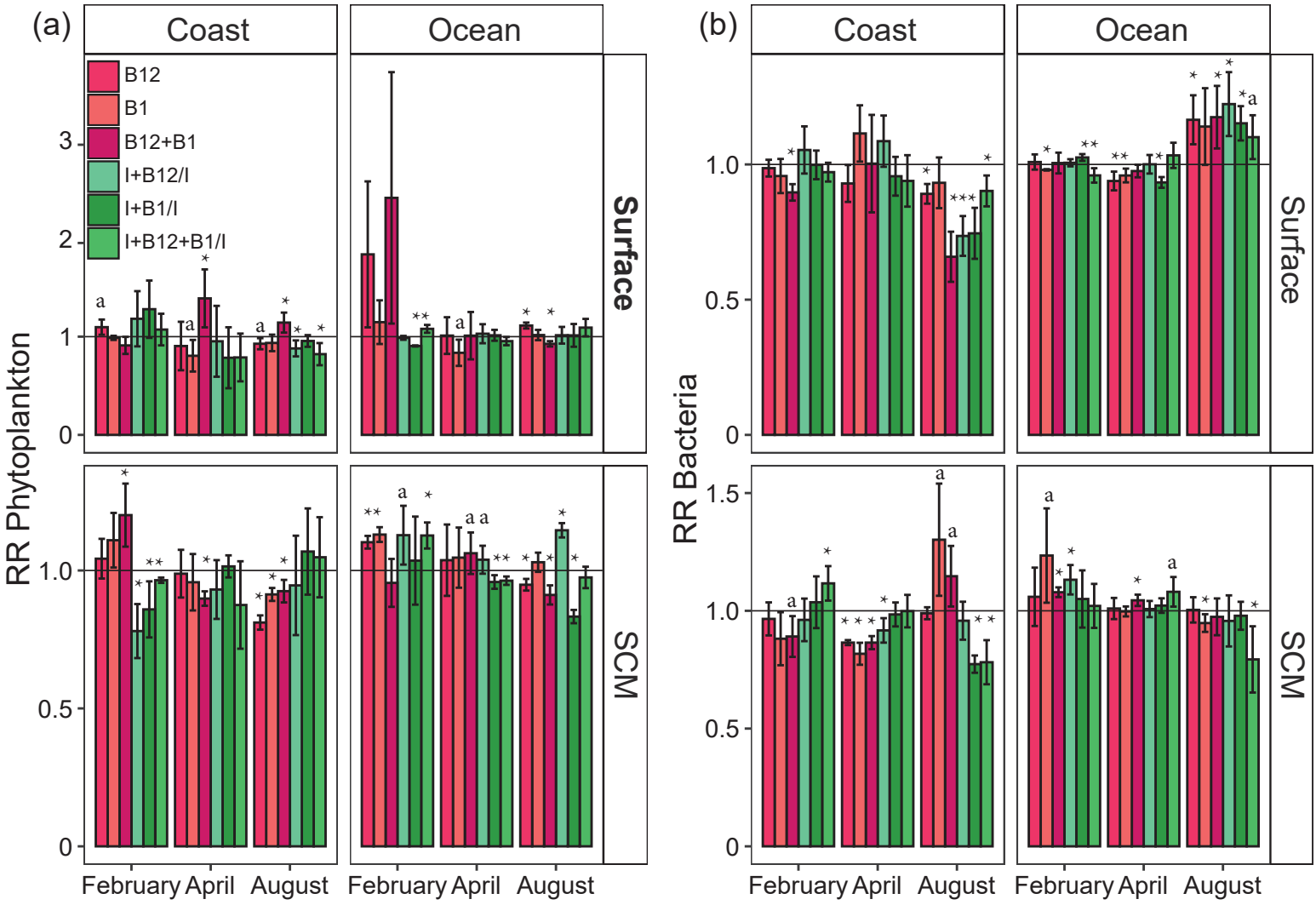
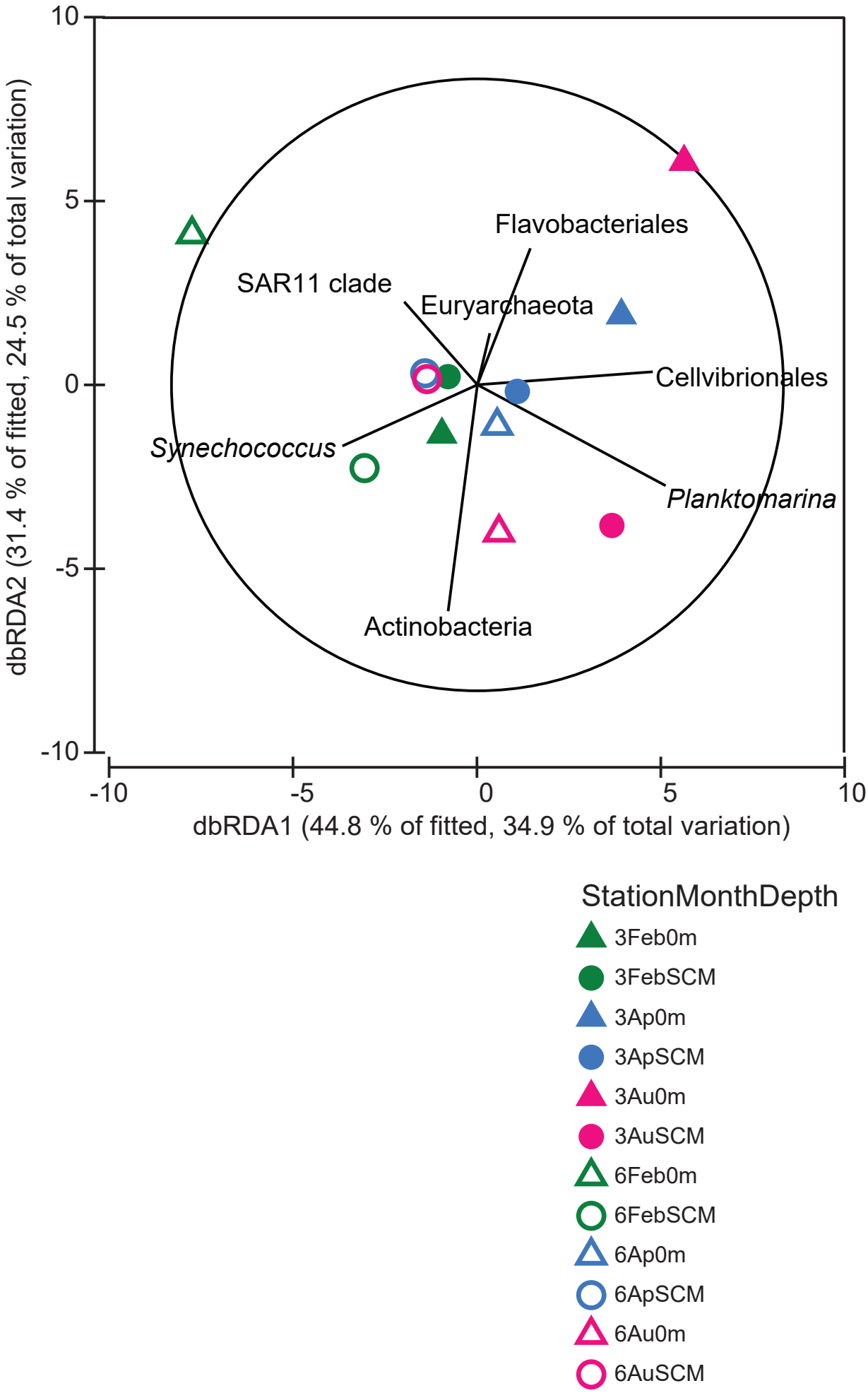

(a)
(b)
Coast
Ocean
Surface
SCM
B12
B1
B12+B1
I+B12/I
I+B1/I
I+B12+B1/I
RR Phytoplankton
RR Bacteria
February
April
August

fig. 08


10
5
0
-5
-10
-10
-5
0
5
10
dbRDA2 (31.4 % of fitted, 24.5 % of total variation)
dbRDA1 (44.8 % of fitted, 34.9 % of total variation)
Flavobacteriales
SAR11 clade
Euryarchaeota
Cellvibrionales
Synechococcus
Planktomarina
Actinobacteria
StationMonthDepth
3Feb0m
3FebSCM
3Ap0m
3ApSCM
3Au0m
3AuSCM
6Feb0m
6FebSCM
6Ap0m
6ApSCM
6Au0m
6AuSCM

Supplement information

Table S1: concentration of hydroxocobalamin (OHB12) and cyanocobalamin (CNB12) in seawater samples corresponding to the initial time of the experiments. Abbreviations: Not detected (nd) and lower concentration of the quantification limit (<LOQ).

Sample ID	Station	Depth	Month	OHB12 pM	CNB 2 pM
1602_st3_d1_p1	coast	surface	February	0.21	nd
1602_st3_d3_p1	coast	surface	February	0.20	nd
1602_st3_d5_p1	coast	surface	February	0.26	nd
1604_st3_d1_p1	coast	surface	April	0.47	nd
1604_st3_d3_p1	coast	surface	April	0.66	nd
1604_st3_d5_p1	coast	surface	April	0.23	nd
1608_st3_d1_p1	coast	surface	August	0.30	nd
1608_st3_d3_p1	coast	surface	August	0.38	nd
1608_st3_d5_p1	coast	surface	August	0.19	nd
1602_st3_d1_p2	coast	SCM	February	0.36	nd
1602_st3_d3_p2	coast	SCM	February	0.10	nd
1602_st3_d5_p2	coast	SCM	February	0.41	nd
1604_st3_d1_p2	coast	SCM	April	0.32	nd
1604_st3_d3_p2	coast	SCM	April	0.27	nd
1604_st3_d5_p3	coast	SCM	April	0.15	nd
1608_st3_d1_p2	coast	SCM	August	0.46	nd
1608_st3_d3_p2	coast	SCM	August	0.21	nd
1608_st3_d5_p2	coast	SCM	August	0.39	nd
1602_st6_d1_p1	ocean	surface	February	0.31	nd
1602_st6_d3_p1	ocean	surface	February	0.09	nd
1602_st6_d5_p1	ocean	surface	February	0.06	nd
1604_st6_d1_p1	ocean	surface	April	0.13	nd
1604_st6_d3_p1	ocean	surface	April	0.09	nd
1604_st6_d6_p1	ocean	surface	April	0.04	nd
1608_st6_d1_p1	ocean	surface	August	0.20	nd
1608_st6_d3_p1	ocean	surface	August	0.09	nd
1608_st6_d6_p1	ocean	surface	August	0.14	nd
1602_st6_d1_p3	ocean	SCM	February	0.21	0.55
1602_st6_d3_p2	ocean	SCM	February	0.08	nd
1604_st6_d1_p2	ocean	SCM	April	nd	nd
1604_st6_d3_p2	ocean	SCM	April	0.07	nd
1604_st6_d6_p2	ocean	SCM	April	0.05	nd
1608_st6_d1_p2	ocean	SCM	August	0.19	nd
1608_st6_d3_p2	ocean	SCM	August	0.09	nd
1608_st6_d6_p2	ocean	SCM	August	0.16	nd

Table S2: Summary of initial conditions for each experiment (expt). Sampling months were February (Feb), April (Apr) and August (Aug).

Station	Depth	Month	Expt	Temp °C	Sal	NO_3^- µM	NO_2^- µM	NH_4^+ µM	HPO_4^{2-} µM	DIN:P µM	SiO_4^{2-}	Chl-a µg l^{-1}	BB µgC l^{-1}
Coast	surface	Feb	3a	13.75	35.02	2.86	0.19	0.35	0.17	19.65	3.62	1.39	1.84
			3b	13.22	34.27	4.89	0.36	0.51	0.33	17.25	6.77	0.73	1.91
			3c	13.43	34.21	4.63	0.19	0.09	0.18	27.68	8.57	4.86	3.45
		Apr	3a	12.96	34.58	2.21	0.24	0.32	0.19	14.55	5.24	2.73	7.88
			3b	13.31	34.25	12.46	0.36	0.54	0.41	32.73	12.57	1.40	9.17
			3c	14.04	31.83	4.18	0.16	0.55	0.19	25.90	10.52	2.18	4.30
		Aug	3a	14.14	35.60	0.50	0.10	0.84	0.12	11.77	1.11	5.73	14.64
			3b	14.36	35.61	0.81	0.08	1.08	0.20	9.95	0.28	5.52	6.39
			3c	13.66	35.16	3.93	0.17	0.12	0.33	12.78	3.86	5.64	10.61
	SCM	Feb	3a	13.73	35.71	3.58	0.14	0.04	0.31	12.13	5.25	0.21	1.30
			3b	13.91	35.27	4.16	0.15	0.07	0.37	11.91	4.63	0.99	1.83
			3c	13.45	34.66	2.94	0.09	0.10	0.17	18.37	6.13	4.98	2.36
		Apr	3a	12.80	35.34	3.22	0.34	0.46	0.28	14.34	4.39	0.99	5.90
			3b	13.22	35.28	0.24	0.07	0.12	0.04	10.19	2.83	2.15	9.47
			3c	13.92	34.95	0.21	0.07	0.10	0.06	6.52	3.41	2.18	9.51
		Aug	3a	13.58	35.62	0.91	0.13	0.23	0.15	8.32	1.68	20.75	12.71
			3b	13.82	35.61	1.40	0.16	0.14	0.23	7.49	1.40	20.07	1.73
			3c	13.38	35.63	5.29	0.13	0.14	0.41	13.47	3.93	4.63	9.21
Ocean	surface	Feb	6a	13.98	30.20	1.32	0.18	0.11	0.16	10.07	3.23	0.82	2.38
			6b	14.16	35.86	0.90	0.11	0.04	0.12	9.15	2.29	1.20	2.98
			6c	14.10	35.40	1.03	0.15	0.13	0.16	8.43	2.97	2.08	2.92
		Apr	6a	13.44	35.68	0.95	0.11	0.06	0.12	9.63	2.31	1.51	6.58
			6b	13.59	35.66	0.47	0.11	0.06	0.08	8.33	2.71	1.29	7.37
			6c	13.93	35.57	0.12	0.03	0.06	0.04	4.90	2.08	0.75	11.76
		Aug	6a	15.97	35.61	0.05	0.01	0.06	0.02	4.88	1.46	0.65	39.38
			6b	16.04	35.59	0.26	0.01	0.09	0.05	7.46	3.21	0.99	11.46
			6c	15.34	35.53	0.45	0.04	0.05	0.07	7.38	1.37	1.30	5.63
	SCM	Feb	6a	14.08	35.75	1.73	0.20	0.04	0.18	11.18	3.47	0.88	2.28
			6b	14.10	35.76	1.60	0.19	0.02	0.15	11.75	2.86	1.22	3.18
			6c	14.13	35.82	1.13	0.18	0.12	0.16	9.17	2.92	2.39	3.49
		Apr	6a	13.28	35.69	1.63	0.31	0.10	0.18	11.51	3.16	1.61	5.38
			6b	13.28	35.68	1.45	0.33	0.12	0.16	11.88	2.42	1.50	6.96
			6c	13.72	35.60	0.03	0.06	0.07	0.05	3.01	1.89	1.45	11.74
		Aug	6a	14.90	35.60	0.00	0.04	0.10	0.03	4.20	1.44	0.84	26.55
			6b	15.95	35.60	0.27	0.00	0.07	0.05	6.45	2.79	1.11	6.04
			6c	15.41	35.62	0.35	0.06	0.06	0.07	6.51	1.66	1.41	5.45

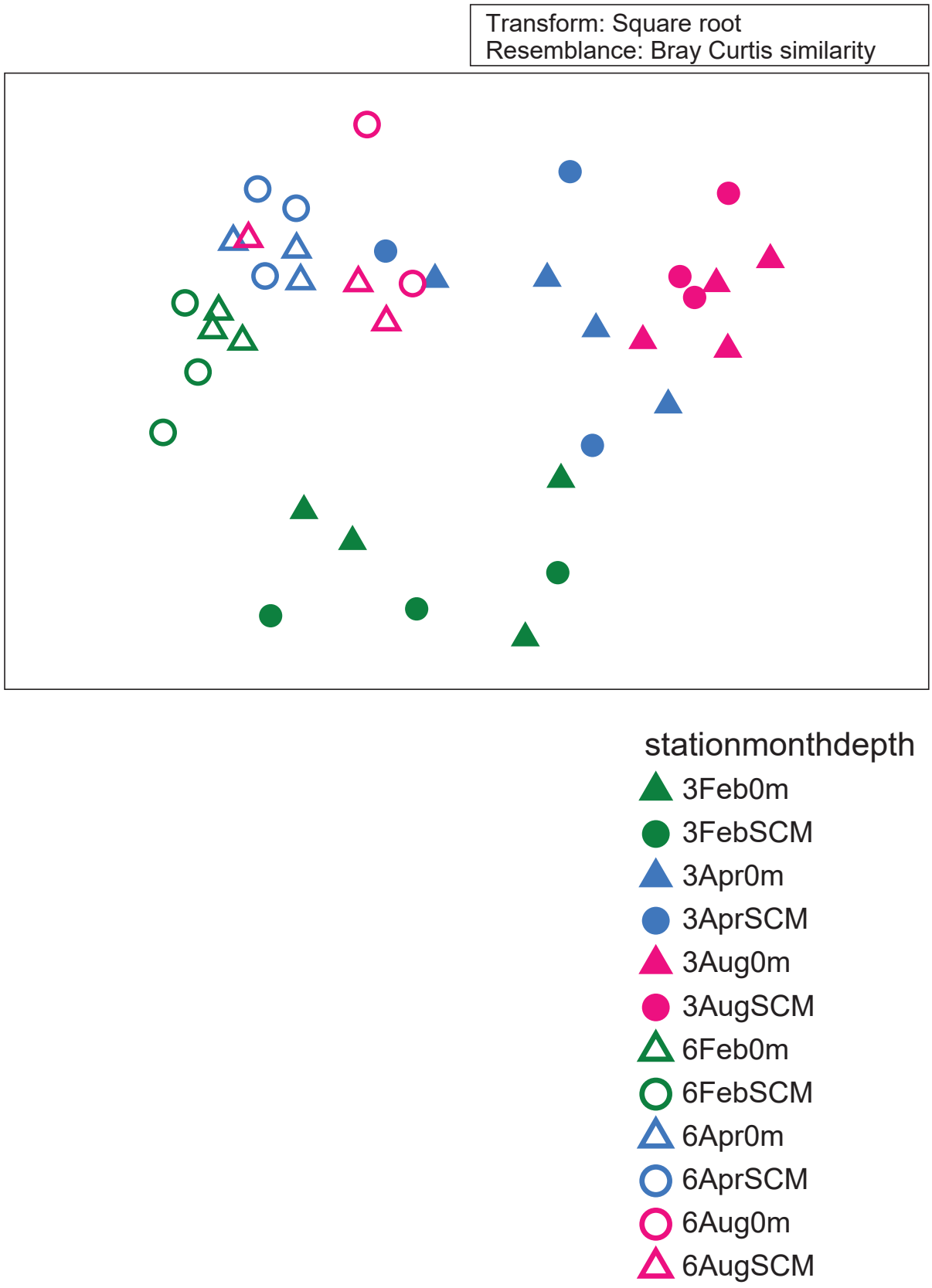
Figure S1: A multidimensional scaling (MDS) showing the distance according to similarity in the microbial plankton composition at the beginning of each experiment (each symbol). Filled and open symbols represent samples from coastal and oceanic station, respectively, numbers correspond to the sampling station, triangles and circles represent samples from surface and SCM, respectively, and colours correspond to the months: (green) February, (blue) April and (pink) August.

Figure S2: Response ratio (RR) to inorganic nutrient addition (averaged biomass at the end of the experiments by the averaged value in the control) of total phytoplankton community (smooth bars) and of bacterial biomass (striped bars) at (a) coastal and (b) oceanic station. Each bar corresponds to one of the 3 experiments (a, b or c) performed in each depth and station during February, April and August. Colours represent samples from (light grey) surface and (dark grey) SCM. Horizontal line represents a response equal to 1, that means no change relative to control. Asterisks indicate phytoplankton significant response relative to control (t-test; * $p < 0.05$) and circle indicate bacterial significant response relative to the control (t-test; ^{0} $p < 0.05$). Note that different scales were used.

Figure S3: 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;

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

Figure S1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Resemblance: Bray Curtis similarity
stationmonthdepth
3Feb0m
3FebSCM
3Apr0m
3AprSCM
3Aug0m
3AugSCM
6Feb0m
6FebSCM
6Apr0m
6AprSCM
6Aug0m
6AugSCM

Figure S2

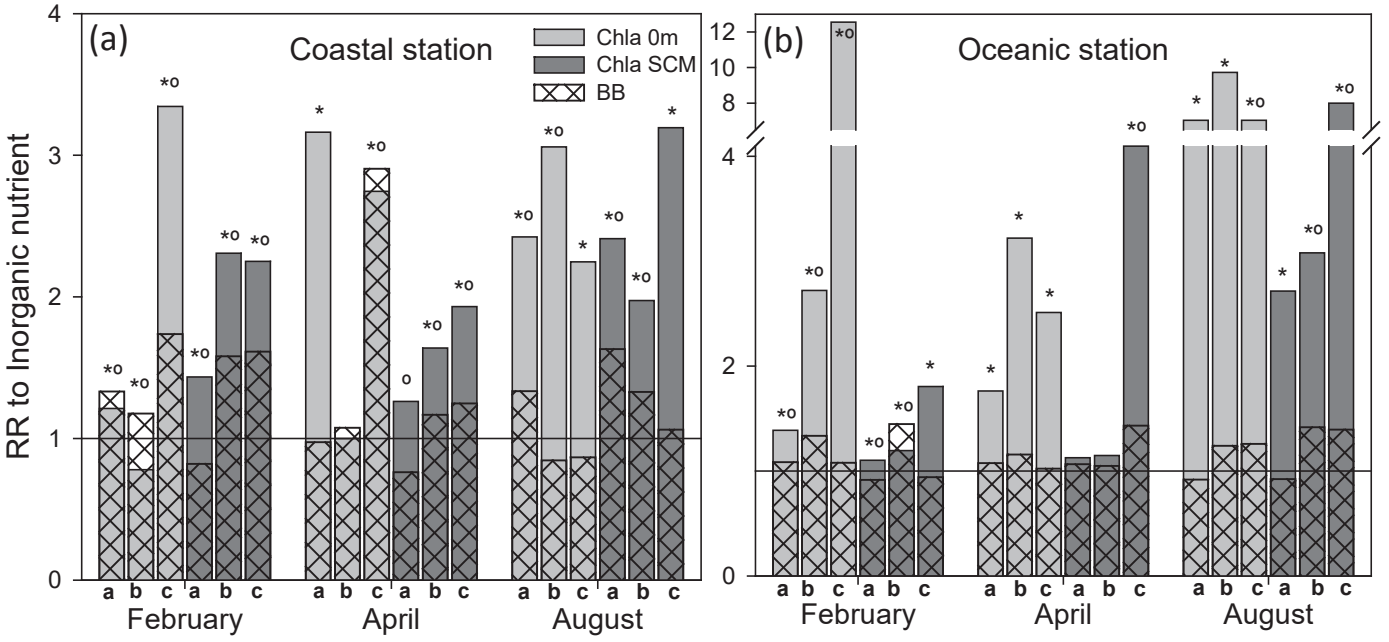
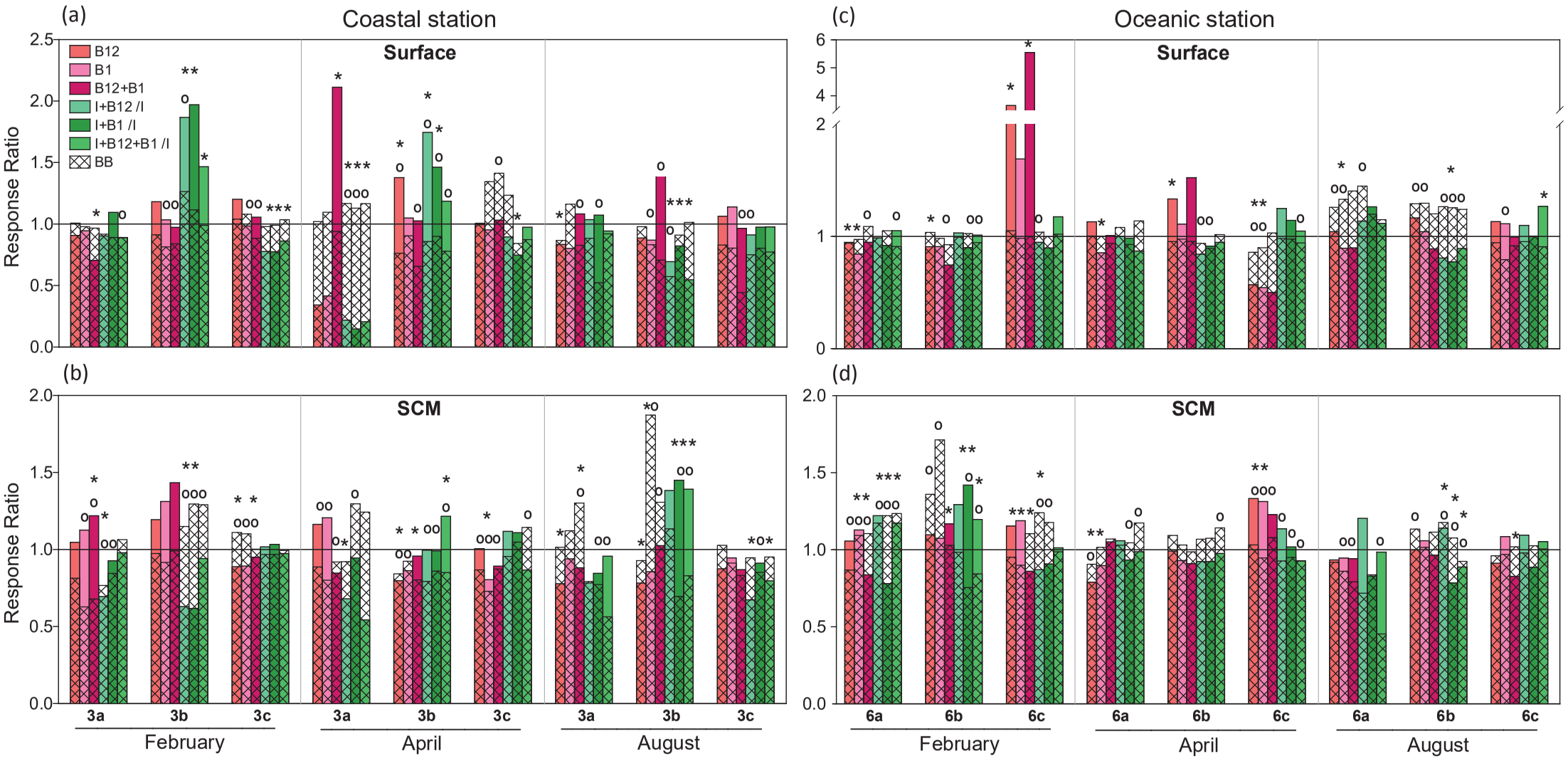

(a)
Coastal station
Chla 0m
Chla SCM
BB
RR to Inorganic nutrient
(b)
Oceanic station
a b c a b c a b c a b c a b c a b c
February
April
August

Figure S3


(a)
Coastal station
Surface
B12
B1
B12+B1
I+B12 /I
I+B1 /I
I+B12+B1 /I
BB
Response Ratio
(b)
SCM
(c)
Oceanic station
Surface
(d)
SCM
3a
3b
3c
6a
6b
6c
February
April
August