Vanessa Joglar

Biological Oceanography Group

Koji Suzuki

Associate Editor

Biogeosciences

Vigo, 27th February 2020

Dear Koji

Please find attached a new 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-Ruiz, Emilio Fernández and Eva Teira.

We would like to appreciate the extensive and constructive comments of the anonymous referees, which clearly helped us to improve the overall quality and understanding of the manuscript. We have considered all the issues raised by the reviewers, and a detailed response to all comments is attached. In the individual responses to referee comments, the suggestions and comments of the reviewers are in plain font and our responses are in italic and blue font. The revised version of the manuscript with marked changes is also provided. The major changes are summarized below:

- The language has been revised and improved.
- A table (Table 1) has been included to facilitate the understanding of the experimental treatments.

- The quality of the figures has been improved by changing the layout and/or the colours when appropriate (e.g. figure 5, figure 6, figure 7, figure S3 and figure S4).
- Figure S3 has been modified and replaced by new Fig. S3 and Fig. S4.
- As one of the reviewers required, we have carefully revised the manuscript, including the text and figures, clearly indicating that the response variable is chlorophyll-a, not phytoplankton biomass.
- We have reviewed the manuscript replacing bacterial biomass to prokaryote biomass, as archaea were also included in the cytometer counts.
- The station numbers have been replaced by coastal and oceanic station.
- We reviewed the discussion eliminating speculative statements and toning down some of our conclusions.

Looking forward to hearing from you,

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Referee #1

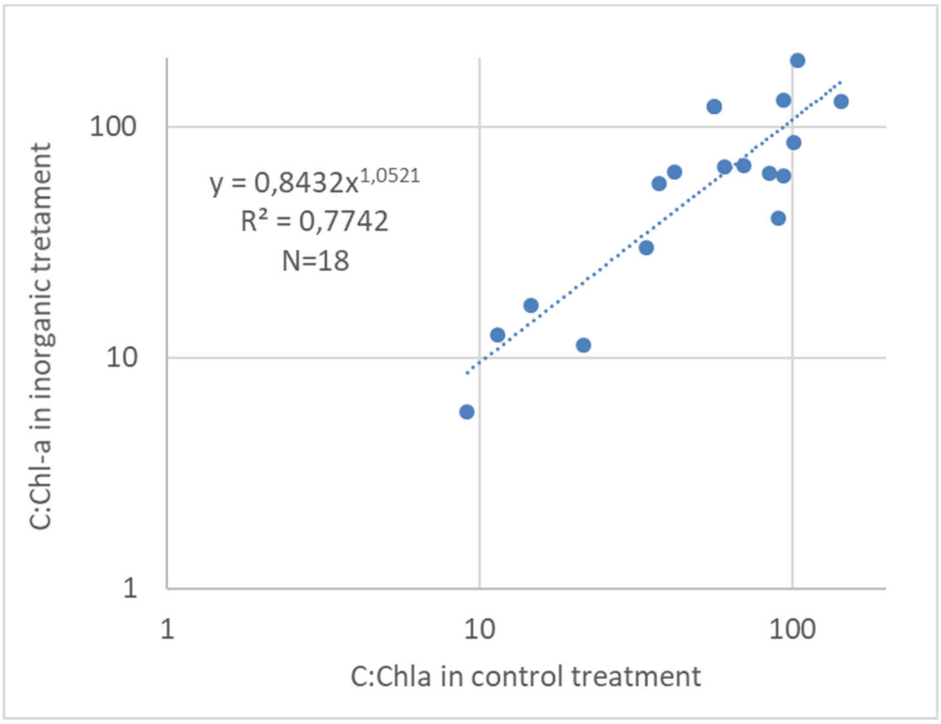
While the authors have successfully addressed many of my specific comments, they have not addressed the global issue of the manuscript being vague and unspecific to the point where it is not clear that the data matches the conclusions the authors are making. It's very possible they do match, but the way the manuscript is written, it is not currently clear. The manuscript still suffers from major flaws that prevent it to be published in its current form. Most concerning of which is the lack of specificity that the authors use in their language. It is impossible for the reader to know exactly what results the authors are referring to.

For example: throughout the manuscript the authors use phytoplankton biomass and chlorophyll concentration interchangeably (e.g., ln 321, 355). The authors measured chlorophyll concentration, not biomass. It is strongly established that chlorophyll concentration to cellular carbon (biomass) ratios are highly variable in phytoplankton, especially across seasonal changes in light and nutrient concentration (which is the context for the authors experiments). It is not valid to say that phytoplankton biomass is being estimated by chlorophyll concentration. I do think that measuring chlorophyll concentration is a valid method of tracking phytoplankton, but the authors need to be specific throughout the manuscript about what they are actually measuring, and make sure that the conclusions they are making can be supported by their actual data. Reporting changes in chlorophyll concentration have a dramatically different physiological and ecological implications than reporting changes in biomass. I can't be sure what they mean with the manuscript in its current form.

In sum, this manuscript has a lot of potential. I was (and am) excited to see this experiment, and I do think the data should eventually be published. However, as it stands currently, the authors have not done their due diligence making sure that the manuscript is clear, specific, and ready for publication.

We appreciate the overall positive comments of the reviewer. We believe that this revised version is now more clear and focused. We are aware that chlorophyll-a can be only used as an estimator of phytoplankton biomass. We had clearly indicated in the methodological section that we use chlorophyll-a concentration as a proxy for phytoplankton biomass (line 176 in the

former resubmitted version). This pigment is universally used in phytoplankton studies and its use as a phytoplankton biomass estimator is common among the scientific community. Moreover, most experimental studies evaluating responses of phytoplankton to nutrient additions used chlorophyll-a concentration as response variable (e.g., Bertrand et al., 2015; Browning et al., 2017; Caron et al., 2000; Martínez-García et al., 2010, Hernández-Ruiz et al 2020). We are also aware that C:Chla ratio varies with light and nutrients. Regarding light, all the treatments were incubated under the same light conditions (simulating the corresponding in situ irradiance). On the other hand, in previous experiments, using the same inorganic nutrient additions in the same sampling area we found a very good linear relationship with slope ca. 1, between the C:Chla ratio in the control and the corresponding ratio in the inorganic treatment (see plot below built with data from Martínez-García et al 2010b and Teira et al 2011), suggesting that at this short time scale, nutrient levels are not significantly affecting C:Chla ratio. As determining phytoplankton carbon biomass is extremely time-consuming, we decided to use only chlorophyll-a to evaluate the response of phytoplankton in this extensive study. Nevertheless, for clarity we have carefully revised the manuscript, including the text and figures, clearly indicating that the response variable is chlorophyll-a, not phytoplankton biomass.



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Referee #2

Review of "Spatial and temporal variability in the response of phytoplankton and bacterioplankton to B-vitamin amendments in an upwelling system" by Joglar et al.

General comments

This manuscript covers a very interesting and highly relevant topic, which the authors focuses on in the manuscript. Dynamics of B-vitamins in the worlds ocean is not often studied and this manuscript attempts to provide important information on this topic. The sampling campaign is definitely impressive, as well as the work that went in to the study. I don't agree with all the comments from the previous reviewers, for instance I find the use of response ratios very informative and a great display of the results. With that said, I have some comments and concerns with the manuscript that need to be addressed.

One main problem with the manuscript is that only one of the vitamins investigated is analyzed. I realize this may be due to problem quantifying B1 in natural sea water, which the authors can state more clearly. It would also have been very interesting to have quantified the cellular content in the two size fractions, but unfortunately this was not done.

Generally, is there any benefit of using st3 and st6 instead of coastal and oceanic station? I feel the readability would increase if you used coastal and oceanic instead.

When referring to figures, state which of the figures, a, b or c. you refer to. Also, look over all figures so that all are labelled a, b, c …. For fig. 2 and 3 I'm having troubles seeing the benefits of having several a's, several b's etc. I would like to see labelling a-r instead for fig. 2, then you can refer to the specific mosaic.

I would like to see a more accurate reporting of statistics. Please provide statistics value (t, F, df) when appropriate.

In my opinion, the results should be presented as averages, per station and cruise and ignore 3a, 3b, 3c etc. I understand that a tremendous amount of work has gone into this experiment, but I believe that the paper would benefit from a more succinct and concise result section.

We very much appreciate the extraordinarily constructive and extensive review made by the referee, which undoubtedly and substantially improved the manuscript. We now clearly state that

we were unable to measure dissolved B1 concentration due to the very low concentration in the water and the reduced pre-concentration volume (ca 1 L) (Lines 245-246). Even though we filtered 2 L of seawater sample for B-vitamin determinations, we could only pre-concentrate 1 L in the C18 columns, as the columns became systematically clogged. We have changed the denomination of the two sampling sites and now we use coast and ocean. We have carefully revised the format of figures including the lettering to refer to the different plots. We also provide now the t or F value and the df when appropriate.

We agree that the big picture emerge when averaging the three experiments in each sampling site, and for this reason we only represent the average response ratio in figure 7. We maintain the raw data represented in figure 5 and 6 as other reviewers required to include those figure in the manuscript. Nevertheless, the results description and discussion is mostly based on averaged responses.

Specific comments

Abstract

L15; "… unimportant, …" – I would suggest changing wording, as you cannot know if it is unimportant or not. Maybe "slight" or "limited"?

This has been corrected (L15)

L15; how can an "unimportant" variability lead to the assumption that there are factors operating at other scales? Requires clarification.

We have clarified this sentence. We conclude that the availability of B-vitamins might be, in part, controlled by seasonal processes given that inter-season variability was larger than inter-day variability (L15-17)

L20; change "alone" to solely?

This has been changed (L21)

L22-24; auxotrophy is also high in phytoplankton, causing the argument to halter a bit. I would suggest mentioning this as well and combine it with bacteria dependence.

Phytoplankton B1 auxotrophy has been considered in the abstract (L25-28)

Introduction

L34; state which toxic episodes you refer to.

This has been clarified (L39-40)

L60-61; I would suggest reading Cruz-Lopez et al. 2016.

This reference has been included (L67)

L69-74; I would suggest reviewing if you really need all references to say what you want to say. In a relatively short sentence, you use 13 references.

Several references have been removed (L77-80)

L100 & L110; decide if you use numbers or text, 36 or thirty-six, and use throughout.

This has been corrected (L106)

L105; change "synthetize" to synthesize.

This has been changed (L111)

Methods

L119; What is the timeframe between a, b and c? Looking at fig 1 I realized I can figure it out, but it is a very unclear way to present samplings.

The timeframe between experiments (a, b and c) was 2 or 3 days. This can be observed in figure 1b, 1c, and figure 2 (black points represent the initial time of each experiment). This information is also now included in the text (L126-129)

L120-123; To increase clarity, I would recommend to state that surface is 5m deep more clearly.

This has been clarified (L130-133)

L123; State which occasion this sampling failed.

This has been indicated (L134)

L128-129; do you refer to the t0 for each experiment (a, b and c)? Needs clarification.

Yes, we refer to t0. This has been clarified (L140).

L129-132; Does the UI provide you with important information?, now sentence feels a bit dropped in the text.

UI indicates the upwelling intensity helping to understand the initial hydrographic conditions. We have also included now the source of precipitation data (L144-146). We agree that this information did not fit very well in the experimental design section. We have renamed this section as "Sampling strategy".

L133-134; What about small zooplankton, copepodites and nauplii? Did you check for this, if so it should be stated. If not, the potential impact of these should be taken into account for.

Seawater samples were pre-filtered through a 200 µm mesh to exclude large zooplankton. 18S sequence data revealed no presence of these small zooplankton groups.

L138-144; This is a very confusing way to present the treatments. I would suggest providing all of this important information in a table instead. Additionally, the rationale behind the levels of nutrients and vitamins should be given.

A table (Table 1) has been included to facilitate the understanding of the treatments administered. So, the description in the text has been summarized (L154-160). The rationale behind the levels of nutrient and vitamins added is also provided in the revised versions (L160-167).

L147-150; This is unclear to read. First it is natural conditions, then the conditions were reproduced? How was this done? What screens are you referring to?

Incubation was performed on deck, and therefore under natural solar radiation. The incubation bags were submerged in incubation tanks filled with constantly circulating surface seawater to maintain a temperature similar to that of the surface mixed layer. The tanks where the SCM samples were incubated were covered with several layers of a neutral mesh to attenuate the incident light and simulate the irradiance at the corresponding SCM. We used radiometers to determine the number of layers needed to attenuate incident light. We have revised this paragraphs for clarity (L169-173)

L152; change to "t0"?

This has been changed (L175).

L160-162; Revise sentence. Suggestion "Samples were incubated 20 min for the fixative to act on cells, immersed in liquid nitrogen for 15 min before being frozen at -80ºC."

This has been corrected (L184-185).

L169-170; Could the usage of two different factors cause a problem in the interpretation of the data, when comparing coast and oceanic station?

Given the small biovolume of prokaryotes, the difference between the two conversion factors would be <20%. We decided using two different factors as there were clear differences in the prokaryotic community composition between the coastal and oceanic station.

L173; "… first place…" before all other variables? If so, please clarify.

This has been clarified (L198)

L174-177; Revise sentence. Suggestion “Polyethylene bottles (50 ml, pre cleaned with 5% HCl were filled with the sample using contamination-free plastic gloves and immediately frozen at –20°C until analysis, using standard colorimetric methods with a Bran-Luebbe segmented flow analyzer (Hansen and Grasshoff 1983).” Or did I misunderstood “free-contamination”?

This has been clarified (L199-201).

L182; Unfortunately, you only have samples for dissolved B12. This should be specified.

This has been specified (L245-246).

L183; Specify when the fifth or sixth day was sampled, as it can influence the results.

This has been specified (L208-210).

L188; Do you refer to leftover water? If so, change wording. If not, clarify.
This has been clarified (L215-217)

L199-200; State which values apply for length, inner diameter and particle size of column.

This has been indicated in the text (L227-228).

L211; You have not used subscript before, change to B12.

This has been removed (L240).
L211-212; State which congener had which LOD.
LODs have been described correctly (L239-241).

L212; If the case, state that 0.05 is for cyanocobalamin, CNB12. Also, change to hydroxocobalamin.

This has been corrected (L241-242).

L214; You have not stated what CNB12 is.

This has been described (L241)

L219; Why was plankton community sampled day 1, 2, 4, 6, while B12 was sampled day 1, 3, 5/6?

We have corrected this as there was a confusion with the denomination of the sampling days. We now use the same nomenclature throughout the manuscript, that is, the first day of the cruise was denominated day 0, and so on. During the ENVISION cruises, due to the large

work load, the sampling for plankton community at the coastal and oceanic stations was done only at day 0, 1, 3, and 5 during the three cruises. For the coastal station, samples for B vitamin were also taken at day 1, 3, and 5, while the oceanic station was only sampled for vitamins only at the t0 of the experiments (day 1, 3 and 6). The rationale behind was that we concentrated B vitamin sampling efforts in the coastal station, sampling every second day 5 depths. By contrast the oceanic station was only sampled at those depths and days coinciding with the t0 of an experiment.

L222; Change "litters" to liters.

This has been corrected (L253)

L237-238; Can you update with the accession numbers?

The accession numbers have been updated (L268-269).

L245-247; How can this be? Is it fragments of cells going through the 3 µm filter? Would benefit from an explanation for this.

The filter used to separate size fractions had a diameter of 3 µm. There are numerous organisms which cell size range include the 3 µm (for example a given specie may have cells ranging in size from 2-4 µm), thus during filtration some cells will be retained in the 3 µm filter, and some will pass through. In addition, depending on their morphology, some cells may cross the 3 µm filter and others cannot. For example, cylindrical cells will pass through the filter depending on their position. A short explanation has been included (L277)

L251-252; Please provide the rationale for this procedure.

When the centered log ratio (clr) transformation is applied to relative abundance data. Zero values must be replaced (Aitchison, 1982). *We replaced zeros by the minimum value that is larger than 0 divided by 2 as it is a common practice to replace zeros with a number less (e.g. 50%) than the detection limit* (Martín-Fernández *et al.*, 2003). *This has been clarified (L282-284).*

L265; "… if necessary to attain normality". Was this not always the case, do you have some samplings where the data was not normalized and some where it is? If so, you should state when this was the case and discuss how this might affect the results and conclusions drawn from them.

We transformed all datasets that did not comply with normality. This has been clarified

(L297-298).

L266; When "standardizing", do you refer to using the corrected p value?

Due to the small number of samples, p value was corrected as recommended by Good, 1982 (L299-300). This is

$$p\ value \times \sqrt{(\frac{N}{100})}$$

being
N: number of observations (samples)

L267-273; Why using ANOVA and Z-test? The reasoning behind this choice should be given.

ANOVA was used to assess differences in the response ratios to nutrient or vitamin additions between stations, seasons, and depths. As we found a significant effect of the three factors, and given that the CV (coefficient of variation) between replicate experiments (i.e. a, b, c) was relatively low, we decided to average the replicate experiments for each station, sampling depth and season. This average RR are represented in figure 7.

The significance of these averaged response ratios (represented in Fig. 7) was evaluated by comparing the averaged value with "1" using a Z-test. Thus, the Z-test was only used to test if averaged RRs were significantly different form "1". This has been clarified (L311-313).

L276-281; how was this data normalized? Change to "chl-a and bacterial biomass".
To normalise a variable, we subtracted its mean and divided by its standard deviation. A normalised variable has a mean of zero, a standard deviation of 1 and (therefore) a variance of 1.

The suggested changes were made (L318). Also bacterial biomass has been changed throughout the manuscript to prokaryote biomass, to account for the presence of archaea, that are also included in the flow cytometer counts.

L283; How many permutations were performed? Should be stated.
999 permutations were performed (L324)

L285; I would suggest using bacterioplankton prior to this. Use already in introduction over bacteria.

This has been changed were appropriate. For our results we now refer to prokaryotes. (L61, L66).

L287; "… selection criteria)…". Remove ")".

This has been corrected (L328)

L291; change “responses” to limitations?

This fragment has been removed as it referred to an analysis that we finally did not include (L329-333).

Results

L294-312; This part is very descriptive, it would benefit from being shortened, to get to the more interesting findings of you paper.

This has been shortened (L351-353)

L294; Here and elsewhere, when referring to figures, state which of the figures, a, b or c. you refer to. See general comment.

This has been corrected.

L296; change meters” to m?

This has been changed (L339)

L310; change “an” to and.

This has been changed (L354)

L313-320; why not presenting DIP values by themselves, but only in DIN:DIP ratio? L319; add 16:1 to Redfield ratio. (…Redfield ratio (16:1))

16:1 has been added (L363)

DIP values are presented in Table S2.

L321; change “greatly varied” to varied greatly?

This has been corrected (L365)

L323-324; “cruise” is redundant.

This has been corrected (L368)

L325; change “bacterial biomass” to BB, as you state this in L323.

This has been corrected, see also response to comment L285 (L367, 370, 371)

L332; Information on MDS analysis is missing from statistics section. Please add information regarding this analysis.

MDS analysis has been included (L305-311)

L332-333; Please clarify. Suggestion "… relatively reduced variability within period".

This has been corrected (L378)

L338; *Mamiellophyceae* is not included in the legend in. As they are the first once you mention, I would suggest including them in the figure 4.

Mamiellophyceae is a class included in the phylum Chlorophyta, dominated in our samples by two genera: Ostreococcus and Micromonas. We now indicate that we refer to these two genera represented in figure 4 (L384).

L342; Explain what MALV refers to.

This has been clarified (L388)

L343; Change to "Flavobacteriales and Rhodobacteriales…"

"Flavobacteriales" has been corrected (L389 & 392) however, Rhodobacterales is the correct name.

L343; The reference to fig 4b is incorrect. See general comment regarding labelling of figure and mosaics.

The labelling has been corrected throughout the manuscript.

L345; See comment L338. Also, which cyanobacteria are you referring to?

This has been clarified (L393)

L346; See comment L343.

The labelling has been corrected throughout the manuscript.

L347; See comment L338, regarding Archaea.

This has been clarified in the text (L394)

L349; change "Mean" to Average?

This has been changed (L397)

L350; Here and elsewhere, provide t value.

The t and F values, as well as df have been added (L398-399).

L351; There is no fig 4c. See general comment regarding labelling of figure and mosaics.

The labelling has been corrected throughout the manuscript.

L354; change “evolution” to development?

This has been changed (L403)

L356; “… in most …” Too general. Please specify the proportion at least.

This has been indicated (L406)

L361-365; This section does not relate to response ratios (even if stated in L361). Please rephrase.

We believe that there is some confusion here. In this section we deal with responses based on both the raw data figures and the response ratios (Figs. 5, 6, 7 and S2). The ANOVA was done with RR data. This has been clarified (L411-413).

L362-363 & 367; Here and elsewhere, provide F value and df.

This has been corrected throughout the manuscript (L415, L420).

L367-369; Revise English.

This has been revised (L420-425)

L369; Here and elsewhere, provide F value and df.

This has been corrected throughout the manuscript.

L369-372; Revise English.

English has been revised (L425-428).

L373-375; Maybe state in which experiments this happens? Similar to L387-390.

The significant experiments are indicated by asterisks in figures S2, S3, and S4. We believe that the result section is rather complex as to also include this detailed information for replicate experiments. The reader can go to figures S2, S3 and S4 and check which experiments and treatments were significant.

L373-383; I would suggest restructuring for clarity. As now it is very difficult to understand when different responses occurred.

We agree that this paragraph describing the 36 experiments is a bit difficult to follow, and this was the reason of averaging the replicates. We have revised this fragment for clarity (L430-441).

L377-378; Maybe state in which experiments this happens? Similar to L387-390.

This information was added as specifically required by a reviewer, but we believe that the

reading of this fragment is already complex as to add such details on replicate experiments.

L391-395; This part appears to belong in Material and Methods section.

We believe that the calculation of a coefficient of variation is simple, and thus, there should be no need to explain that in the method section.

L395; 4 sites? 2 stations and 2 depths? Please clarify.

This has been clarified (L453)

L397-400; To me, these results are the most interesting. I would suggest restructuring the result, putting emphasis on the response ratios.

We agree. The description of figure 7 has been moved to section 3.2, which is the one dealing with the responses (L460-474)

L405; "Most positive…". State proportion (%).

Proportions haves been indicated (L463).

L418-422; This part appears to belong in Material and Methods section.

The description of the RELATE analysis is already in the Methods section, we decided to explain the procedure here as well for clarity.

L422-423; What was Spearman Rho correlation with eukaryotic community composition.

Eukaryotic community composition did not correlate with the B-vitamin responses (Spearman Rho = 0.054, p = 0.39).

L426; Where does the 78% originate from? State each dimensions contribution.

This value is the % cumulative variation of the DistLM (L501-502).

L430-431; State each dimensions contribution to the 59.4%.

This is now indicated (L505)

L431-433; Revise English. Also, I'm struggling to see that the stations are actually separated.

This has been revised and toned down (L506-507).

L434; "… highly and positively correlated…". Revise English.

English has been revised (L509)

Discussion

L443-445; As you don't have measurements on B1, this statement is not fully true. Please

tone down this statement.

This statement has been toned down (L518-519)

L446; What expectations are you referring to? These should be stated more clearly before.

Considering the high short-time variability of the hydrographic conditions in the area (Álvarez-Salgado et al 1996), we expected a large inter-day variation in the responses to B vitamin amendments. This information has been added (L522-524)

L448-452. What about predation pressure? Cellular demand of B vitamins? Actual cellular content of B vitamins? Should be expanded to include more potential explanations.

As we have neither measured cellular content of B vitamins nor predation pressure we believe that expanding explanations here would be a largely speculative exercise. In addition, all the suggested explanations (predation pressure B vitamin cellular demand, etc.) are likely related to the seasonal succession of microbial plankton species.

L452-454; In my opinion, this should have been done for all of the results. I understand that a tremendous amount of work has gone into this experiment, but I believe that the paper would benefit from a more succinct and concise result section.

We tried to reduce as much as possible the part of the results related to raw data, and centered the discussion mostly in the general patterns that emerge when averaging replicate (a, b, c) experiments. We decided to keep figures 5 and 6 and describe also the raw data as it was specifically suggested by other reviewers. We believe that this revised version represents a good balance among all the received comments and suggestions.

L456 "… frequent but relatively moderate…". What does this mean, please clarify.

The significant responses were small, that is, the averaged increase of chl-a or prokaryote biomass after B vitamin additions did not exceed 1.3-fold. We have corrected an error detected here, as the 2.4-fold increase in figure 7 is not significant (L534-536)

L461-464; What results are this statement based on?

This is based on data presented in figure 7 and 8. This has been stated in the revised version (L542-543).

L497-500; Highly speculative. Please rephrase to tone down this statement.

This has been toned down (L578-580)

L521; change “potentially” to potential

This has been corrected (L601)

L522-546; I would suggest reading Fridolfsson et al. 2018 and 2019, as well as Sylvander et al. 2013 to provide additional depth to the discussion on B1 and B12 amendments.

We very much appreciate the suggested reading. We have included the study by Fridolfsson et al. 2019 to expand the discussion on B1 (L628-632)

L563-566; Shouldn’t dinoflagellates pop out in the analysis then?

We also expected dinoflagellates contributing to explain the responses, however, the RELATE analysis found no correlation between eukaryote community and responses to B vitamins (RELATE, Rho = 0.054, p = 0.39). Also, the correlation between the clr abundance of dinoflagellates and the different B vitamin responses were not significant (analyses finally not included in the manuscript).

L567; Why “strikingly”?

This has been changed (L653)

L570; change “revel” to reveal?

This has been changed (L656)

L576; Which “predation” are you referring to? Please clarify.

This has been clarified (L663)

L582-583; What about uptake rates? I would suggest reading Koch et al. 2011, 2012, 2013 and discuss.

This has been discussed (L670-676).

L588; “… B12 producers and B1 consumers.” This is extremely generalized and implies that you can determine this in your paper. This is not fully true, especially for B1 as you don’t have measurement for this B vitamin.

This has been rephrased (L681).

L590; “… cope with B vitamin shortage…”. See L588. Once again, it is unfortunate, but you don’t have measurements for B1 so your conclusions regarding this B vitamin should be toned down.

We are aware that we do not have B1 measurements, but it is very likely that the concentration of B1 is also very low in the area, as both B vitamins tend to follow very

similar patterns (see e.g. Suffridge et al 2018). Nevertheless, this has been rephrased (L685).

Figure captions

Please make sure that everything in your graphs can be identified. E.g fig 1, that cruises is illustrated by lines (in 1c legend), dots in fig 2, what 16:1 line refer to in fig 3.

Also, Generally, is there any benefit of using st3 and st6 instead of coastal and oceanic station. I feel the readability would increase if you used coastal and oceanic instead.

The station numbers have been replaced by coastal and oceanic station.

L937-941; Change "µmol l^{-1}" to µM? Pinpoint that axes are broken. Specify what SCM means.

This has been specified (L1058-1061).

L942-945; If so, state that it refers to t0. Also, what are the error bars showing?

No, the community composition showed in this figure is the averaged composition during the cruises. Error bars have been explained (L1066-1067).

L946-949; Suggestion, …(estimated as Chl-a concentration (µg l^{-1})). Change "time-zero" to t0. Change "final-time" to endpoint. Pinpoint that axes are broken. Also, what are the error bars showing?

This has been changed (L1070-1074)

L950-952; Change "time-zero" to t0. Change "final-time" to endpoint. Pinpoint that axes are broken. Also, what are the error bars showing?

This has been corrected (1077-1080).

L953-960; I would suggest using more mosaics, a-d.

The suggestion has been considered. More mosaics have been included.

L961-968; change "… microbial plankton…" to microbial bakterioplankton, as it is only prokaryotes? Should be stated in the beginning and not at the end of the figure caption

The Bray- Curtis similarities were built considering the responses of both phytoplankton and prokaryotes responses. This has been clarified (L1094).

Figures

Figure 1; Generally, is there any benefit of using st3 and st6 instead of coastal and oceanic

station. I feel the readability would increase if you used coastal and oceanic instead.

Station IDs have been changed

Figure 2; When referring to figures, state which of the figures, a, b or c. you refer to. Also, look over all figures so that all are labelled a, b, c …. For fig. 2 and 3 I'm having troubles seeing the benefits of having several a, several b etc. I would like to see labelling a-r instead for fig. 2, then you can refer to the specific mosaic.

Labelling has been modified for clarity.

Figure 3; See comment for fig 2. For fig. 2 and 3 I'm having troubles seeing the benefits of having several a, several b etc. For the legend, the depth is stated as 0m and SCM, change to "surface (5m) and SCM", as you did not sample 0m, correct? Also, state what the 16:1 line refers to. Also, I would suggest providing an average per station and cruise, and not all 3a, 3b and 3c etc, see general comments.

All the suggested changes have been made.

We agree that there is much information, but still we strongly believe that most readers will like to see the raw data, to corroborate that the short-term changes were rather limited.

Figure 4; change mosaics to cover a-c, as stated in the main text. On the x-axes, the depth is stated as 0m and SCM, change to "surface (5m) and SCM", as you did not sample 0m, correct? You do not use a consistent taxonomy level, some are species whilst other groups are a combination. Could this affect your results? If not, I would still reconsider the different taxonomical levels presented.

The suggested changes have been made.

The depicted taxonomic groups were carefully defined based on their abundance and relevance. Focusing only at a given level (e.g. phylum, class, order) would omit and put together rather distinct functional groups. For example, within the Alphaproteobacteria class, SAR11 and the order Rhodobacterales occupy different niches, with SAR11 being representative of more oligotrophic conditions. Also within the Rhodobacterales, we found three genera that showed on average high abundance and that appeared to have different dynamics (Amylibacter, Ascidiaceihabitans, Planktomarina). In the case of cyanobacteria, they were dominated by the genus Synechococcus, which we find much more inofrmative. In the case of eukaryotes, the Clorophyta was dominated by ASVs belonging to the order Mamiellales, including two distinct genera (Ostreococcus and Micromonas) that also show

different dynamics in this coastal site (see Hernández-Ruiz et al 2018). The criteria to subdivide a taxonomic level was that the relative abundance of the resulting subgroups (lower taxonomic levels) was higher than 5 % in at least one occasion. No subdivisions were made for kingdoms or phyla with low relative abundance (e.g Planctomycetes, Verrucomicrobia, Fungi, Rhizaria).The use of different taxonomic levels is a common practice to depict community composition as functional microbial groups do not always match with taxonomic levels.

Figure 5 and Figure 6; The colors are very difficult to distinguish. Also, I would suggest providing an average per station and cruise, and not all 3a, 3b and 3c etc, see general comments.

We have corrected the colours for clarity. We decided to keep the figures 5 and 6 showing the raw data (replicate experiments a, b, c). Nevertheless, we focus the description of the results and the all the discussion on figures 7 and 8, based on the averaged data (see also the response to figure 3 comment).

Figure 7; I would suggest changing the layout, to something used frequently when presenting fold change. You don't need to show 0, as every finding is around 1. See oversimplified suggestion below.

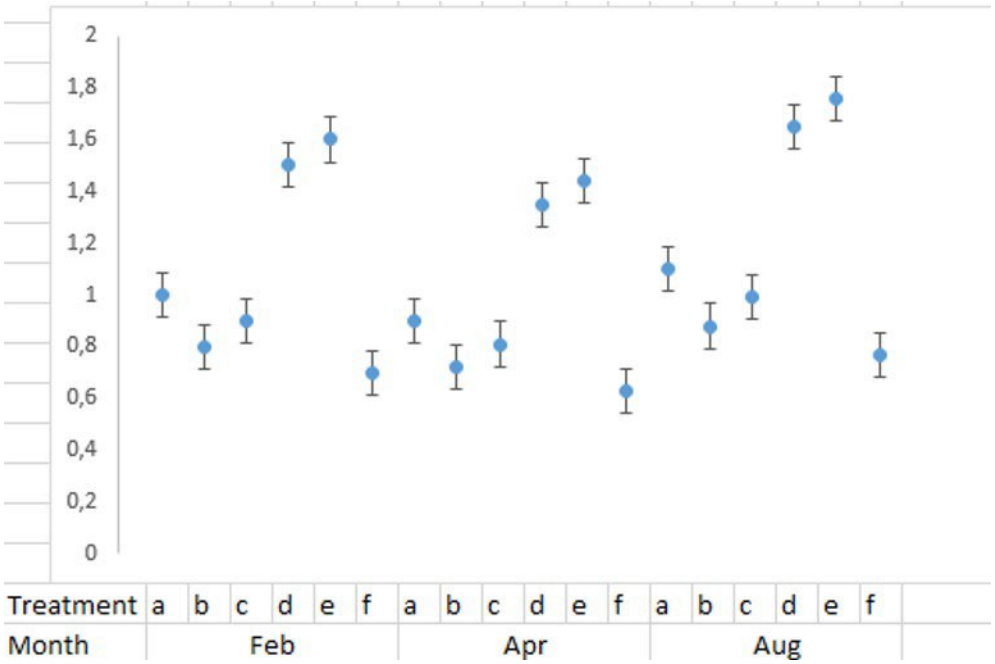

Figure 7 has been modified following the suggestions made by the reviewer.

Figure 8; You do not use a consistent taxonomy level, some are species whilst other groups

are a combination. Could this affect your results? If not, I would still reconsider the different taxonomical levels presented. The legend needs formatting prior to publication, much too large as it is now. The depth is stated as 0m and SCM, change to "surface (5m) and SCM", as you did not sample 0m, correct?

Legend has been modified. For this figure we used the 12 most abundant prokaryote groups as depicted in figure 4. We can only introduce 12 explicative variables as inputs in the DITLM model as we only have 12 data points (2 station x 2 depth x 3 seasons). See also the response to figure 4 comments.

Supplement information

Table S2; This information is the same as in fig 3, correct? To me, this is redundant. If to be included, abbreviations in column names should be explained.

Table S2 shows all information taken from t0 of each experiment while Figure 3 only shows initial biomasses, DIN (which is the sum of nitrate, ammonium and nitrite) and ratio DIN:DIP. Column names have been added.

L18-27; "… experiments by the averaged…". Add divided? Change "that means" to which implies. Pinpoint that axes are broken.

This has been modified.

Figure S1; Shouldn't axes present statistics?, Percentages? The legend needs formatting prior to publication, much too large as it is now.

Axis of this graph do not included values because it is a non-metric multidimensional analysis (MDS). The MDS significance was tested by ANOSIM (analysis of similarity) (L 305--311).

Legend has been modified (Fig. S1)

Figure S2; I propose including this graph over Fig 5 and 6. If included, it must be formatted to conform to the palette the authors have used, for clarity. How was these stats performed, as RR already considers the control. Clarify.

This figure shows the ratio of chlorophyll-a or prokaryote biomass in the inorganic treatment divided by the corresponding variable in the control at the end-point for the 36 experiments (2 months x 2 stations x 2 depths x 3 experiments (a, b, c)).

Figure 5 and 6 show the raw chlorophyll-a and prokaryote biomass at t0 and at the end-point for each treatment (control, inorganic nutrients, B12, B1 and all combinations) in the 36 experiments. Therefore, we do not see how to include this graph (RRs) over figures 5 and 6

(raw data). The significance of each RR in figures S2, S3 and S4 was tested using a t-test, comparing averaged (from the three replicates) values between two treatments.

We represented the response ratios to inorganic nutrient separately as we wanted to focus the attention on the responses ratios to vitamins added either solely (vitamin treatment/control) or in combination with inorganic nutrients (vitamin treatment/inorganic). The response ratios to vitamins are represented in figures S3 and S4. The averaged RRs to vitamins are represented in figure 7 in the manuscript.

Figure S3; I would suggest changing the layout, to something used frequently when presenting fold change. You don't need to show 0, as every finding is around 1. See comment for figure 7. As it is now, it is impossible to get any valuable information from the figure.

The layout of Figure S3 has been modified to facilitate its understanding. Now, two figures (Fig. S3 and Fig. S4) have been created for better clarity.

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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 experimentally evaluated the temporal (inter-day and inter-season) and spatial variability in microbial plankton responses to vitamins B12 and/or B1 supply (~~also~~ solely or in combination with inorganic nutrients) in coastal and oceanic waters of the northeast Atlantic Ocean. Phytoplankton and, to a lesser extent, ~~bacteria~~ prokaryotes were strongly limited by inorganic nutrients. Inter-day variability in microbial plankton responses to B-vitamins was limited compared to inter-season variability~~unimportant~~, suggesting that B-vitamins availability ~~was~~ might be partially controlled by factors operating at ~~larger temporal~~seasonal scale~~s~~. ~~Phytoplankton~~ Chlorophyll-*a* (Chl-*a*) concentration and ~~bacteri~~prokaryote biomass (PB) ~~a positively responded~~significantly increased after ~~to~~ B-vitamin amendments in 13 % and 21 %, respectively, of the 216 cases (36 experiments x 6 treatments). Most of these positive responses were produced by treatments containing either B12 solely~~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. Negative responses, implying a decrease in Chl-*a* or PB, represented 21 % for phytoplankton and 26 % for prokaryotes. Growth stimulation by B1 addition was more frequent on ~~bacteria~~ prokaryotes than in phytoplankton, ~~which is coherent with their widespread dependence on exogenous sources of this growth factor,~~ suggesting that B1 auxotrophy in the sampling area could be more widespread in prokaryotes than in phytoplankton. 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, such as those ~~i~~associated to the proliferation of toxic-producing species, 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 (Bertrand and Allen,

2012; Carlucci and Bowes, 1970; Haines and Guillard, 1974; Helliwell et al., 2011). 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 bacterioplankton~~a~~ 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 or B12 and B1 dependent phytoplankton and bacterioplankton~~a~~ (Croft et al., 2005; Amin et al., 2012; Cooper and Smith, 2015; Cruz-López and Maske, 2016).

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

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

Within this context, the aim of our study was to explore spatial (horizontal and vertical) and temporal (inter-day and inter-season) variability patterns in B12 and B1 vitamin limitation in relation to the prevailing initial abiotic (e.g., nutrient and B12 concentrations) and biotic (eukaryote and prokaryote community composition) conditions in this productive ecosystem. We conducted a total of thirty-six ~~36~~ microcosm

bioassays in February, April, and August 2016 to evaluate the response of heterotrophic bacteria and phytoplankton biomasses to the addition of B12 and/or B1.

Considering that a large fraction of eukaryotic phytoplankton and bacterial taxa require exogenous B-vitamins and considering the different requirements and capabilities to synthesize~~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 Sampling strategy~~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 (C)~~(st3)~~ (42º N, 8.88º W) and one oceanic station (Oc)~~(st6)~~ (42º N, 9.06º W) (Fig. 1a), 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 (C~~3~~ a, ~~3b~~ C-b & ~~3c~~ C-c and ~~6a~~Oc-a, ~~6b~~ Oc-b & ~~6c~~Oc-c, respectively) with water from two different depths. Each experiment began on the first (day 0), third (day 2) and sixth (day 5) of each cruise for the coast and on the second (day 1), fourth (day 3) and seventh (day 6) of each cruise for the ocean (Fig. 1b, c). Water was collected using 20 l Niskin metal-free bottles. Surface (5 m) and sub-surface chlorophyll

maximum (SCM) (between 10 m and 50 m according to the CTD data) 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. 2a-f). We failed to sample the SCM on two occasions (C-a in February and C-a in April), 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~~ chlorophyll-a (Chl-*a*), ~~and bacterial~~ prokaryotic biomass (PB)~~es~~, dissolved nutrient concentration, including vitamin B12, and microbial plankton community were collected at the beginning (time zero, hereafter referred to as t0) of each enrichment 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). Precipitation data was obtained from the Regional Weather Forecast Agency-Meteogalicia (http://www.meteogalicia.gal) in the meteorological station Illas Cies (ID 10125).

2.2. Experimental design

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 (see Table 1 for details). Inorganic nutrients were added to avoid that inorganic nutrient limitation masked the responses to B vitamins. The nutrient concentrations of the additions were the same as previously used in similar enrichment experiments in the sampling area (Martinez-García et al 2010a). ~~. Inorganic nutrients were added to avoid that inorganic nutrient limitation masked the responses to B vitamins.~~ The amount of B12 and B1 vitamin experimentally added approximated maximum concentrations previously observed in coastal areas (Okbamichael and Sañudo-Wilhelmy 2004, 2005, Sañudo-Wilhelmy et al 2006). 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~~ was reproduced by submerging the bags in tanks filled with constantly circulating surface seawater. To simulate light intensity at the SCM the incident light was attenuated by covering the tanks with mesh screens. ~~connected to the surface-water pump system, and covered with screens simulating the light intensity at the sampling depth.~~

2.~~3~~2 Chlorophyll-*a*

Chlorophyll-*a* (Chl-*a*) concentration was measured at ~~time-zero~~t0 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~~4 Flow cytometry

Samples for ~~heterotrophic bacteria~~ prokaryote 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, immersed in liquid nitrogen for 15 min, and frozen at -80ºC. ~~after 15 min. immersion in liquid nitrogen.~~ Abundance of ~~heterotrophic bacteria~~ prokaryotes 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 Moran (2006) were used to estimate ~~the~~ cell 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.~~5~~4 Nutrients

Aliquots for inorganic nutrient determinations (ammonium, nitrite, nitrate, phosphate, and silicate) were collected ~~in first place~~before all other variables and directly from the Niskin bottle in order to avoid contamination. Polyethylene bottles (50 ml) precleaned with 5 % HCl ~~5 %~~ were filled with the sample ~~employing~~ using ~~free~~ contamination-free plastic gloves and immediately frozen at –20°C until analysis ~~by~~ using 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.

2.6~~5~~ Vitamin B12

Seawater samples for dissolved vitamin analysis were taken at surface and SCM depth on day 1, day 3 and day 5 in the coastal ~~on day 1, day 3 and day 5,~~ and on day 1, day 3 and day 6 oceanic station ~~on the first, third and fifth (or sixth) day~~ of each cruise (Table S1 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. Gas pressure was ~~initialy~~initially set at 5 PSI and was slowly increased to 15 PSI until 300-500 µl of sample remained. ~~Residual water~~The concentrated samples ~~behind (300-500 µl)~~ ~~was~~ were frozen at -20ºC until further analysis using liquid chromatography coupled to mass spectrometry system.

The concentrate was filtered again through a cellular acetate membrane 0.2 µm (Phenomenex) prior to the analysis. Ultra Performance Liquid Chromatography tandem Mass Spectometry 3Q (UPLC-MS/MS) methodology was adapted from Sañudo-Wilhelmy et al. (2012), Heal et al. (2014) and Suffridge et al. (2017). 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 inned diameter × 50 mm length, 1.8 µm particle size) 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 for hydroxocobalamin (OHB12) and 0.01 pmol l^{-1} ~~pM~~ for cyanocobalamin (CNB12), while the LOQs values were 0.05 and 0.025 pmol l^{-1} ~~pM~~ for ~~hidroxocobalamin (~~OHB12~~)~~ and CNB12, respectively. 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. We failed to detect B1 vitamin in the pre-concentrated samples, likely due to a low ambient concentration and low pre-concentration volume.

2.~~6~~7 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 (~~day 0,~~), second (~~day 1~~)~~, ~~fourth (~~day 3~~)~~ and ~~sixth day (~~day 5~~)~~ 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 li~~t~~ters 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 PRJEB36188 ~~XXXXXX~~ (16S rDNA sequences) and PRJEB36099~~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 (e.g. those having a cell size range including 3 µm), we combined datasets derived from the 0.2 and the 3 µm filters for eukaryotic community analyses. As explained

279 in Hernández-Ruiz et al. (2018), we normalized the reads from each filter size by the filter
280 DNA yield, as recommended in Dupont et al. (2015), obtaining 2293 unique ASVs. The
281 sequence abundances of the subsampled ASV tables were transformed using the centered
282 log ratio (clr) (Fernandes et al., 2014; Gloor et al., 2017). Before clr transformation,
283 ~~Z~~zeros were replaced by the minimum value that is larger than 0 divided by 2 (Aitchison,
284 1982; Martín-Fernández et al., 2003).

285 **2.8~~7~~ Statistical analysis**

286 To compare the effect of different nutrient additions on the response variables,
287 ~~phytoplankton~~ chlorophyll-*a* concentration and prokaryote~~bacterial~~ biomass~~es~~, we
288 calculated response ratios (RR) by dividing each observation (mean of triplicates) of each
289 treatment by the respective control treatment mean. A value equal to 1 implies no
290 response, a value < 1 implies a negative response and a value > 1 implies growth
291 stimulation after nutrient addition. Secondary limitation by B vitamins was calculated by
292 dividing the mean ~~biomass~~ value in the inorganic nutrients and B vitamin combined
293 treatment by the mean ~~biomass~~ value in the inorganic nutrient addition treatment. In the
294 same way, a value < 1 implies a negative effect of B vitamins and a value > 1 implies
295 ~~growth stimulation~~stimulation positive effect of ~~by~~ B vitamin treatment through
296 secondary limitation.

297 Normal distribution was tested by a Kolmogorov-Smirnov test and non-normal variables
298 were log transformed ~~if necessary~~ to attain normality. All statistical analysis were
299 considered significant at the 0.05 significance level and p-value was standardized as
300 proposed by Good (1982) in order to overcome the low number of replicates. Differences
301 between station and depth (spatial variability) and among sampling months (temporal
302 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. Non-metric multidimensional scaling (MDS) was used to analyze the similarities between the samples based on microbial assemblage structure using the PRIMER6 software (Clarke and Warwick, 2001; Clarke and Gorley, 2006). The similarities were evidenced in a multidimensional space by plotting more similar samples closer together. Analysis of similarity (ANOSIM) was used to verify that microbial community composition from the same season and station were more similar to each other than to communities from a different season and station. Z-test was used to ~~evaluate the significance~~ test i~~o~~f ~~the~~ averaged B vitamins response ratios ~~for each period, sampling site and depth~~were significantly different from 1. 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, chl-*a* ~~chlorophyll a~~ and ~~bacterial~~ prokaryote 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 (999 permutations). In order to highlight which specific taxonomic groups are associated to changes of microbial plankton (~~bacterioplankton~~ prokaryote plankton 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~~limitations) 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 (Fig. 1c) combined with relaxed winds ~~(Fig. 1)~~ caused a halocline at 10 ~~meters~~ m depth (Fig. 2m). 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 (Fig. 2a). 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 (Fig. 2d).

Strong precipitation during the April cruise (Fig. 1c) caused a persistent surface halocline at the coastal station (Fig. 2n). Maximum Chl-*a* concentrations ranged from 0.99 to 2.73 µg l^{-1}, declining from day 5 onwards (Fig. 2b), 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 (Fig. 2e).

In August, strong thermal stratification was observed at both stations (Fig. 2i and Fig. 2l). At the beginning of the cruise, high Chl-*a* concentration (close to 20 µg l^{-1}) was observed

in subsurface water (Fig. 2c). ~~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, and increased by the end of the sampling period (Fig. 2f) as a consequence of an upwelling event (Fig. 1b), 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. 3i). 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 (16:1). Phosphorous limitation (DIN:DIP > 16) was frequent in coastal ~~sub~~surface waters in February and April (Fig. 3j and Fig. 3k).

~~Phytoplankton biomass, estimated as~~ Chl-*a* concentration ~~greatly~~ varied greatly between stations and seasons but was always higher at the coastal ~~(st3)~~ than at the oceanic ~~(st6)~~ station (Fig. 3a-c). ~~Bacterial~~ Prokaryote biomass (~~BB~~PB) 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 (Fig. 3a), while ~~bacterial biomass~~ ~~BB~~PB remained very low throughout this sampling period (Fig. 3d). In April, both ~~BB~~PB and Chl-*a* were similar in the ocean and the coast, and showed reduced temporal variability (Fig. 3b and Fig. 3e), irrespective of the observed nutrient variability (Fig. 3h). 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. 3c). At the beginning of the sampling period, ~~BB~~PB was higher in the ocean than in the coast, and tended to decline by the end of the cruise (Fig. 3f).

A MDS analysis revealed that microbial community composition showed a relatively reduced variability within period ~~variability~~, with samples clustering according to the sampling period (ANOSIM, p = 0.001) and station (ANOSIM, p = 0.001) (Fig. S1 in the Supplement). Consequently, we averaged the microbial community composition for each period and sampling site. The sampling period-averaged composition of the eukaryote community showed a clear variability among sampling dates, while differences between sampling locations and depths were less pronounced (Fig. 4a). At the coastal location, Mamiellophyceae (*Ostreococcus* and *Micromonas*) 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 marine alveolates (MALV) groups (MALV-I and MALV-II) were most representative in February at both locations. Flavobacteriales and Rhodobacterales were the dominant prokaryotes (Fig. 4b) in coastal waters, particularly in August, when both represented more than 80 % of sequences, while the Cyanobacteria *Synechococcus* were mostly present in February and April. In oceanic waters, Flavobacteriales and ~~Cyanobacteria~~ *Synechococcus* were the dominant prokaryotes. SAR11 clade and Archaea (Euryarchaeota and Thaumarchaeota) were most abundant in February at both sampling locations.

B12 concentration was low, ranging from 0.06 to 0.66 pmol l^{-1} ~~pM~~ (Table S1 in the Supplement) ~~Mean~~ Average B12 concentration was significantly higher in the coast (0.30±0.13 pmol l^{-1} ~~pM~~) than in the ocean (0.15±0.12 pmol l^{-1} ~~pM~~) (t-test, t = 3.17, gl =

10,~~,~~ p = 0.01), and showed less variability at the coastal than at the oceanic station (Fig. 4c).

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

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

The response ratios (RRs) of Chl-*a* and prokaryote biomass were calculated as a measure of ~~T~~the magnitude of phytoplankton and ~~bacteria~~ prokaryote responses to nutrient and vitamin treatments (Fig S2, S3 and S4 in the supplement). The RRs ~~(i.e., the response ratios) to the different addition treatments~~ differed between sampling stations (ANOVA, F (1,502) = 18.059, p < 0.001) and among sampling periods (ANOVA, F (2,501) = 6.54, p = 0.002). The most prominent responses of phytoplankton, compared to the control treatment, occurred after inorganic nutrient amendments, especially in surface oceanic waters (Fig. 5c and Fig. S2b, f and j in the Supplement). The magnitude of the phytoplankton response to inorganic nutrients was significantly higher in oceanic than in coastal waters (ANOVA, F (1,34) = 5.22, p = 0.028). ~~Bacteria~~Prokaryotes responded less than phytoplankton to inorganic nutrients and, in addition, ~~bacterial~~heterotrophic prokaryote ~~-~~responses to inorganic nutrients were similar between coastal and oceanic

waters (ANOVA, F (1,34) = 1.68, p = 0.203). ~~Bacteria responded comparatively less than phytoplankton to inorganic nutrients (Fig. 6) 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~~Chl-*a* in 31 out of the 36 experiments (Fig. 5 and Fig S2 in the supplement), while ~~bacteria~~ prokaryotes increased their biomass in ~~and in~~ 19 out of 36 experiments ~~in bacterial biomass~~ (~~Fig 5,~~ Fig. 6 and Fig. S2 in the Supplement).

The addition of B12 stimulated phytoplankton ~~growth~~ in 5 out of 36 experiments (Fig. 5 and Fig. S3 in the Supplement) ~~while~~ and ~~bacteria~~ prokaryotes ~~responded positively to B12~~ in 6 experiments (Fig. 6 and Fig. S~~4~~3 in the Supplement). ~~Phytoplankton biomass~~Chl-a ~~-~~increased in 3, and ~~bacterial~~prokaryote biomass in 7 out of 36 experiments after adding B1 (Fig. 5 and Fig. 6). B vitamins also caused negative responses of phytoplankton (Fig. 5 and Fig. S3 in the Supplement) and ~~bacterial~~prokaryote biomass (Fig. 6 and Fig. S~~4~~3 in the Supplement). The addition of vitamins induced decreases of ~~phytoplankton biomass~~Chl-*a* ~~-~~in 6 experiments (4 after adding B12 and 2 after adding B1) and ~~bacterial~~ prokaryote 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, Fig. 6 and Fig. S3 and Fig. S4 in the Supplement). In the case of ~~phytoplankton~~Chl-*a*, secondary limitation by B-vitamins was found in the C-~~3~~b-surface, ~~6~~Oc-a-SCM and ~~6b~~Oc-b-SCM experiments in February, in the C-~~3~~b-surface and

C-~~3~~b-SCM experiments in April, and in the C-~~3~~b-SCM, Oc-~~6~~b-SCM and ~~6c~~Oc-c-surface experiments 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~~2 stations and 2 depths ~~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).

When averaging the responses within each sampling point (Fig. 7), some general patterns emerge. Both phytoplankton and prokaryotes showed more negative than positive responses to B1 and/or B12 amendments. Most positive responses occurred at the oceanic station (83.3%), while negative responses dominated in the coast (61.5%). 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. 7a). The largest significant increase in Chl-a (ca. 1.4-fold) occurred in April after the combined addition of B12 and B1 in coastal surface waters. Significant positive prokaryote responses mainly occurred in August, when the largest increase (ca. 1.3-fold) occurred in coastal subsurface waters after B1 amendment (Fig. 7b). Most positive responses were associated with treatments containing B12 either alone or combined with B1 (Fig. 7b). Phytoplankton primary B1 limitation was only found at the oceanic SCM in February

(Fig. 7a), while prokaryote primary B1 limitation only occurred at the coastal SCM in August. In addition, prokaryote secondary B1 limitation occurred in oceanic surface waters in February and August.

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. 7a). 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. 7b). Most positive responses were associated with treatments containing B12 either alone or combined with B1 (Fig. 7b). Phytoplankton primary B1 limitation was only found at the oceanic SCM in February (Fig. 7a), 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* (24%), Actinobacteria (14%), SAR11_clade (8.2%), Cellvibrionales (8.5%), Euryarchaeota (8.7%), Flavobacteriales (9%) and *Synechococcus* (6.1%). 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 (34.9%) and db-RDA2 (24.5%) was 59.4 %, both represented as x and y axis, respectively (Fig. 8). The db-RDA1 axis ~~tended to~~ separated, to some extent, coastal samples, 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~~prokaryotes ~~biomass~~ to vitamin B12 and/or B1 addition. The 36 experiments developed in this study

~~allowed a detailed eva~~contributed ~~luation of~~to increase our ~~knouledge~~ understanding of the role of vitamins B12 and B1 at different spatial and temporal scales.

~~Contrary to our expectations~~Considering the high short-time variability of the hydrographic conditions in the area (Alvarez-Salgado et al., 1996), we expected a large inter-day variation in the responses to B vitamin amendments. By contrast, inter-day variability of microbial responses to B vitamins and microbial plankton community composition was relatively small (Fig. 5, Fig. 6, ~~and~~ Fig. S1 and Fig. S2 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~~ prokaryote ~~–~~growth enhancement in at least one B vitamin treatment was frequent but relatively ~~moderate~~ small in this productive ecosystem, showing 1.1 to ~~2~~1.~~4~~3-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. 4c) 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~~prokaryotes (Fig. 7), which appear to be mostly explained by the prokaryotic community composition (Fig. 8).

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~~prokaryotes) 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 overall low and stable concentration of B12 at both sampling locations suggests a high turnover time of this compound in these productive, well-lit waters. Rapid cycling of B12 in surface waters may occur due to high biological uptake rates (Taylor and Sullivan, 2008; Koch et al., 2012) and/or photochemical degradation (Carlucci et al., 1969;

570 Juzeniene and Nizauskaite, 2013; Juzeniene et al., 2015). The measured B12
571 concentrations were in the lower range reported for coastal sites, and similar to that found
572 in the upwelling system off the California coast in the San Pedro Basin during winter,
573 spring and summer (Panzeca et al., 2009).

574 The increase of ~~phytoplankton biomass~~Chl-*a* was mostly associated to B12 amendments,
575 which is consistent with the known incapability of eukaryotes to synthesize this vitamin
576 (Croft et al., 2005; Tang et al., 2010; Sañudo-Wilhelmy et al., 2014). Considering the
577 very low concentration of B12 in the sampling area, the relatively limited phytoplankton
578 response to B vitamins ~~is consistent with~~suggests that the existing ~~present~~ species ~~that~~
579 ~~may~~ might have adapted to overcome B12 ~~limitation in the environment by using~~
580 ~~alternative enzymes~~shortage. For example, changes in external B12 availability may
581 cause shifts from vitamin B12-dependence to vitamin B12-independence in taxa
582 possessing the vitamin B12-independent methionine synthase (MetE) gene (Bertrand et
583 al., 2013; Helliwell et al., 2014). Other strategies used by phytoplankton to cope with low
584 cobalamin concentration include, increased cobalamin acquisition machinery, decreased
585 cobalamin demand, and management of reduced methionine synthase activity through
586 changes in folate and S-adenosyl methionine metabolism (Bertrand et al., 2012). The
587 available data on B12 half-saturation constants for phytoplankton (0.1-10 p~~M~~mol l^{-1})
588 (Droop, 1968, 2007; Taylor and Sullivan, 2008; Tang et al., 2010; Koch et al., 2011) are
589 similar or higher than the B12 concentrations measured here (0.3 pmol l^{-1}~~M~~ in the coastal
590 and 0.15 pmol l^{-1}~~pM~~ in the oceanic waters, on average), reinforcing the hypothesis of a
591 phytoplankton community adapted to B12 limiting concentrations in this upwelling
592 system.

593 The positive responses of phytoplankton in surface oceanic waters in February seemed to
594 be associated with high abundance of *Synechococcus* and SAR11 (Fig. 4~~a~~b and Fig. 8).

595 *Synechococcus* produce a B12 analog known as pseudocobalamin, where the lower ligand
596 base adenine replaces 5,6-dimethylbenzimidazole (DMB) (Helliwell et al., 2016). In
597 natural conditions, pseudocobalamin is considerably less bioavailable to eukaryotic algae
598 than other cobalamin forms (Helliwell et al., 2016; Heal et al., 2017). SAR11 do not
599 require B12 and do not have pathways for its synthesis (Sañudo-Wilhelmy et al., 2014;
600 Gómez-Consarnau et al., 2018), suggesting that B12 synthesis could be limited in oceanic
601 waters in winter, due to the low abundance of potential~~ly~~ B12 producers.

602 Microbial responses to B vitamins in subsurface oceanic waters in February were
603 associated to high abundance of *Synechococcus* and, to some extent, of Actinobacteria
604 (Fig. 8). In these experiments, positive effects of B1 addition on phytoplankton and
605 ~~bacteria~~prokaryotes were observed (Fig. 7). While *Synechococcus* is capable of B1
606 synthesis (Carini et al., 2014; Sañudo-Wilhelmy et al., 2014; Gómez-Consarnau et al.,
607 2018), Actinobacteria seems to have a strong dependence on this vitamin (Gómez-
608 Consarnau et al., 2018). Among the sequenced eukaryote genomes, only Stramenopiles
609 contain genes codifying for the synthesis of thiamine monophosphate (Sañudo-Wilhelmy
610 et al., 2014; Cohen et al., 2017) . While Stramenopiles, dominated by Bacillariophyta,
611 were ubiquitous in the sampling area, their relative contribution was lower in oceanic
612 waters (Fig. 4a). The simultaneous stimulation of phytoplankton and ~~bacteria~~prokaryotes
613 by B1 addition in subsurface oceanic waters in winter suggest a strong demand for this
614 compound under these particular conditions, however what triggers the observed
615 responses remain unclear.

616 Even though B1 caused a significant effect on phytoplankton only in subsurface waters
617 in winter, half of the positive responses of ~~bacteria~~prokaryotes were associated to B1
618 supply (Fig. 7b). This pattern is consistent with the recently described widespread
619 dependence of bacterioplankton on external B1 supply (Paerl et al., 2018). B1 stimulated

~~bacterial~~ prokaryote ~~-~~growth in subsurface coastal waters and surface oceanic waters in summer (Fig. 7b), 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 of prokaryotes to vitamin treatments in surface oceanic waters in summer, when the ~~bacterial~~ prokaryote biomass was high and dissolved inorganic nitrogen concentration was very low (Fig. 3i), suggest that ~~bacteria~~prokaryotes may have an advantage in the uptake and assimilation of B vitamins under nitrogen limiting conditions. This is consistent with the observation of small (0.7–3 µm) ~~-size fraction of~~ plankton cells containing more B1 than larger ~~size fraction~~cells (Fridolfsson et al., 2019). Following this, it has been speculated that bacteria and small phytoplankton can transfer B1 to large cells through predation by acting as an important source of this compound in the marine environment (Fridolfsson et al., 2019).

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~~ prokaryote responses after vitamin amendments in the coast during summer (~~Fig. 6,~~ Fig. 7b~~, and Fig. S3 in the Supplement~~), when nutrient concentrations were low (Fig. 3), suggest either a strong competition between phytoplankton and ~~bacteria~~prokaryotes or a stimulation of predation. Dinoflagellates were particularly abundant in summer at both sampling sites and depths. Many dinoflagellate species are auxotrophs for B1 and/or B12 (Croft et al, 2006; Tang et al., 2010), and also many of them are phagotrophs (Stoecker and Capuzzo, 1990; Smayda, 1997; Sarjeant and Taylor, 2006; Stoecker et al., 2017), 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, resulting in an increased predation pressure on ~~bacteria~~prokaryotes.

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

~~bacteria~~prokaryotes when mineral nutrients were added. This hypothesis contrasts with previous studies reporting that B12 and B1 vitamin uptake is dominated by picoplankton (Koch et al., 2011, 2012), strongly suggesting that bacteria could outcompete larger phytoplankton for vitamin uptake. By contrast, Koch et al. (2014), found that carbon-specific B12 uptake by large phytoplankton was significantly higher during non-bloom (low nutrient concentration) compared to bloom conditions (high nutrient concentration), which suggest better competitive ability under nutrient-rich conditions.

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~~ previously reported major role as B12 producers and B1 consumers. Even though we lack data on B1 concentration, ~~T~~the overall moderate responses ~~in terms of biomass~~ together with the low ambient B12 concentration, suggest that the microbial plankton community in this area ~~is~~ could be 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.

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

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6 Tables and Figures

Table 1: Eight different treatments were applied consisting of: (1) control treatment (C): no nutrients added; (2) inorganic (I) nutrient treatment: 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 treatment: 100 pmol l^{-1}; (4) vitamin B1 treatment: 600 pmol l^{-1}); (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.

Figure 1: (a) The NW Iberian margin (rectangle) and locations of the stations that were sampled in the Ría de Vigo (C) ~~(st3)~~ and on the shelf ~~(St6)~~(Oc) (diamonds), (b) distribution of daily coastal upwelling index (~~Iw~~UI) and (c) registered precipitations during each sampling period showing the initial time of each experiment (C-~~3~~a, C-~~3~~b, ~~3c~~ C-c and ~~6a~~Oc-a, ~~6b~~Oc-b, ~~6c~~Oc-c). ns: no sampling day.

Figure 2: Vertical distribution in the coastal station of (a) ~~chlorophyll~~Chl-*a* (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) ~~chlorophyll-a~~Chl-*a* (µg l^{-1}), (e) temperature (ºC) and (f) salinity (PSU) over time for February, April and August. Dots show the t0 of the experiments. Chl-*a*: Chlorophyll-a concentration.

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, b, c), Chl-*a*, total Chl-*a* (µg l^{-1}). Note that the y-axis is broken; (d, e, f) PB, ~~bacterial~~ prokaryote biomass (µg C l^{-1});

(g, h, i) DIN, dissolved inorganic nitrogen (µmol l^{-1}) and (j, k, l) DIN:DIP, ratio inorganic nitrogen:phosphate. The blue line shows the Redfield ratio (16:1) and SCM refers to the sub-surface chlorophyll maximum. Chl-*a*: Chlorophyll-a concentration.

Figure 4: ~~(a)~~ Averaged relative contribution of reads to the major taxonomic groups of (a) eukaryotes and (b) prokaryotes at surface and SCM in the coastal and oceanic station in February, April and August. (c) Averaged B12 concentration (pmol l^{-1} ~~pM~~) at surface and SCM in the coastal and oceanic station in February, April and August. Error bars represent standard error.

Figure 5: ~~Phytoplankton biomass (estimated as Chlorophyll-a concentration)~~ Chlorophyll-a concentration (µg l^{-1})~~)~~ in the t0 of each experiment (striped bars) and in the ~~final time~~ endpoint of each treatment (colored bars) in the experiments conducted at (a) 5 m and (b) SCM in the coastal and at (c) surface and (c) SCM in the oceanic station in February, April and August. Error bars represent standard error. Note that the y-axis is broken. SCM: sub-surface chlorophyll maximum.

Figure 6: Prokaryote ~~Bacterial~~ biomass (µg C l^{-1}) in the t0 of each experiment (striped bars) and in the endpoint~~final time~~ of each treatment (colored bars) in the experiments conducted at (a) surface and (b) SCM in the coastal and at (c) surface and (d) SCM in the oceanic station in February, April and August. Error bars represent standard error. Note that the y-axis is broken. SCM: sub-surface chlorophyll maximum.

Figure 7: Monthly averaged response ratio (RR) of (a) ~~phytoplankton~~ Chl-*a* or (b) prokaryote biomass at surface and SCM in the coastal and oceanic station ~~and monthly~~

~~averaged RR of (b) bacterial at surface and SCM in the (c) coastal and (d) oceanic station~~. Horizontal line represents a response equal to 1, that means no change relative to control in the pink bars (treatments with vitamins alone) and no change relative to inorganic (I) treatment in the green bars (vitamins combined with I treatments). Asterisks indicate ~~phytoplankton or bacterial significant response relative to control or I~~averaged RRs that were significantly different from 1 (Z-test; * $p < 0.05$) and "a" symbols indicate averaged RRs that were marginally significant~~response with a level of significance between 0.05 and 0.06~~ (Z-test; [a] $p = 0.05$-0.06). SCM: sub-surface chlorophyll maximum.

Figure 8: Distance based redundancy analysis (dbRDA) of B vitamin responses by ~~microbial~~ phytoplankton and prokaryotes ~~plankton~~ based on Bray-Curtis similarity. Only prokaryotic taxa that explained variability in the B vitamin responses structure selected in the DistLM model (step-wise procedure with adjusted R^2 criterion) were fitted to the ordination. 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. ~~Only prokaryotic taxa that explained variability in the B vitamin responses structure selected in the DistLM model (step-wise procedure with adjusted R^2 criterion) were fitted to the ordination.~~ SCM: sub-surface chlorophyll maximum.

Table 1

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

Figure 01

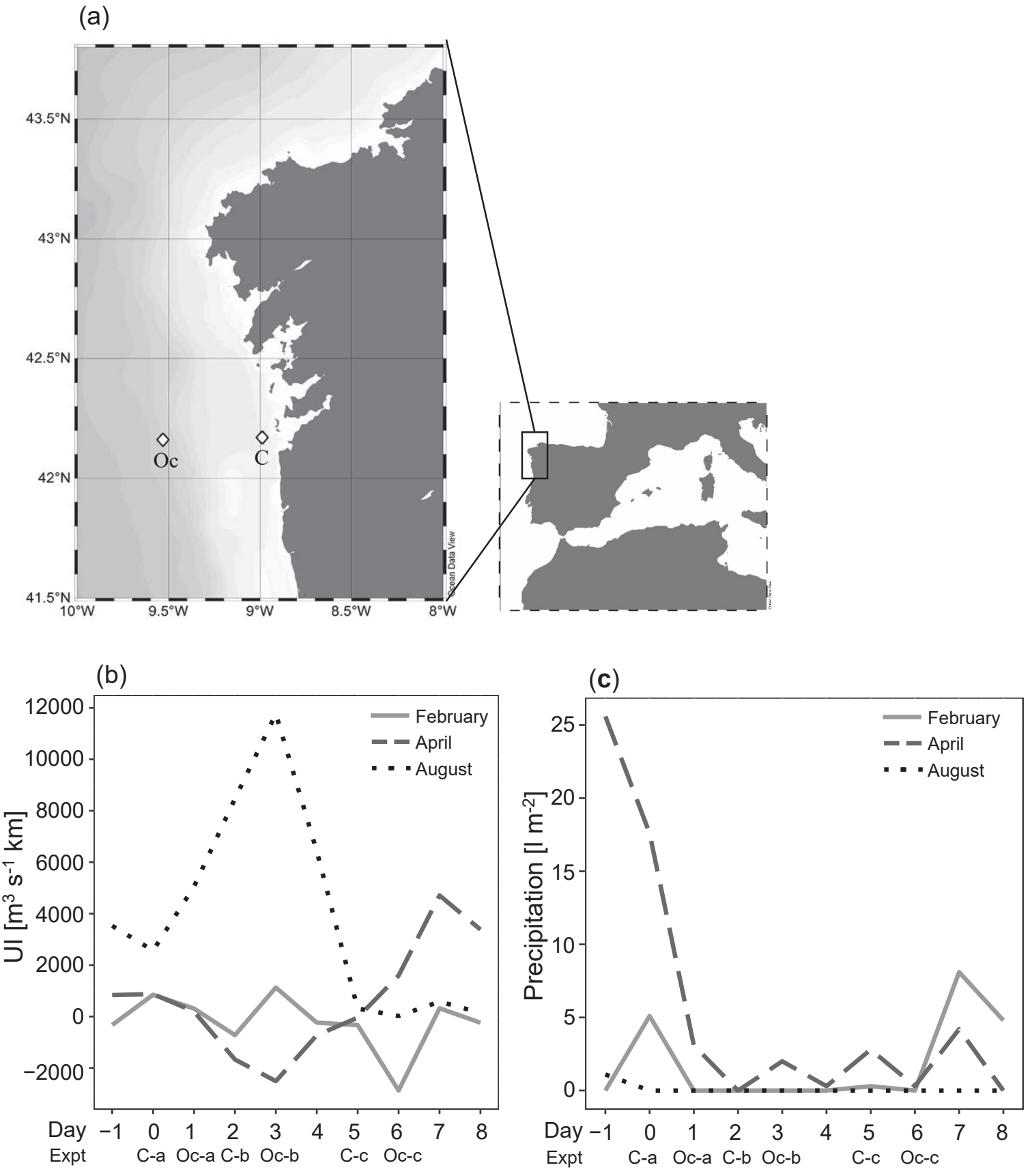

(a)
43.5°N
43°N
42.5°N
42°N
41.5°N
10°W
9.5°W
9°W
8.5°W
8°W
Oc
C
Ocean Data View
(b)
UI [m3 s-1 km]
12000
10000
8000
6000
4000
2000
0
−2000
February
April
August
Day −1 0 1 2 3 4 5 6 7 8
Expt C-a Oc-a C-b Oc-b C-c Oc-c
(c)
Precipitation [l m-2]
25
20
15
10
5
0
February
April
August
Day −1 0 1 2 3 4 5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

Figure 02

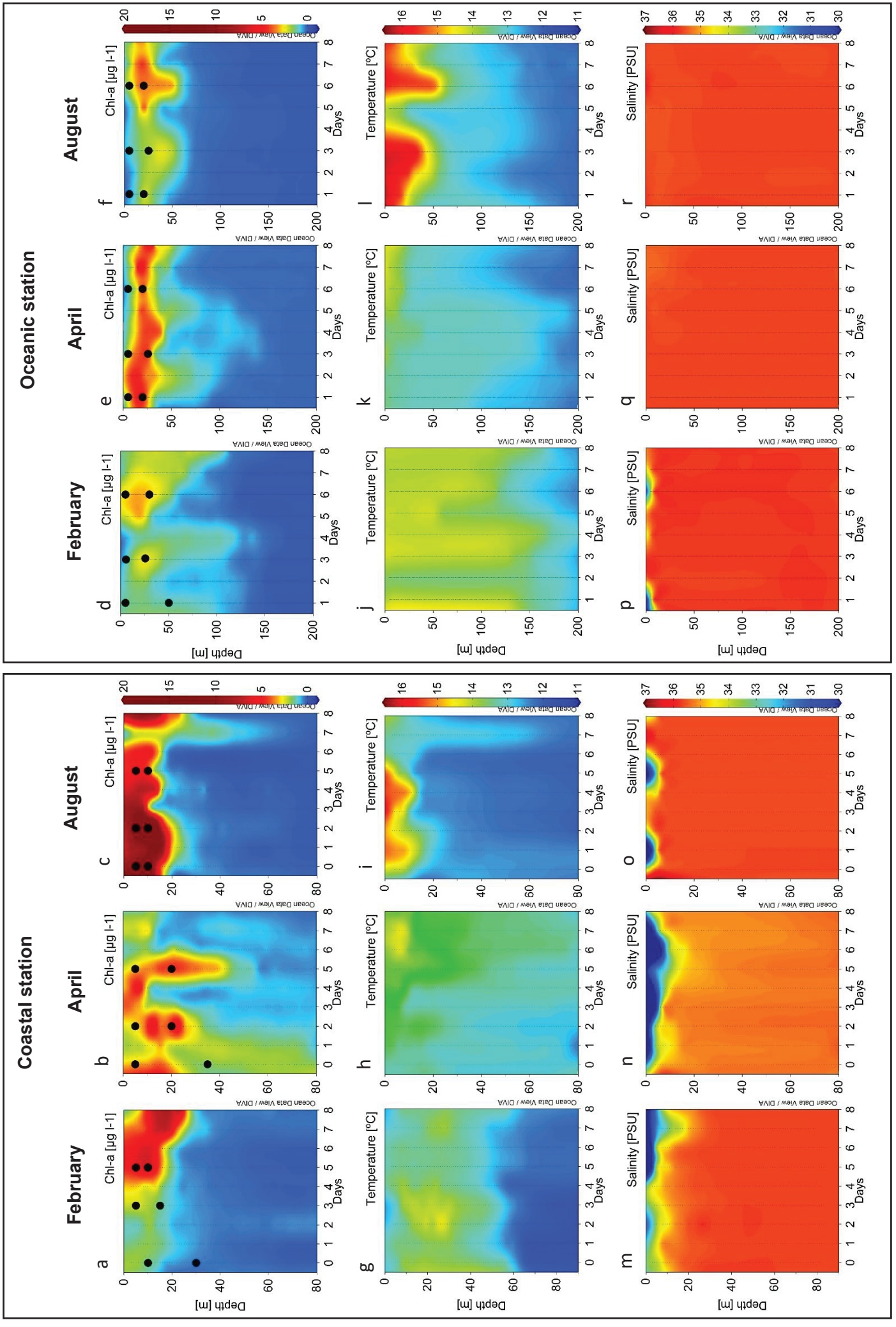

Coastal station
Oceanic station
February
April
August
Chl-a [µg l-1]
Temperature [°C]
Salinity [PSU]
Depth [m]
Days
Ocean Data View / DIVA
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r

Figure 03

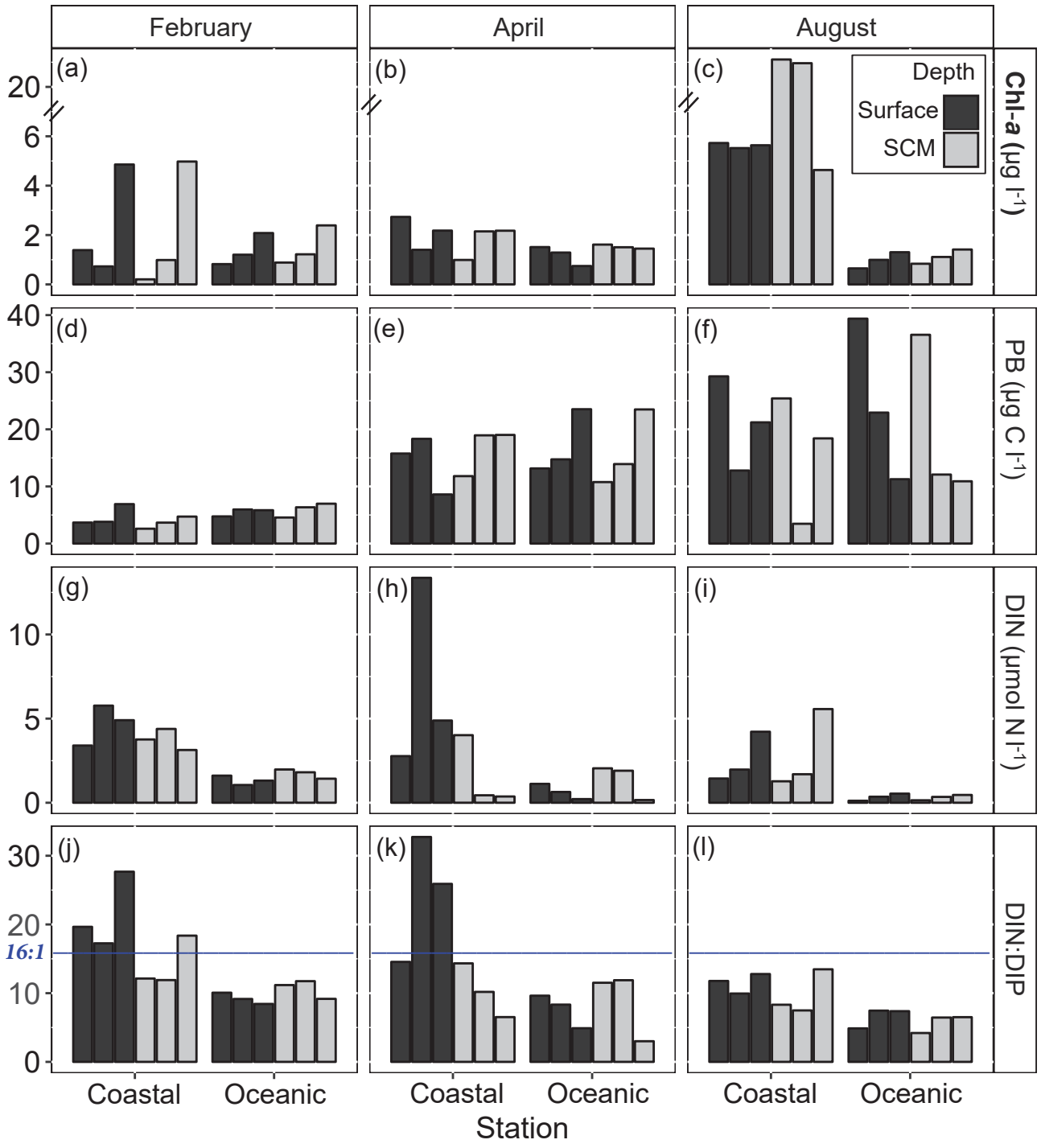

February
April
August
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
Depth
Surface
SCM
Chl-a (µg l-1)
PB (µg C l-1)
DIN (µmol N l-1)
DIN:DIP
16:1
Coastal
Oceanic
Station

Figure 04

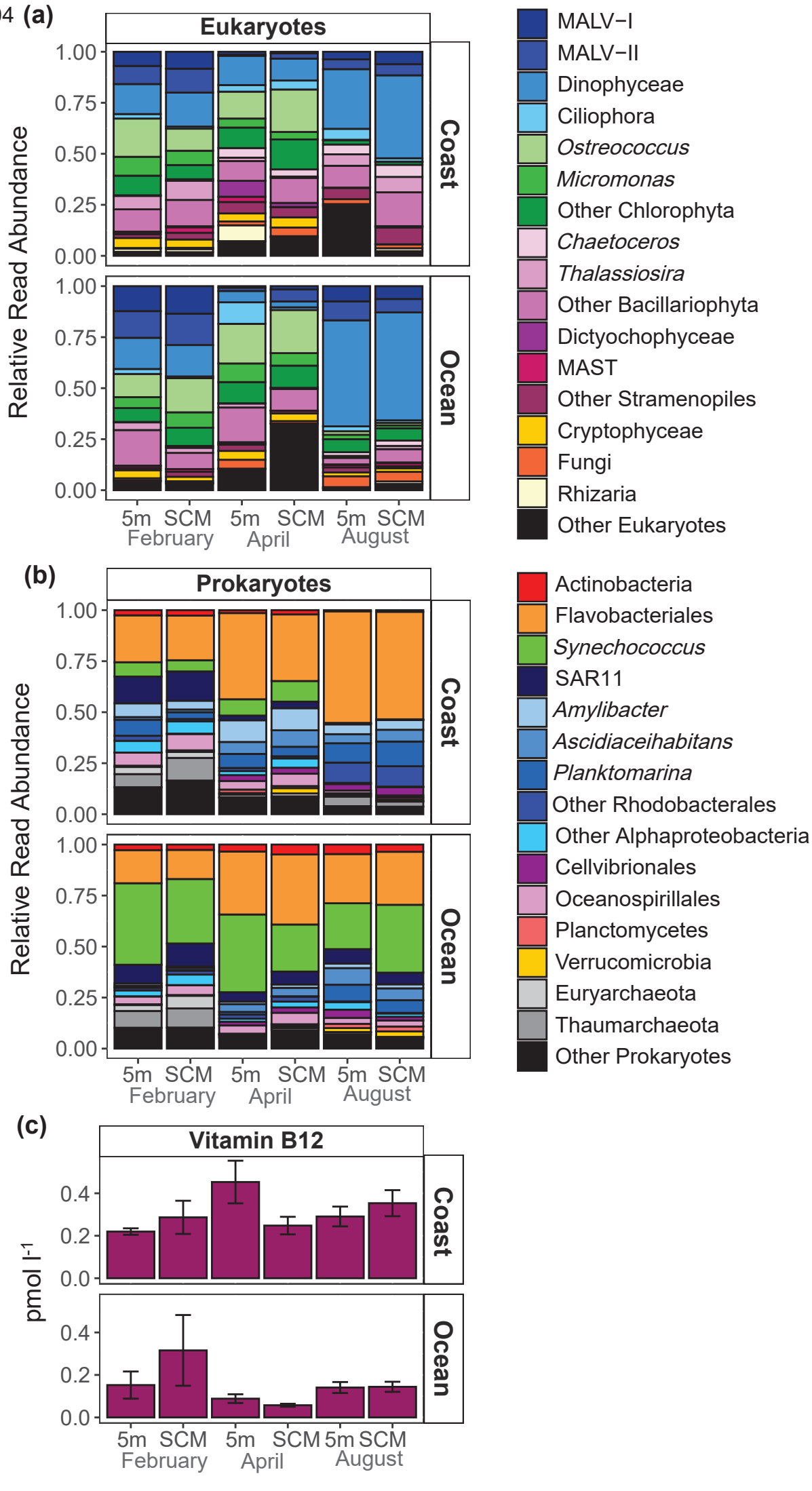

(a)
Eukaryotes
Coast
Ocean
Relative Read Abundance
1.00
0.75
0.50
0.25
0.00
5m SCM 5m SCM 5m SCM
February April August
MALV−I
MALV−II
Dinophyceae
Ciliophora
Ostreococcus
Micromonas
Other Chlorophyta
Chaetoceros
Thalassiosira
Other Bacillariophyta
Dictyochophyceae
MAST
Other Stramenopiles
Cryptophyceae
Fungi
Rhizaria
Other Eukaryotes
(b)
Prokaryotes
Actinobacteria
Flavobacteriales
Synechococcus
SAR11
Amylibacter
Ascidiaceihabitans
Planktomarina
Other Rhodobacterales
Other Alphaproteobacteria
Cellvibrionales
Oceanospirillales
Planctomycetes
Verrucomicrobia
Euryarchaeota
Thaumarchaeota
Other Prokaryotes
(c)
Vitamin B12
pmol l-1
0.4
0.2
0.0

Figure 05

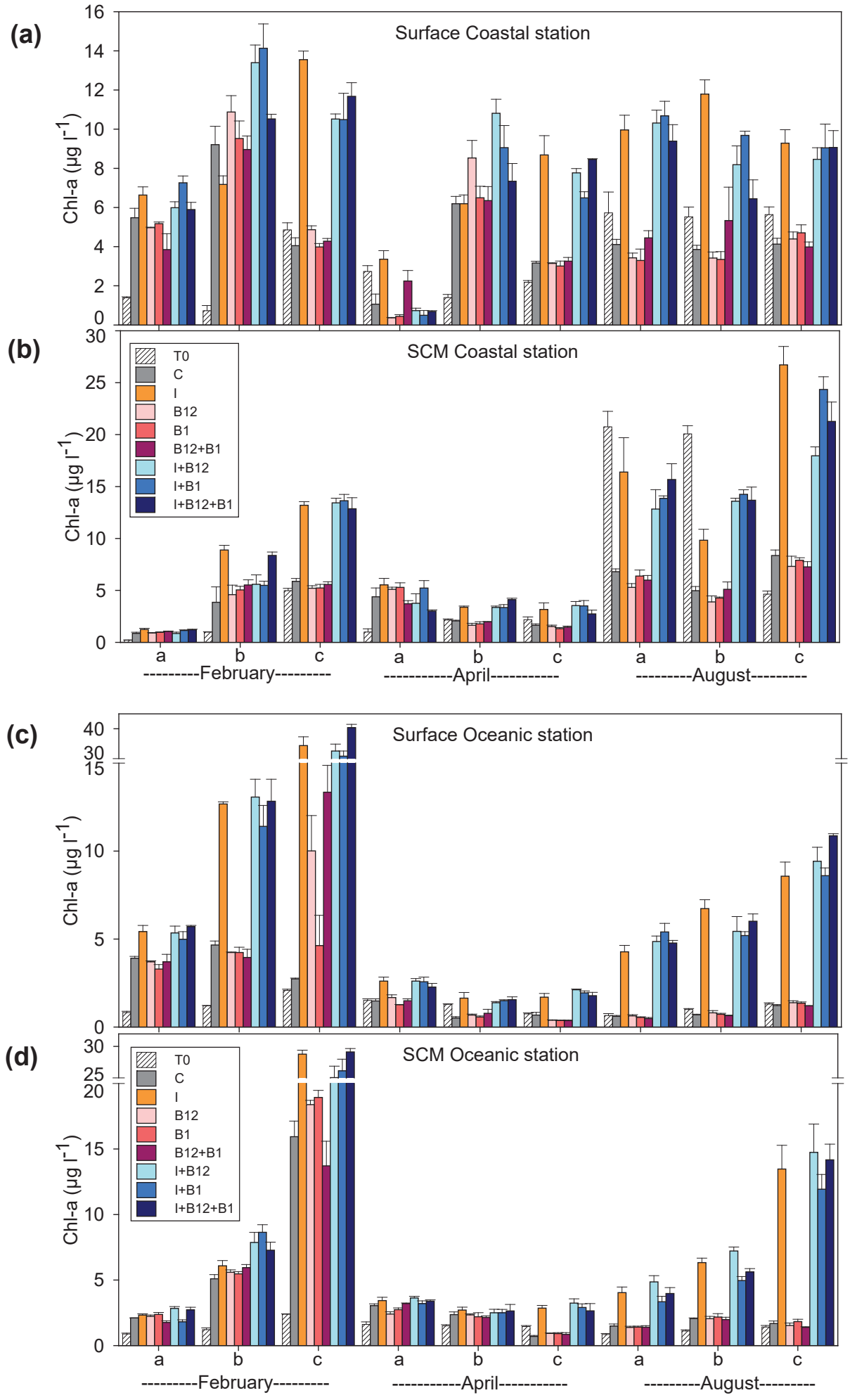

(a)
Surface Coastal station
Chl-a (µg l-1)
(b)
SCM Coastal station
T0
C
I
B12
B1
B12+B1
I+B12
I+B1
I+B12+B1
a
b
c
---------February---------
----------April----------
---------August---------
(c)
Surface Oceanic station
(d)
SCM Oceanic station

Figure 06

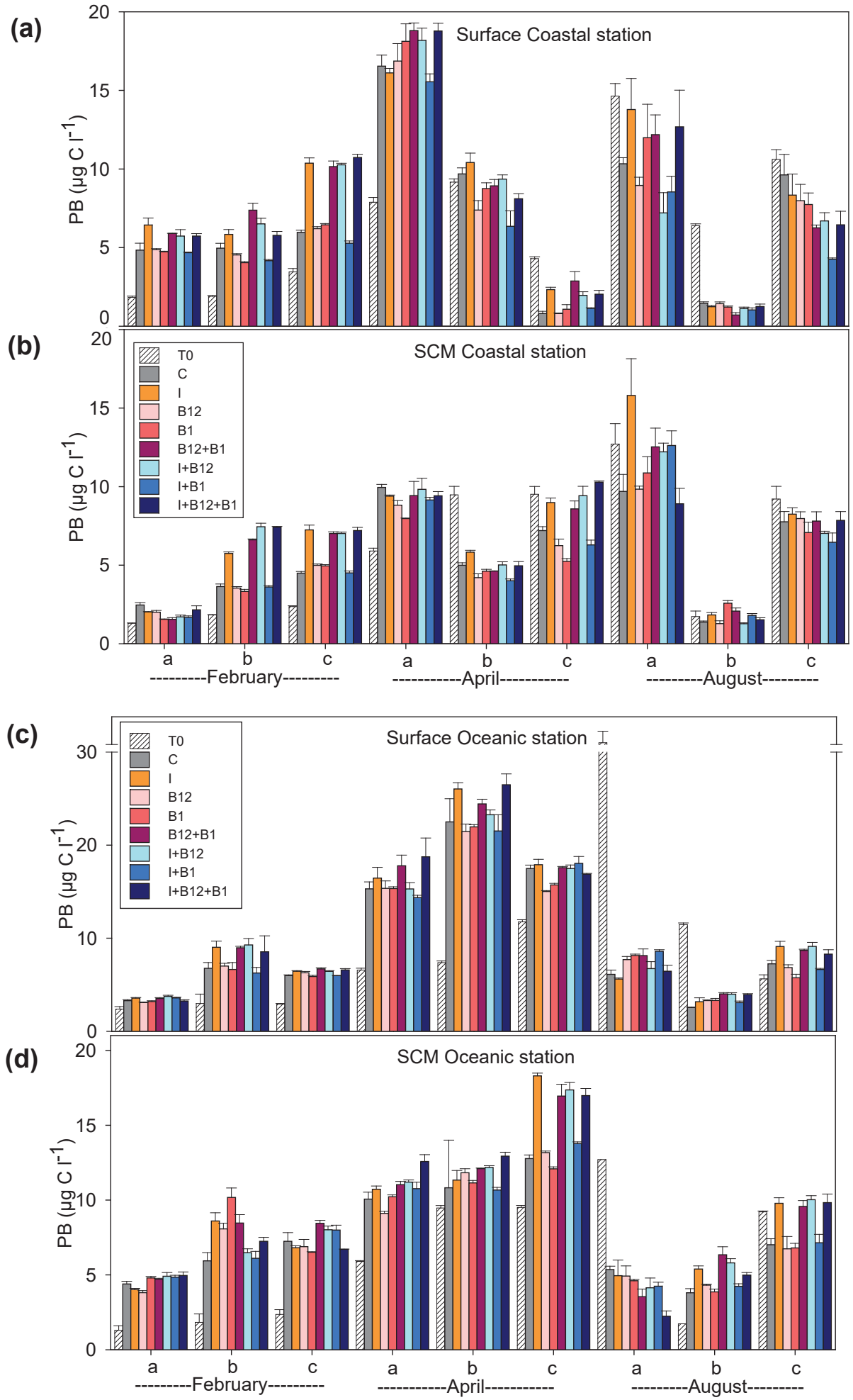

(a)
Surface Coastal station
PB (µg C l-1)
(b)
SCM Coastal station
T0
C
I
B12
B1
B12+B1
I+B12
I+B1
I+B12+B1
a b c a b c a b c
---------February--------- ----------April---------- ---------August---------
(c)
Surface Oceanic station
(d)
SCM Oceanic station

Figure 07

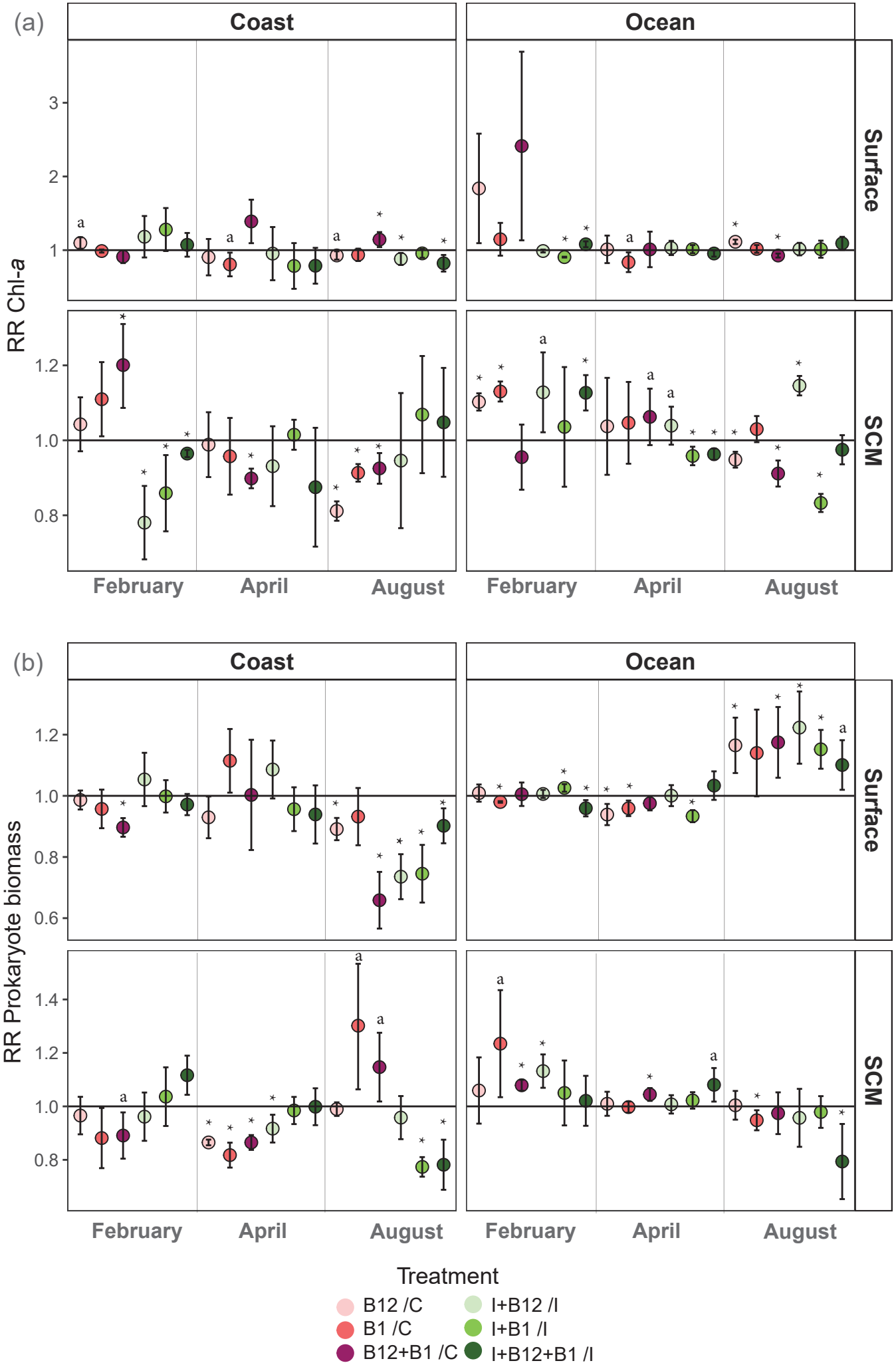
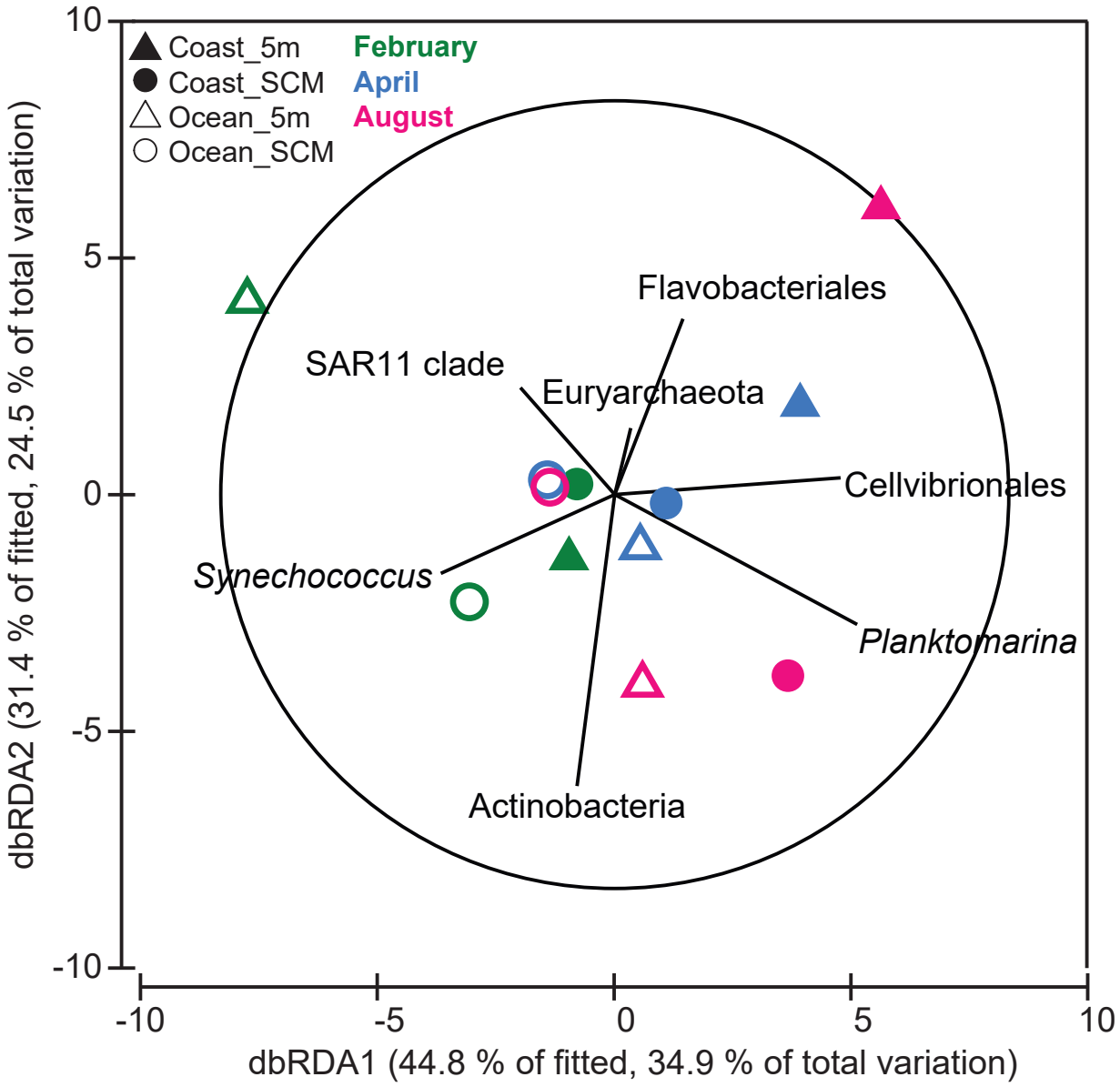

(a)
Coast
Ocean
Surface
SCM
RR Chl-a
3
2
1
1.2
1.0
0.8
February
April
August
(b)
RR Prokaryote biomass
1.4
0.6
Treatment
B12 /C
B1 /C
B12+B1 /C
I+B12 /I
I+B1 /I
I+B12+B1 /I

Figure 08


Coast_5m
Coast_SCM
Ocean_5m
Ocean_SCM
February
April
August
Flavobacteriales
SAR11 clade
Euryarchaeota
Cellvibrionales
Synechococcus
Planktomarina
Actinobacteria
dbRDA2 (31.4 % of fitted, 24.5 % of total variation)
dbRDA1 (44.8 % of fitted, 34.9 % of total variation)
10
5
0
-5
-10
-10
-5
0
5
10

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 pmol l^{-1}	CNB12 pmol l^{-1}
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) at both coastal and oceanic stations (Stn). Sampling months were February (Feb), April (Apr) and August (Aug). The variables measured at t0 were temperature (Temp), salinity (Sal), nitrate (NO_3^-), nitrite (NO_2^-), ammonium (NH_4^+), phosphate (HPO_4^{2-}), ratio inorganic nitrogen:phosphate (DIN:P), silicate (SiO_4^{2-}), Chlorophyll-*a* (Chl-*a*) and prokaryote biomass (PB).

12 **Table S2**

Stn	Depth	Month	Expt	Day	Temp °C	Sal	NO_3^-	NO_2^-	NH_4^+	HPO_4^{2-}	DIN:P	SiO_4^{2-}	Chl-*a*	~~BB~~PB
							µmol l^{-1}					µmol l^{-1}	µg l^{-1}	µg C l^{-1}
Coast	surface	Feb	3~~a~~	0	13.8	35.0	2.86	0.19	0.35	0.17	19.7	3.6	1.39	1.84
			3~~b~~	2	13.2	34.3	4.89	0.36	0.51	0.33	17.3	6.8	0.73	1.91
			3~~c~~	5	13.4	34.2	4.63	0.19	0.09	0.18	27.7	8.6	4.86	3.45
		Apr	3~~a~~	0	13.0	34.6	2.21	0.24	0.32	0.19	14.6	5.2	2.73	7.88
			3~~b~~	2	13.3	34.3	12.46	0.36	0.54	0.41	32.7	12.6	1.40	9.17
			3~~c~~	5	14.0	31.8	4.18	0.16	0.55	0.19	25.9	10.5	2.18	4.30
		Aug	3~~a~~	0	14.1	35.6	0.50	0.10	0.84	0.12	11.8	1.1	5.73	14.64
			3~~b~~	2	14.4	35.6	0.81	0.08	1.08	0.20	9.9	0.3	5.52	6.39
			3~~c~~	5	13.7	35.2	3.93	0.17	0.12	0.33	12.8	3.9	5.64	10.61
	SCM	Feb	3~~a~~	0	13.7	35.7	3.58	0.14	0.04	0.31	12.1	5.2	0.21	1.30
			3~~b~~	2	13.9	35.3	4.16	0.15	0.07	0.37	11.9	4.6	0.99	1.83
			3~~c~~	5	13.4	34.7	2.94	0.09	0.10	0.17	18.4	6.1	4.98	2.36
		Apr	3~~a~~	0	12.8	35.3	3.22	0.34	0.46	0.28	14.3	4.4	0.99	5.90
			3~~b~~	2	13.2	35.3	0.24	0.07	0.12	0.04	10.2	2.8	2.15	9.47
			3~~c~~	5	13.9	34.9	0.21	0.07	0.10	0.06	6.5	3.4	2.18	9.51
		Aug	3~~a~~	0	13.6	35.6	0.91	0.13	0.23	0.15	8.3	1.7	20.75	12.71
			3~~b~~	2	13.8	35.6	1.40	0.16	0.14	0.23	7.5	1.4	20.07	1.73
			3~~c~~	5	13.4	35.6	5.29	0.13	0.14	0.41	13.5	3.9	4.63	9.21
Ocean	surface	Feb	6~~a~~	1	14.0	30.2	1.32	0.18	0.11	0.16	10.1	3.2	0.82	2.38
			6~~b~~	3	14.2	35.9	0.90	0.11	0.04	0.12	9.2	2.3	1.20	2.98
			6~~c~~	6	14.1	35.4	1.03	0.15	0.13	0.16	8.4	3.0	2.08	2.92
		Apr	6~~a~~	1	13.4	35.7	0.95	0.11	0.06	0.12	9.6	2.3	1.51	6.58
			6~~b~~	3	13.6	35.7	0.47	0.11	0.06	0.08	8.3	2.7	1.29	7.37
			6~~c~~	6	13.9	35.6	0.12	0.03	0.06	0.04	4.9	2.1	0.75	11.76
		Aug	6~~a~~	1	16.0	35.6	0.05	0.01	0.06	0.02	4.9	1.5	0.65	39.38
			6~~b~~	3	16.0	35.6	0.26	0.01	0.09	0.05	7.5	3.2	0.99	11.46
			6~~c~~	6	15.3	35.5	0.45	0.04	0.05	0.07	7.4	1.4	1.30	5.63
	SCM	Feb	6~~a~~	1	14.1	35.8	1.73	0.20	0.04	0.18	11.2	3.5	0.88	2.28
			6~~b~~	3	14.1	35.8	1.60	0.19	0.02	0.15	11.7	2.9	1.22	3.18
			6~~c~~	6	14.1	35.8	1.13	0.18	0.12	0.16	9.2	2.9	2.39	3.49
		Apr	6~~a~~	1	13.3	35.7	1.63	0.31	0.10	0.18	11.5	3.2	1.61	5.38
			6~~b~~	3	13.3	35.7	1.45	0.33	0.12	0.16	11.9	2.4	1.50	6.96
			6~~c~~	6	13.7	35.6	0.03	0.06	0.07	0.05	3.0	1.9	1.45	11.74
		Aug	6~~a~~	1	14.9	35.6	0.00	0.04	0.10	0.03	4.2	1.4	0.84	26.55
			6~~b~~	3	16.0	35.6	0.27	0.00	0.07	0.05	6.5	2.8	1.11	6.04
			6~~c~~	6	15.4	35.6	0.35	0.06	0.06	0.07	6.5	1.7	1.41	5.45

Figure S1: A non-metric multi-dimensional 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. SCM: sub-surface chlorophyll maximum.

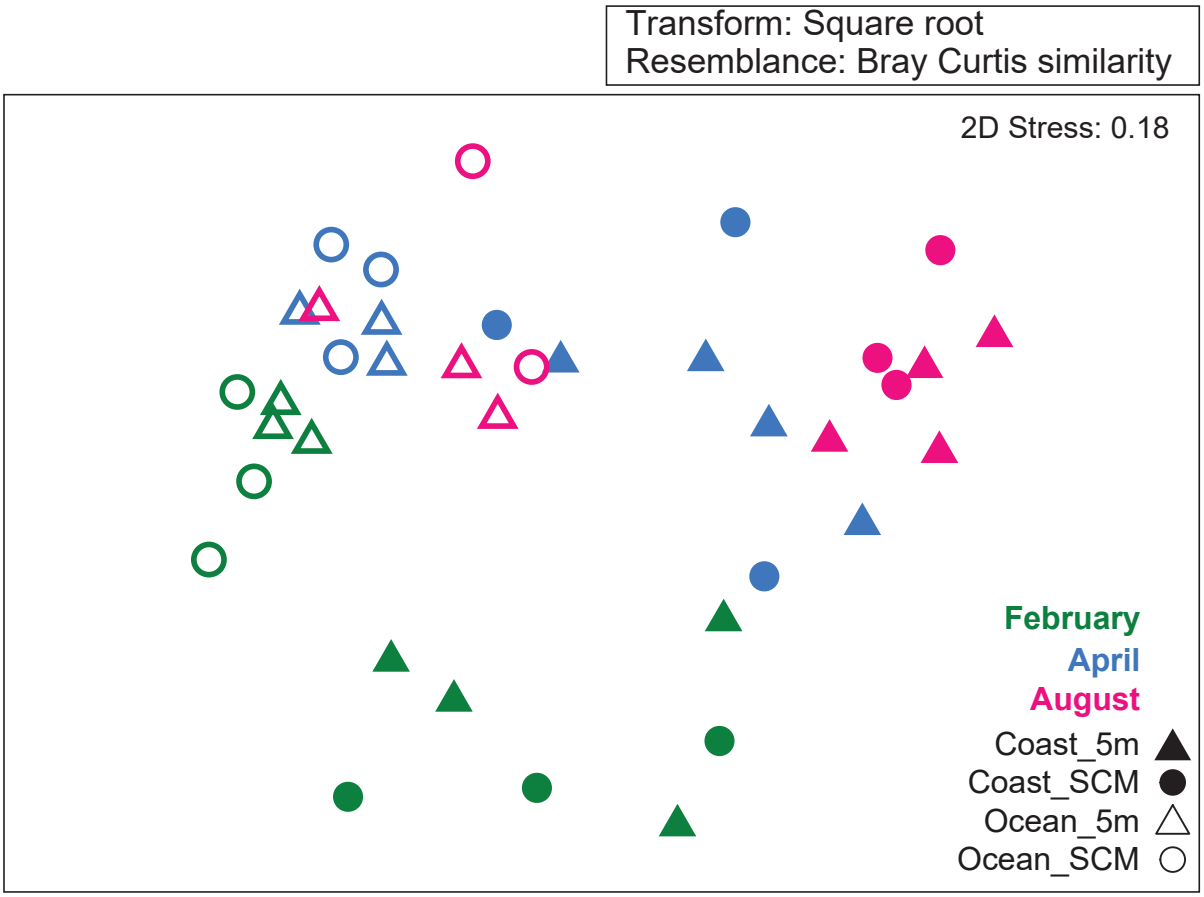
Figure S2: Response ratio (RR) to inorganic nutrient addition (averaged biomass at the end of the experiments divided by the averaged value in the control) of total phytoplankton community (smooth bars) and of ~~bacterial~~ prokaryote biomass (PB) (striped bars) at (a) coastal and (b) oceanic station. 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~~which implies 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. Note that y-axis in Fig. S2 b is broken. SCM: sub-surface chlorophyll maximum.

Figure S3: Response ratio (RR) of total phytoplankton ~~community (smooth bars) and of bacterial biomass (striped bars)~~ at surface and SCM in the coastal station and at surface and SCM in the oceanic waters in (a-d) February, (e-h) April and (i-l) August. Treatments represented are: B12/C; B1/C; B12+B1/C in pink tones and I+B12/I; I+B1/I; I+B12+B1/I in green tones. Pink ~~bars simbols~~symbols represent primary responses to B vitamins and green ~~bars simbols~~symbols represent secondary responses

to B vitamins. Horizontal dotted-~~-~~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 S4: Response ratio (RR) of prokaryote biomass at surface and SCM in the coastal station and at surface and SCM in the oceanic waters in (a-d) February, (e-h) April and (i-l) August. Treatments represented are: B12/C; B1/C; B12+B1/C in pink tones and I+B12/I; I+B1/I; I+B12+B1/I in green tones. Pink ~~simbols~~symbols represent primary responses to B vitamins and green ~~simbols~~symbols represent secondary responses to B vitamins. Horizontal dotted-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 prokaryote significant response (t-test; * $p < 0.05$).

Figure S1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Resemblance: Bray Curtis similarity
2D Stress: 0.18
February
April
August
Coast_5m
Coast_SCM
Ocean_5m
Ocean_SCM

Figure S2

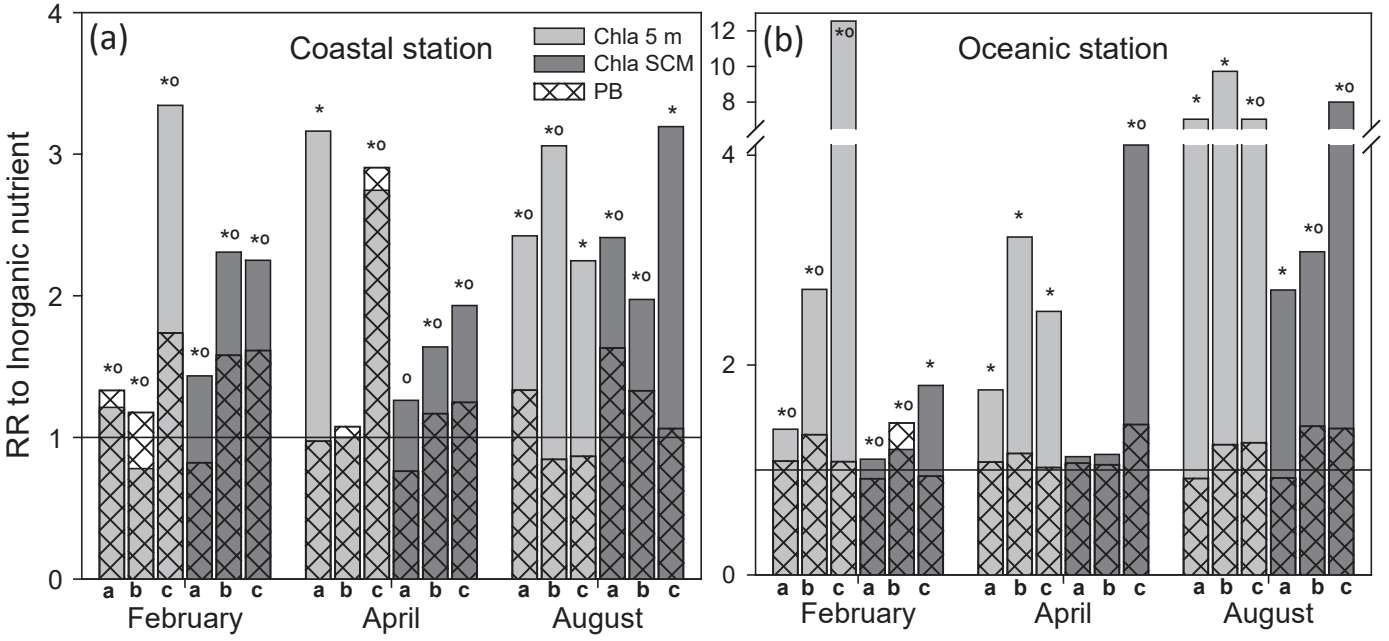

(a)
Coastal station
Chla 5 m
Chla SCM
PB
RR to Inorganic nutrient
(b)
Oceanic station
a b c a b c
February
April
August

Figure S3

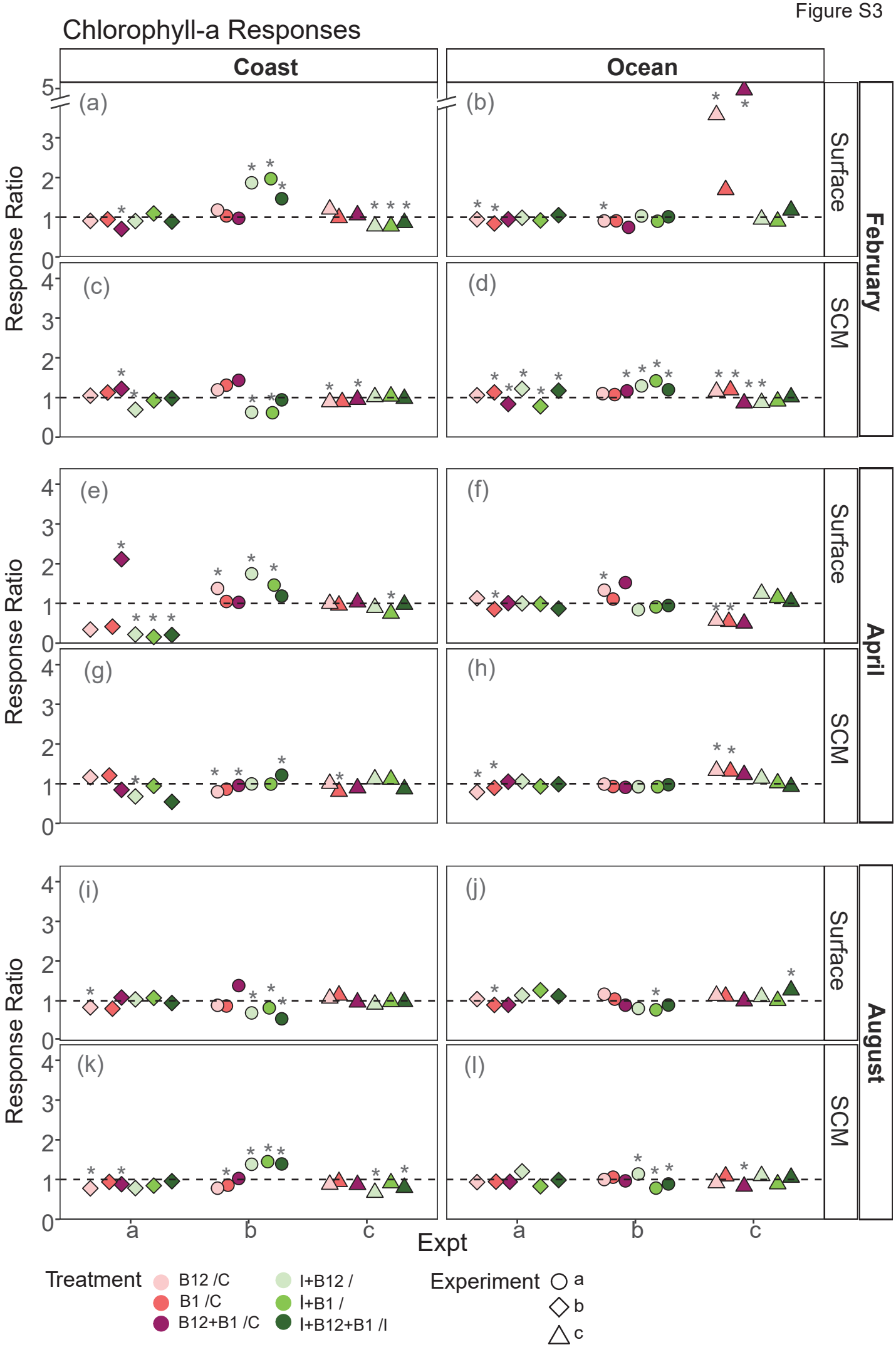
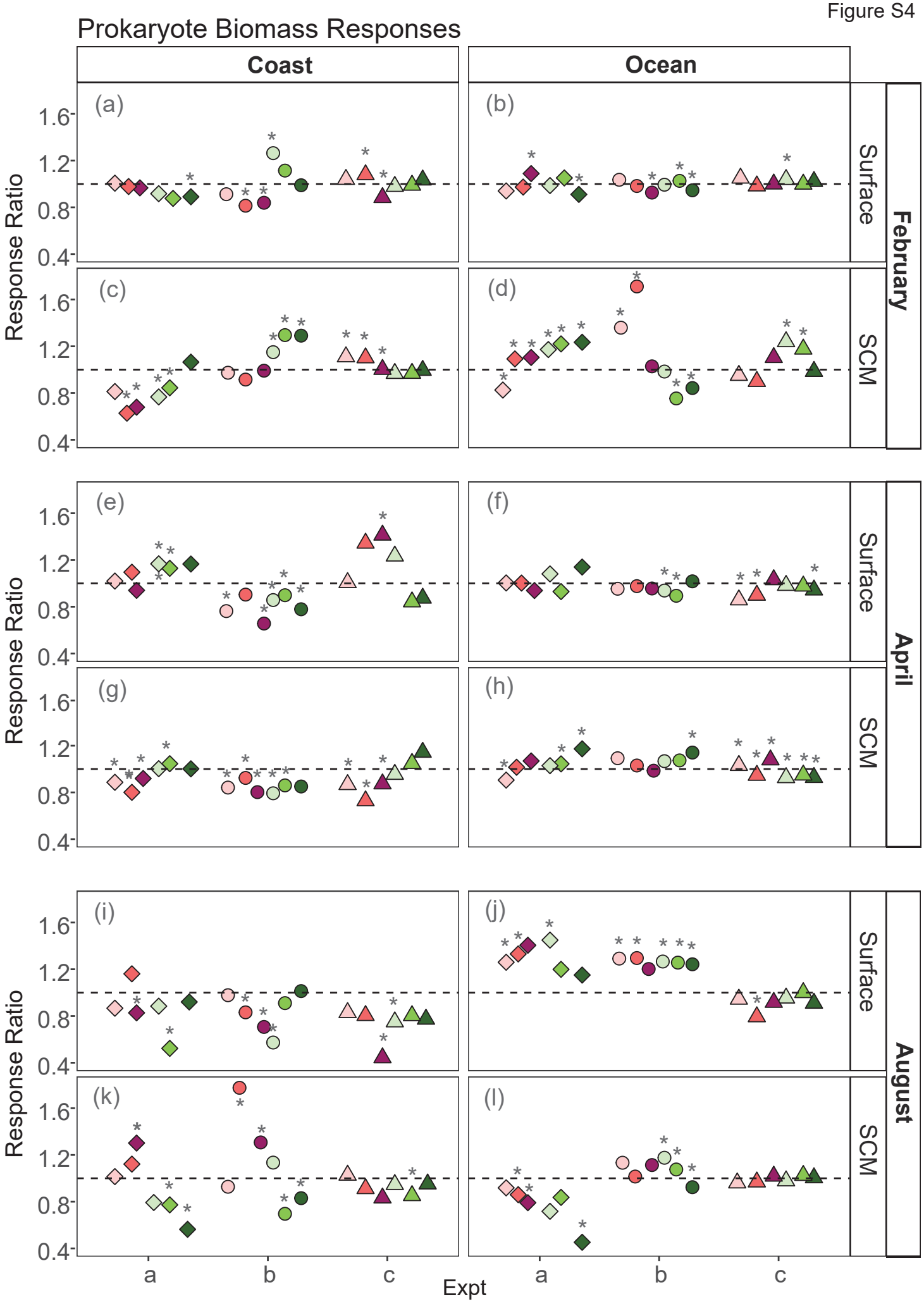

Chlorophyll-a Responses
Coast
Ocean
Surface
SCM
February
April
August
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
Response Ratio
Expt
a
b
c
Treatment
B12 /C
B1 /C
B12+B1 /C
I+B12 /
I+B1 /
I+B12+B1 /I
Experiment
a
b
c

Figure S4


Prokaryote Biomass Responses
Coast
Ocean
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
(k)
(l)
Surface
SCM
February
April
August
Response Ratio
1.6
1.2
0.8
0.4
a
b
c
Expt