

RC1

MAJOR COMMENTS

My main concern is the use of data from only 3 time-space points (3 stations in spring 2015 and water collected only from DCM) to make conclusions on the importance of copepods in N and P recycling for the whole Western Tropical South Pacific (title, p. 13 lines 22 to 24). These 3 stations are located (stations coordinate not given) on a transect > 1300NM long, thus covering a very vast area. To support the potential in situ applicability of their conclusions, the authors should give strong evidence (based on other manuscripts of the same issue or at least based on previous bibliography) about the temporal and spatial (horizontal and vertical) homogeneity of this vast area.

My second concern, which enhances the first one, is using an experimental ratio of copepods to bacteria > 13:1 compared to the one in the field (p.12 lines 2 & 3), to make conclusions on the in situ effect of zooplankton on the microbial community. It is fully understandable that high experimental densities of copepods are necessary to obtain a signal in nutrients within a few hours. However, it is doubtful that if the nutrients available to bacteria per time unit were much less, that the remineralisation effects and shifts in bacterial composition would be the same (especially considering also the role of the other player - phytoplankton).

In brief, the transfer of lab observations under such experimental conditions and from only 3 space-time points to processes occurring in the field at a very different copepod:bacteria ratio and over vast spatio-temporal scales is not at all straightforward. My suggestion is to rewrite conclusions in a much more conservative way. Then the manuscript including title should be adapted in consequence.

Attending to the general comments:

First, the title has been modified to better fit to the paper content which mostly focuses on comparisons of remineralization and bacterial response in three contrasted areas.

The conclusion has been rewritten and now focused on the observations made at each station, avoiding general conclusions for the whole study area. These stations were selected for their different biogeochemical characteristics and chosen along an oligotrophic gradient, so that our study was performed over a heterogeneous and contrasting region. Studies in this region are very scarce and even scarcer for the role of zooplankton in nutrient recycling, thus comparing our results with other studies become difficult. Even that, we are confident that our work provides key insight and information about the environmental significance of nitrogen and phosphorus regeneration as mediated by marine copepods.

One of the difficulties in our experimental studies is simulating the in situ conditions in a more realistic way. Research on zooplankton, especially on copepods, commonly use experimental densities much higher than those encountered in the field. In our case, since the nutrient concentrations in the study area were quite low, particularly for ammonium, it was necessary to concentrate the number of copepods in incubations. We have now deeper discussed this issue. Although we are aware that our results could be overestimated, our

conclusions are also supported by a set of previous works in the field (Perez-Aragon et al., 2011; Valdes et al., 2017; Valdes et al., 2017).

2) SPECIFIC COMMENTS

2a) Introduction:

p.2 lines 20, 25, 26. Copepods may excrete much more than 53% of their body nitrogen in the form of ammonia and this percentage is highly variable (as said in line 25). There are many papers on this subject, including review papers.

We agree. We have now provided a better assessment of the subject and improve citations.

p.3 lines 16-17: How is the presence of an oligotrophic gradient supported? Nutrient values from the only three stations are insufficient. Nutrient and/or Chla data from more stations would be helpful.

This paragraph was rephrased. An explanation about the gradient selection and its support was given in detail in the Methods section. Briefly, the three LD stations were chosen along a regional gradient in oligotrophy and they were selected using satellite imagery, altimetry and Lagrangian diagnostic (Moutin et al., 2017). A complementary approach included the abundance of selected diazotrophs *nifH* gene copies (on board; Stenegren et al., 2017).

In the revised text, we removed the oligotrophic gradient from the description of the study area and now focus only in the general study area: WTSP. Details on how the oligotrophic gradient was determined was added to the Methods section.

2b) Methodology:

General remark for the experimental set up: an additional control with copepods only would have being very helpful.

p.3 line 22: please define "long duration"

We have now defined long duration stations in Material and Methods section.

p.3 line 22: please add coordinates of the stations

Coordinates were added. Additionally, we have now added a map of the transect during the cruise with the stations.

p.3 lines 24-25: please add maximum and minimum values of chlorophyll-a

Details about the chlorophyll values and about sampling strategy during OUTPACE cruise are available in Moutin et al., 2017. We have now provided the reference for this in Methods section as well as the chlorophyll-a values in the DCM in Table 1, which is the depth in which we took the seawater for our experiments.

p.4 lines 2-4: please specify if tow was vertical (or oblique), tow speed, net diameter

We have now provided these sampling details in “Mesozooplankton sampling” section.

p.4 line 13: the field composition of copepods and other zooplankton should be also shown in Table S1

More details about the compositions of copepods and other zooplankton in the field were provided in Carlotti et al., (this issue), and the abundance of copepods in the study area was provided in the second paragraph of Discussion section.

p.4 lines 23-24: please specify % of mortality

We have now provided the percentage of mortality during the incubations in the “Preparation of the microcosm and experimental setup” section.

p.8 lines 21: check that Redfield ratio of organic nutrients is < 16:1

Results were checked and modified accordingly.

p.9 lines 7-8: unclear meaning

These lines were clarified.

2c) Results:

General remark. Do not repeat values that can be found in tables or figures unless necessary.

Table 1: use either 2 or 3 decimals depending on the precision of the method for each parameter

Done.

Table 1: add Temperature, Chla, DON, DOP, values at DCM

The information was added. DON-DOP and Chla were extracted from Moutin et al., (this issue) and from Dupoy et al., (this issue), respectively.

p.7 line 25: "first treatment", replace by "treatment with copepods"

Done.

p.8 line 4: "significant difference in time,". Please add results of statistical test

We added the information to the text.

2d) Discussion:

General remark. Do not repeat detailed description of results, but only briefly giving outcome in connection with related literature.

p.10 line 15 and line 17: Table 1 shows that MA stations (LD A & LD B) are characterized by higher inorganic nitrogen but not higher inorganic phosphorus concentration than SG (LD C).

The information was checked and corrected accordingly.

p.10 line 25: "influence on biogeochemical variability": not clear.

We have now clarified this section in the text.

p.10 lines 31-32: Since there was no significant difference between treatments in LD A for bacterioplankton abundance (p.8 lines 26-27), a conclusion on an effect of zoo- plankton cannot be made.

This issue was discussed in the discussion section.

p.11 lines 7 to 10. LD B is not in the South Pacific Gyre (p.1 line 24). Check and eventually move this part to discussion on LD C which is in the South Pacific Gyre.

We have now restructured this section to make it pertinent to the station sampled.

p.13 lines 33-34: Too general conclusion on the role of zooplankton metabolism, not supported by manuscript's observations.

We have now rewritten our conclusion, which focuses more on our observations.

2e) Bibliography:

References to articles in preparation should be avoided

We modified the text accordingly.

3) TECHNICAL CORRECTIONS

p.2 line 2, p.8 line 18, p.10 line 12, p.10 line 15, p.12 line 32, p.13 lines 8-9: English grammar mistakes

These sections have been revised and modified accordingly.

p.2 line 10: change order of references

We have now modified the order of the references.

p.3 line 3: ". . .in a phosphorus limited. . ."

Corrected.

p.3 line 5: ". . .Gasol, 2007"

Corrected.

p.3 line 8: ". . .zooplankton-produced DOM by bacterial communities. . .": unclear expression

We have now clarified this expression.

p.4 line 14: "acclimation" instead of "acclimatization"

Corrected.

p.4 line 21: "such that 6 bottles were added in that case": not clear

We have now clarified this sentence.

p.5 line 23: correct "u sing"

Corrected.

Please correct typing mistakes and journal names abbreviations in bibliography

Done.

RC2

The authors present a study that simultaneously examines the role of zooplankton and bacteria on nitrogen and phosphorus cycling in low-nutrient systems. Few studies have tried to assess these factors simultaneously to link the two processes. Although the authors provide a lot of interesting results, I am not convinced that their data back up the larger assertions of the role of copepod excretory products play in spurring growth of microbes. Also, I am not convinced that in situ conditions were accurately enough recreated to directly apply these results to the in situ setting.

More specifically:

1) It is challenging to know how much N and P is being utilized by the bacteria (and in what forms) when you don't have baseline data for N and P excretion rates for copepods in the absence of microbes. Without this information, you are unable to say the actual impact either party (copepods or microbes) is having on buildup or drawdown of different N or P compounds.

2) In many cases, there were not significant or clear differences between the controls and copepod treatments. For instance, the bacterial community structure for LD A and LD B stations grouped more by time point than by treatment. That implies an effect of the incubation itself, and not due to extra nutrients brought on zooplankton excretion. Also, in 2 out of the 3 experiments, it appears that bacterial abundance was higher in the controls at the end of the experiment. If they were using the nutrients to grow, we would expect to see significantly higher abundances in the copepod bottles.

3) Copepod densities in the bottles were much higher than in situ conditions. While high densities of zooplankton are definitely necessary for detectable changes in excretory products, this also meant that the change in N and P facilitated by zooplankton is likely to be much larger within this experiment than what bacteria experience in the field.

4) When looking at the dendrogram results in Figure 8, it appears that the DNA samples taken from the in situ environment show a very different community structure when compared to the communities that were present in the incubation bottles at time T0. If the community structure in the bottles at the beginning of the experiment isn't representative of the local community, the changes observed might not reflect what would occur in situ.

5) I am concerned that there are significant differences between copepod and control treatments at T0. The methods say that the organisms were placed in new incubation water at the beginning of the excretion step. I assume initial samples were taken immediately. While the authors attempt to explain the differences seen in DON (p. 11, line 26), there is no information addressing the differences in bacterial community structure at T0 for several of the experiments.

Additionally, the entire document needs to be reviewed for use of proper English grammar. Most of the issues are minor, but do make the paper a bit harder to read and understand. I will point out several below in my comments, but there are certainly more within the document.

Attending to the general comments:

(1) It is true that the "microbe free" effect of copepod excretion on free-living microorganism's metabolism is difficult to address and is certain not the scope of our studies. We consider the copepod as a hole considering also its own microbiome and potential microbial epibionts. Have a "microbe free" experiment is also not realistic itself if we try to determine nutrient fluxes and potential role of copepod. Our approach is based on a series of previous studies about the excretion of copepods and its impact on microbial communities (Perez-Aragón et al., 2011; Valdés et al., 2017a; Valdés et al., 2017b). We have a robust data base regarding the type of compounds excreted by copepods in central-southern Chile. In addition, the excretion of copepods has been studied for several decades and there is an important amount of works and reviews about this topic (Bidigare, 1983; Ikeda et al., 2001; Steinberg and Saba 2008; Hernandez-León et al., 2008; Smith and Whitley 1977; Pérez-Aragón et al., 2011). Several of these studies have demonstrated that the main compounds excreted by copepods are DON, ammonium, and DOP, although the latter has been much less studied than nitrogen compounds.

(2) We agree with the reviewer that incubation time was an important factor and that the enrichment associated with copepods could be evidenced mainly at the beginning of the incubation, since a later bacterial response could be associated with microbial metabolisms. However, our results indicate that presence of copepods and its metabolisms end products enhance the activity of different microbial communities in very short time response (<2h). We identify examples of active

bacteria in our incubations such as LDA, known to avidly react to the different compounds potentially provided by copepods. References associated, for example, to Alteromonadales were included in the discussion of the manuscript. Moreover, the reviewer can check for example in Figure 10 that different active microbial communities were observed in all the stations between the treatment and controls. Therefore, the effect of the copepod metabolism can't be discarded either. The fact that this "activity enhancement" did not result in significant increase in abundance also indicates that copepods interaction with bacterioplankton could be more complex than expected potentially generating shifts among microbial groups, since some bacteria presented a reduction in the copepod treatments.

- (3) We agree in this point, and also this issue was taken in consideration in the Discussion section.
- (4) Figure 8 shows the RNA samples not DNA, and thus the active bacterioplankton community. The most abundant communities present in situ and during the experiments are the same, the changes respond to its contribution. Here we study the activity of the microbial communities and as explained in the point (2) has been reported that some opportunistic microorganism as Gammaproteobacteria increase their activity in a very short time in response to enrichment experiments. DNA in situ was used to identify groups present in the study area which could however not usually be the most active ones. But T0 in the graphs were obtain from the activity of bacterioplankton using RNA so is not unexpected to have a different composition particularly in the case of cyanobacteria or photoautotrophs. Moreover, its common that the incubation experiments the microbial communities differs (in diversity and composition) from the field, since the incubation s may favor microorganism rare in nature but featuring opportunistic that allow for a more rapid adaptation to changes in environmental conditions and hence, outcompete other that are originally more abundant. This explanation and references were included in the Discussion section.
- (5) The initial samples were taken once all the bottles were incubated both with or without copepods. The time delay between the first bottle put in incubation and the beginning (time T0) of the experiments was around 20 minutes. This time window can explain the differences found in DON concentrations at the initial sampling points and also the variations in contribution of the most abundant groups. This information has been now added in Methods section.

Specific Comments:

Introduction:

*P. 2, line 7-8: Citation needed for sentence about thermal stratification and global warming decreasing the nutrient supply

We have now provided the proper reference.

*P. 2, lines 19-23. There has been a lot of work on zooplankton excretion and the various products. A more comprehensive summary should be provided about N cycling of body nitrogen and support for phytoplankton needs in oceanic environments.

We have now added a more comprehensive summary, with new references.

*P. 3, line 1: “enhance” should be “enhances.”

Corrected.

Methods:

*P. 3. Second sentence of section 2.1: Should read “The transect began west of New Caledonia and ended near Tahiti.”

Corrected.

*P. 3, section 2.1: You should include latitude/longitudes for your LD stations. Also, you should include a map of the transect and your specific sites used for the LD experiments. Latitude and longitudes were now included in the first section of methods along with a map with the transect during the cruise and stations sampled.

*P. 3, line 26: it says that you used a CTD rosette to collect water. Further information on hydrographic parameters should be included. Especially on chlorophyll concentrations within the DCM.

Temperature and Chl-a values have been included in the Table 1 on the new version of the MS.

*P. 4, line 7: was the incubation seawater also collected from the DCM? I assume so, but that is not clearly stated.

Yes, the incubation seawater was obtained from the DCM. We have now clarified this in section 2.3 of the new version of the MS.

*P. 4, line 8: isn't the acclimatizing step number 1?

Yes, acclimation step is number 1. We have now corrected.

*P. 4, line 8-9: If you were using the filtered seawater, it should read “seawater (22.5 L) was immediately filtered through a 0.7 um filter....”

Corrected.

*P. 4, line 10: 25C doesn't really seem cold. Perhaps “temperature controlled room” is a better term than “cold room?”

Thanks for the observation, we have now modified to “temperature controlled room”.

*P. 4, line 18: by controlled temperature, is that also in the 25 degrees C room? You should specifically say the temperature.

We have now provided temperature values throughout the MS.

*P. 4, line 21: what 6 bottles were added? Were they all controls? You need to explain this further. Tit is confusing as currently written.

We have now clarified this in “preparation of the microcosm and experimental setup” section.

*P. 4, line 24: should be “to ensure” instead of “of ensuring”

Corrected.

*P. 4, line 26: suggest specifying “During the excretion phase...”

We agree, thanks for the suggestion.

*P. 5, line 12: I am not familiar with the wet oxidation method, so I looked up the paper you cited. It looks that paper assesses PON and POP. What modifications need to be made to look at dissolved materials?

The wet oxidation method is used for both particulate and dissolve compounds, the differences is that the oxidation procedures uses seawater samples previously filtered through pre-combusted GF/F filter in Teflon flask for dissolved face and for the particulate sample, the filter is used in the Teflon flask for the oxidation procedures. Details are provided in “dissolved inorganic and organic measurements” section.

*Sections 2.5-2.6: My expertise is in zooplankton physiology, so I cannot assess the suitability of the genetics and bacterial methods.

*P. 5, line 22: “u sing” should be “using”

Corrected.

Results:

*Generally speaking, the authors did a good job displaying a very complicated data set. However, there are some places where the clarity of the results presentation can be improved with some minor modifications. I provide some of those suggestions below for Table S2 and Figures 2-5 and 8.

*P. 7, line 21: If these are ANOVA results, you should say so.

Done for the entire Results section.

*P. 7, line 24: “notorious” is not a correct term to use. “Notable” instead?

We agree, we have now modified.

*P. 7, line 30: I don’t understand why there is a difference at time T0. Is there a gap between when you started the experiment and took your initial sample? If that is the case, you need to specifically mention that in the methods.

Yes, there is a gap between the moment we started the experiments and the moment we took the initial sample. The initial sample (T0) was taken after all the bottles were conditioned (when all the bottles had the copepods and/or the seawater). The gap of the time was around of 15-20 minutes. We have now added this information in Methods section.

*P.7, line 30-31: Your final sentence appears to contradict itself. If there were nonsignificantdifferences, wouldn’t the p-value need to be >0.05?

We have now corrected. Thanks for the observation.

*P. 8, line 1-2: Consider re-wording for clarity. Specifically, all of the statistical test results should be together within parentheses.

We have now clarified this sentence and all the statistical tests were in parentheses.

*P. 8, line 6: missing a “(“ before the “LD B: : :”
Corrected.

*P. 8, line 7: “trough” should be “through”
Corrected.

*P. 8, line 8: “estimated” does not make sense here. Perhaps “significant differences were only observed between: : :”
Thanks for the observation, now it was modified accordingly.

*P 8, second paragraph:

(1) You should be very specific here about which N:P ratio you are discussing at any given time. It is confusing at the beginning when you define it only using nitrate/nitrite and phosphate, but then discuss inorganic and organic ratios.

We modified the nomenclature to DIN:DIP and DON:DOP for inorganic and organic ratios, respectively.

(2) Would you expect DON and DOP to be comparable to Redfield? Urea (one of the more common forms of DON), actually has 2 N per molecule, so if it is molar ratios that you are using, then it is not a 1:1 comparison that could be made between DON and DOP. Are there other papers that have using the organic N:P ratio? If so, it would be really helpful to bring those in to support your use and interpretation of this ratio.
We did not measured urea, so it is not the molar ratio that we are using. DON: DOP ratio is much less commonly measured than the inorganic ratio and only we have a couple of references with measure of this ratio to compare our interpretation.

(3) Line 18: you say that the DON:DOP is close to Redfield. But, the numbers that you mention in the prior sentences (and those in figure 4) are much higher than Redfield.
We agree and now it was modified.

(4) Line 20-21: The deviation from Redfield appears to be that LD C is much higher, not lower.
We agree and now it was modified.

(5) Line 21: should be “ratio” not “ration”
Corrected.

*P. 8, line 23: should be “shown” not “showed”
Corrected.

*P. 8, line 24: “plankton” is a plural term, so it should be “bacterioplankton were more abundant...”
Done.

*P. 8, line 30: is “and between time” referring to a different time than the sampling times

you mention earlier in the sentence?

Yes, we have now deleted the sentence.

*P. 9, line 14: On the other hand makes it sound like this is contradictory result. The two genetic markers seem to be showing similar trends. "Additionally" might be a better way to begin this thought.

Agree. Now it was modified.

*P. 9, line 27: if LD A and LD B show variability associated with time points, can you draw conclusions then based on presence of copepods? Or is this an artifact of the culturing conditions?

This issue was discussed in the general comment (2).

*P. 10, line 2: do you mean "larger" instead of "longer?"

Yes. We have now modified.

*P. 10, line 12: "show" instead of "showed"

Corrected.

Discussion:

*Section 4.0: Can you say the MA region had higher nutrient concentrations when LD B had the lowest of all of your sites?

The section 4.0 have been now modified, avoiding a general analysis of both regions.

*P. 10, line 22: "region" should be "regions"

Corrected.

*P. 10, line 27: You said there was significant increase in DON. But this was only during one time point. The other one is during timepoint T0, which seem suspicious.

The increase in DON at different time or in only one-time point during the experiments has been reported by other authors (Gardner and Paffenhöfer, 1982; Steinberg et al., 2002). DON excretion is release in a non-continuous way in highly concentrated spurts, while ammonium is release continuously, which is in agreement with our results.

*P. 10, line 31: do you mean "increase" instead of "increment?"

Yes. Thanks for the observation.

*P. 10, line 31: is $22,8 \times 10^3$ correct? Should it be 228 or 22.8? The formatting is a bit odd and makes me think it is a typo.

The formatting is correct. $22.8 \times 10^3 = 22,800$.

*P. 10, line 32: You suggest DON assimilated by growing bacteria. But it is mostly in the control of LD A that the bacterial abundances increase between 1-2 hours.

In average, bacteria increase their abundance but between 1 and at the end of incubation. We have now modified this sentence.

*P. 11, line 10: But in LD B, you said no significant differences were observed over time

(although your reported stats results don't match that - p 7, line 30)

We agree. We have now deleted this sentence.

*P. 11, line 26: How long after adding the copepods did you take your initial measurements? Could it really happen that fast? You need some more specific information to back up this as a reasonable assertion.

The initial sample was taken after all the bottles set for incubation (T0), that is when all the bottles had the copepods and/or the seawater. The gap of the time was around of 15-20 minutes. We have now added this information in methods section.

*P. 11, line 27: "crustaceans" instead of "crustacean"

Corrected.

*P. 12, line 10: Is this supposed to be phosphate or DOP? All the rest of the P references in this paragraph are to DOP, so I wanted to double-check.

Thanks for the observation, now it has been corrected

*Section 4.2, first paragraph:

(1) But it didn't seem like bacterial abundance was increased within the copepod bottles for most of the timepoints.

(2) You said in your bacterial community results that LD B experiments grouped by time primarily, not by treatment.

(1)(2) We agree and we have now rewritten this section.

*P. 13, line 18: "diminished" instead of "diminish"

Ok, corrected.

*P. 13, line 28: Define "substantial amounts." You need to be able to better quantify this. I don't really think this statement is firmly backed up by the data presented

We have now delete "substantial amounts" to avoid misunderstanding. However, our results showed an increase in DOP concentrations at different sampling points during the experiments.

*P. 13, lines 32-34: It didn't really seem like you saw bacterial growth overall. This statement is not directly supported by your results.

We agree, we have now modified this sentence.

Tables and Figures:

*Table 1: Redefine what this N:P ratio includes in the table caption.

We have now clarified in the Table 1, Figure 4 and through the whole manuscript.

*Figure 1:

(1) You mention "in situ temperature" a few times. What is that temperature?

We have now provided temperature values at DCM in Table 1.

(2) What does "*" mean after the 0.25 sampling point?

“*” correspond to the additional sampling time that we took at LD B and LD C stations. We have now clarified in the figure caption.

(3) In the caption, it says the feeding phase lasts for 3 hours. But other places in the document it states 4 hours.

Thanks for the observation. Now it was clarified.

(4) Specify in the caption that each of the 15 bottles contains 10 fed copepods.

Done.

*Table S2: You should include the overall ANOVA results as part of Table S2. It would be much easier for the reader if we could find all of those statistical results in one place. Perhaps on the gray lines?

We try to add all the information in one table, but is difficult to follow the results and is necessary extend the table losing clarity. Thus, we decided not added the information in the Table S2.

*Figures 2-5: The data are really complicated and results are hard to grasp between the text (where ANOVA are reported), table S2 (where post-hoc data are reported), and graphs (where visual trends are reported). Can you find a way to mark statistically significant differences on these graphs? Then the reader can go to these for a summary.

We tried but it's hard to put all these information in the graphs without lose clarity in the figure, since we should add this information between sampling time and treatments. Sorry for this thusdo not agreed with adding the statistical information in the figures.

*Figure 8: Can you use the prefixes in the descriptions of each line? “T1 copepod” or “T2 Control.” It makes it very difficult to see the trends you mentioned when you have to look back at the legend each time you read a line to figure out the color-coded timepoint.

We agree. Now it was modified in the figure.

Nitrogen and phosphorus recycling mediated by copepods and response of bacterioplankton community from three contrasting areas in the Western Tropical South Pacific (20°S)

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Eliminado: .

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Abstract. Zooplankton play a key role in the regeneration of nitrogen and phosphorus in the ocean through grazing and metabolism. This study investigates the role of the organic and inorganic nitrogen and phosphorus compounds released by copepods on biogeochemical processes and on the microbial community composition during the OUTPACE cruise (18 February – 3 April 2015) at three long duration stations (LD). Two LD stations were located in the Melanesian Archipelago region (MA; LD A and LD B) and one in the South Pacific Gyre (SG; LD C), which represent oligotrophic and ultra-oligotrophic regions respectively. At each station, microcosm onboard experiments were performed with locally sampled organisms, comprising a mix of epipelagic copepods fed with their natural food and then incubated along with wild microbial assemblages. In presence of copepods, ammonium and dissolved organic nitrogen showed a significant increase, compared to a control in two situations: in ammonium concentration (rate: $0.29 \mu\text{mol L}^{-1} \text{h}^{-1}$ after 4 h of incubation) in LD C and in dissolved organic nitrogen concentration (rate: $2.13 \mu\text{mol L}^{-1} \text{h}^{-1}$ after 0.5 h of incubation) in LD A. In addition, during the three experiments, an enhanced remineralization (ammonification and nitrification) was observed when adding copepods compared to the controls. A shift in the composition of the active bacterial community was observed for the experiments in LD A and LD B mainly characterized by an increase in Alteromonadales and SAR11, respectively and linked with changes in nutrient concentrations. In the experiment performed in LD C, both groups increased but at different periods of incubation,

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Alteromonadales between 1 and 2 h after the beginning of the experiment, and SAR 11 at the end of incubation. Our results in near *in situ* conditions, show that copepods can be a source of organic and inorganic compounds for bacterial communities, which respond to excretion pulses at different time scales, depending on the initial environmental conditions and on their community composition. These processes can significantly contribute significantly to nutrient recycling in the photic zone of ultra and oligotrophic oceanic regions.

- Eliminado: Finally,
- Eliminado: o
- Eliminado: experimental
- Con formato: Fuente:Cursiva, Sin Resaltar
- Eliminado: epipelagic ecosystem

1 Introduction

The Tropical South Pacific is considered one of the most oligotrophic regions in the world ocean. Its biogeochemical characteristics include nitrogen and phosphate limitation which modulate the low biological productivity (Moutin et al., 2008). In addition, the strong thermal stratification, enhanced by current global warming, decreases the nutrient supply to the euphotic zone (Polovina et al., 2008). The Western Tropical South Pacific (WTSP) was recently recognized as a hot spot of N₂ fixation (Bonnet et al., 2017) and the input of new nitrogen to the surface ocean through this process sustains over 50 % of the primary productivity (Karl et al., 1997; Carpenter et al., 2004). However, it has been reported that predator-prey interactions can provide substantial amounts of nitrogen and phosphorus, and supply an alternative substrate for phytoplankton and heterotrophic microorganisms stimulating the microbial loop in a variety of areas, contributing to the regenerated and new production (Richardot et al., 2001; Vargas et al., 2007; Aristegui et al., 2014; Valdés et al., 2017a, b).

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Metazooplankton metabolism is recognized as one of the main mechanisms controlling concentration, composition and cycling of dissolved organic matter (DOM) in the sea (Sipler and Bronk 2014). Zooplankton may release dissolved organic and inorganic matter through sloppy feeding (Møller, 2004, 2007), leaching of fecal pellets (Hasegawa et al., 2000; Steinberg et al., 2002) and excretion (Saba et al., 2011) and, along with bacterial remineralization, it determines the amount of regenerated nitrogen and phosphorus available for phytoplankton production (Steinberg and Landry, 2017). Copepods excrete up to 53% of their body nitrogen per day, mainly in the form of ammonium, followed by urea and amino acids (Bidigare, 1983), thereby recycling much of the nitrogen in the water column (Ikeda et al., 2001; Steinberg and Saba 2008). The importance of ammonium excretion by mesozooplankton to primary production has been assessed in a variety of areas in the ocean, and the contribution to nitrogen requirements for phytoplankton growth is mainly depending on the productivity of the area (Hernandez-León et al., 2008). Reports indicate that zooplankton could sustain between 40-50% of the nitrogen requirements of phytoplankton in open ocean areas, as in the North Pacific central gyre (Eppley et al., 1973) and this percentage decreases in eutrophic waters, such as upwelling areas (Smith and Whitledge 1977; Pérez-Aragón et al., 2011). Other works indicate that diel vertical migrating zooplankton can sustain between 2% to 19% of the nitrogen requirements by phytoplankton through ammonium excretion at the oligotrophic Sargasso Sea (Steinberg et al., 2002). Furthermore, zooplankton can also excrete substantial amounts of dissolved organic nitrogen (DON), i.e., between 7 to 80% of the total dissolved nitrogen being released (Miller and Glibert, 1998; Conover and Gustavson, 1999; Steinberg et al., 2002; Steinberg and Saba 2008; Pérez-Aragón et

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al., 2011). In fact, the composition and quantity of the excreted compounds are highly dependent on the type of food ingested by zooplankton (Miller and Roman, 2008; Saba et al., 2009). Even less studied than nitrogen is the phosphorus release by zooplankton. It has been reported, that one-third of the phosphorus released by zooplankton (amphipods) was in the organic form (Johannes, 1964; Satomi and Pomeroy, 1965). Isla et al. (2004) estimated that mesozooplankton phosphate excretion can sustain 21.7 % of phytoplankton phosphate requirements in oceanic water of the NW Iberian. Titelman et al. (2008) demonstrated that copepod feeding enhances organic phosphorus uptake by bacterial activity in a phosphorus limited system. However, the impact of zooplankton metabolism on the phosphorus recycling in oceanic areas is largely unknown.

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The importance of quality and quantity of DOM in structuring bacterioplankton community has been increasingly reported (Alonso-Saez and Gasol, 2007). However, the processes and mechanisms through which nitrogen and phosphorus compounds released by zooplankton can determine and influence the food web and the structure of the microbial community are unclear. Recent research has demonstrated that bacterial community composition experiences changes in response to the addition of DOM (Landa et al., 2013; Sarmiento et al., 2013). Studies on the interaction between zooplankton metabolism and bacterial communities are however too scarce. Recent reports demonstrate that the addition of excretory products released by zooplankton (krill) stimulates bacterial growth and production in the Southern Ocean (Aristegui et al., 2014). Specific studies carried out by us in a eutrophic coastal zone off southern/central Chile indicate that the nitrogen excreted by copepods generate a specific response of nitrifying communities and in the active marine bacterioplankton community (Valdés et al., 2017a, b). However, studies of copepod nitrogen and phosphorus excretion impact on microbial community from oligotrophic and ultra-oligotrophic areas can provide valuable information about of the recycling of this nutrients in larger areas of the ocean.

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Herein, we studied the role of organic and inorganic dissolved compounds released by copepods, and its potential effect on their recycling and structuring of the bacterioplankton community, during late austral summer in distinct biogeochemical regions of the WTSP.

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2 Material and methods

2.1 Study area and sampling strategy

Sampling was conducted in WTSP during austral summer 2015 (18 February -3 April), on board the RV L'Atalante. The transect began west of New Caledonia (18° S-159.9° E) and ended near Tahiti (17.56° S-149.05° W; Fig. 1). Experiments were conducted in three long-duration (LD) stations (approximately eight days duration): LD A (27 February; -19.2° S-164.7° E), LD B (17 March; -18.2° S-189.1° W) and LD C (24 March; -18.4° S-194° W). These stations were chosen along a regional gradient of oligotrophy and are located in two geographic regions: LD A and LD B in the Melanesian archipelago (MA) and LD C in the subtropical gyre (SG). These stations chosen as based on the sea surface chlorophyll-a concentration, which was maximum in LD A and LD B, and minimum in LD C station. More details about the sampling strategy and general

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biogeochemical and hydrographic characteristics are described in Moutin et al., (2017) and Moutin et al., (this issue). At each sampling station seawater samples were obtained using Niskin bottles (12 L) arranged on a CTD rosette from the Deep Chlorophyll Maximum (DCM). Seawater was collected for chemical and biological initial characterization, including ammonium (NH₄⁺), nitrate (NO₃⁻), nitrite (NO₂⁻), phosphate (PO₄³⁻), chlorophyll- a, DNA and RNA. The DCM depths correspond to 80 m, 34 m and 140 m in LD A, LD B and LD C, respectively. Details of chlorophyll-a methods are available in Dupouy et al., (this issue).

2.2 Mesozooplankton sampling

Samples were collected by vertical hauls of a Bongo net (70 cm mouth diameter) of 120 µm mesh size, equipped with a non-filtering cod-end to obtain undamaged individuals. Mesozooplankton samples were collected at the three LD stations in the upper 100 m depth at a speed of 1 m s⁻¹ under night conditions. Live samples were immediately transferred to coolers until sorting at the on-board laboratory.

2.3 Preparation of the microcosm and experimental setup

The experimental design had three steps: (1) Copepod acclimation, (2) feeding and (3) copepod+microbial recycling (Fig. 2). Seawater for incubations (30 L) was collected into clean (10% HCl rinsed) polycarbonate carboys from the DCM. For acclimatizing (1) and copepod+microbial recycling (3) steps, seawater (22.5 L) was immediately filtered through a 0.7 µm filter (GF/F; Whatman) using a peristaltic pump. The remaining 7.5 L were used in the feeding phase. Seawater for the different steps was maintained in a temperature controlled room (in situ temperature ~25° C) until the beginning of the experiment.

Undamaged individuals were sorted from the live samples and identified using a stereomicroscope at low light. Copepods samples in the three experiments consisted in a mix of the most representative copepods (adults) in the sample (Table S1; More details about zooplankton composition and abundance are available in Carlotti et al., this issue). In the acclimation phase (1), 15 groups of 10 copepods were incubated in 500 mL Nalgene bottles, maintained for 4-6 h in filtered seawater previously filtered (GF/F Whatman) in darkness and at controlled temperature (in situ). In the feeding step (2) copepods were removed from the bottles using a sieve (20 µm) and maintained in new 500 mL polycarbonate bottles (Nalgene) with <150 µm filtered seawater (polycarbonate membrane) for feeding. This step lasted 4 hours and, as the acclimatization phase, took place under controlled temperature (25°C). Thereafter, each copepod group (15 groups) was placed in a new set of 500 mL polycarbonate bottles (Nalgene) filled with the seawater previously filtered (0.7 µm; Millipore) for the excretion+microbial recycling phase. Also, other 15 bottles without copepods were incubated as control. In total, 30 bottles were used in this step at LD A, whereas additional bottles were included at LD B and LD C, 6 bottles were added at LD B and LD C, 3 with copepods and 3 without copepods as control. The seawater used was filtered to avoid the presence of phytoplankton and small protists, to minimize grazing effect and ensure the presence of natural microbial assemblages. At the end of each step copepods were observed and

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checked by direct observation to ensure swimming behaviour in the bottles. The percentage of mortality at the end of each experiment at each bottle was less than 20%.

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During the excretion+microbial response phase, at time intervals of 0 h (T0), 0.5h (T1: T0+0.5 h), 1 h (T2: T0+1 h), 2 h (T3: T0+2h) and 4 h (T4: T0+4 h) samples were collected for NH_4^+ , NO_3^- , NO_2^- , PO_4^{3-} , DON, dissolved organic phosphorus (DOP), bacterioplankton abundance and RNA. The samples for the initial time (T0) were taken once all the bottles were incubated.

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An additional sampling, corresponding to incubation time of 15 min (T0': T0+0.25h), was added just for having more measurements of NH_4^+ in the experiments of stations LD B and LD C. Six bottles were sacrificed at each time, three for treatment with copepods and three for control (without copepods). The three bottles sacrificed at each time represented triplicates for treatment and control.

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At the end of each observation time, copepod samples were preserved immediately with formalin buffered by sodium borate (5% final concentration) for further zooplankton identification analysis (Table S1).

2.4 Dissolved inorganic and organic nutrients measurements

Nutrient samples (NO_3^- , NO_2^- and PO_4^{3-}) were taken in duplicate in 20 mL HDPE bottles, filtered through 0.7 μm filters (GF/F; Whatman) and poisoned with HgCl_2 to a final concentration of 20 $\mu\text{g L}^{-1}$. Samples were stored at -20 °C and analyzed on board determined by standard colorimetric techniques (Aminot and K erouel 2007), using a SEAL Analytical AA3 HR system (SEAL Analytical, Serblabo Technologies, Entraigues Sur La Sorgue, France). Samples for NH_4^+ determination (40 ml) were taken in duplicate and analyzed on board using a Jasco FP-2020 fluorometer according to Holmes et al., (1999). Samples for DON and DOP (30 mL) were filtered through pre-combusted (450° C, 6h) GF/F filters (Whatman). Samples were collected in Teflon bottles and analyzed immediately on board by the wet oxidation method (Pujo-Pay and Raimbault, 1994). DON and DOP concentrations were determined by sample oxidation (30 min, 120° C) and corrected for NO_3^- , NO_2^- and PO_4^{3-} concentrations, respectively.

2.5 Bacterioplankton abundance, DNA and RNA extraction

Bacterioplankton abundance was determined by flow cytometry. From each bottle 1,350 μL samples were fixed in sterile cryovials with glutaraldehyde (at 0.1% final concentration). The samples were stored at -80° C until laboratory analysis. Samples were analyzed by flow cytometry (FACScan, Becton Dickinson) at the Observatoire Oc anologique de Banyuls sur Mer, France. The abundance of non-fluorescent picoplankton was estimated from samples previously stained with SYBR green I (Molecular probes) according Marie et al. (1997). DNA samples for the initial characterization of bacterial communities were collected at DCM depth of each sampling site. Samples (9-10 L) were filtered through cellulose ester filters (0.22 μm ; Millipore) using a peristaltic pump and stored with RNA later reagent (Ambion) at -20° C until extraction procedures.

DNA was isolated using PowerSoil DNA Isolation Kit (MoBio Laboratories) in accordance with the manufacturer's specifications following Levipan et al., (2014). DNA was quantified by spectrophotometry (NanoDrop ND-1000 Spectrophotometer). RNA samples were collected for initial characterization of active bacterial community at DCM at each LD station and during each experiment to study the active bacterial community composition. Seawater (100 mL) was filtered using a sterilized syringe and 25 mm swinnex through 0.22 µm hydrophilic PVDF filters (Millipore) and the filters were preserved with RNAlater solution (Ambion) and stored at -80° C until RNA extraction procedures.

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RNA samples were extracted using Mirvana kit (AM1560; Ambion) in accordance with manufacturer's instructions including a mechanical disruption step and homogenization using 200 µm diameter zirconium beads (Low Binding Zirconium Beads, OPS Diagnostic) and homogenized twice at ~3,000 rpm for 30 s by using Mini-Beadbeater-8TM (Biospec Products). In order to remove DNA traces, RNA was treated with TURBO DNA-free kit (Ambion). Finally, concentration and quality (A260/A280 ratio) of RNA extracts as well with DNA extracts were determined by spectrophotometry (NanoDrop ND-1000 Spectrophotometer).

2.6 Analysis of the bacterial community structure

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The bacterial community structure was analyzed by Illumina MiSeq sequencing method from 16S rRNA (samples from incubation), using cDNA as template, and 16S rDNA in situ, following Campbell and Kirchman, (2013). cDNA was generated using random primers provided by the ImProm-IITM Reverse Transcription System (Promega). Bacterial 16S rRNA gene libraries from V1-V3 region (27F-519R) were generated at the Molecular Research LP (www.mrdnalab.com, Shallowater, TX, USA).

The 16S rRNA and rDNA gene sequences were processed using Mothur software v1.35.1 (Schloss et al., 2009). Sequencing data sets were curated by quality filtration to minimize the effects of random sequencing errors by eliminating sequence reads <200 bp and trimming of sequences that contained more than one undetermined nucleotide (N) and sequences with a maximum homopolymer length of 8 nucleotides. Chimeric sequences were identified using the Chimera UCHIME algorithm (Edgar et al., 2011) and removed to retain high quality reads.

The 16S rRNA and rDNA gene sequences retrieved were taxonomically classified using the automatic software pipeline SILVAngs available from <https://www.arb-silva.de/> (Quast et al. 2013). In LD A station, a total of 178,097 sequences were analyzed, ranging between 10,831 and 19,703 sequences for each library (Fig. S1). In LD B station 219,019 sequences were analyzed from 15,260 to 20,966 for each library, and in LD C 230,805 sequences were analyzed, ranging between 13,699 and 22,104 sequences for each library. In each of the three LD stations, the highest number of sequences correspond to DNA in situ. Libraries were deposited in the European Nucleotide Archive (ENA) under study accession PRJEB21648 with the following run access numbers: ERS1810581-ERS1810616.

The composition was analyzed at Phyla, Class and Order taxonomic level as abundant (>0.5%) and others (<0.5%) in total sequences retrieved from each library. The Chao, Shannon and Evenness indices were calculated using Past3 software. Previously, since the number of sequences per sample was variable, we normalized the different libraries sizes by subsampling routine in Mothur software version 1.36 (Schloss et al., 2009), to reflect the lowest number of sequences encountered (LD A: 10,831; LD B: 15,260 and LD C: 13,699).

2.7 Statistical analysis

Statistical analysis of treatments effects on chemical and biological parameters were performed using a two-way analysis of variance (ANOVA) after checking normality assumptions (Kolmogorov-Smirnov test) and homoscedasticity (Levene's test). Pairwise multiple comparisons were performed using a Tukey test as a posteriori analysis. Statistical significance was set a $p=0.05$ and analysis was computed using R software. Time point sampled at 15 min was not considered for this analysis, because of too few data.

Changes in bacterial community structure changes were compared using ordination Bray-Curtis similarities and used to build dendrograms by the unweighted pair group method with arithmetic averages (UPMG). For multivariate statistical analysis, the software package PRIMER v.6 and the add-on PERMANOVA+ was used. The permutational multivariate analysis of variance (PERMANOVA) with fixed factor was applied to investigate the differences of bacterial community composition for treatments and control in both experiments. Principal co-ordinate analysis (PCO) was performed to visualize patterns of the bacterial community in response to the different treatments.

3 Results

3.1 Biogeochemical in situ conditions

Inorganic nutrients concentrations, obtained at the DCM depth, varied substantially between the sampling sites (Table 1). Lower concentrations close to the limit of detection of the methods of NH_4^+ , NO_3^- , NO_2^- and PO_4^{3-} were observed in LD B compared to LDA and LD C. The highest concentrations were observed in LD A, particularly for NO_3^- ($1.53 \mu\text{mol L}^{-1}$). The resulting N: P ratio showed greater values in LD A (9.97 ± 0.70) than LD B and LD C, and the lowest N: P ratio (1.21 ± 0.11) was estimated for LD C seawater.

3.2 Changes in inorganic nutrients, DON and DOP during the incubations

Inorganic nitrogen and phosphorus concentrations changed substantially over the course of the experiments at the three LD stations (Fig. 3 and 4). Ammonium concentrations (Fig. 3a, e, i) increased significantly (four- and seven-fold) in the presence of copepods in all experiments (ANOVA, LD A: $F_{1,20}: 93.2, p<0.001$; LD B: $F_{1,18}: 61.7, p<0.001$; LD C: $F_{1,20}: 108.9, p<0.001$).

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The largest and most significant difference in ammonium concentration with respect to the controls was associated with T0 in LD A, T3 in LD B and T4 in LD C (ANOVA and Tukey's test $p < 0.001$; Table S2). Nitrate showed significant differences between the treatment with copepods and controls in LD B and LD C; characterized by notable accumulation at the treatment with copepods (Fig. 3f, j) at T1 in LD B (ANOVA and Tukey's test $p = 0.02$; Table S2), and at T4 in LD C experiments (ANOVA and Tukey's test $p = 0.009$; Table S2). Nitrite (Fig. 3c, g, k) was slightly variable through time in the experiments and no significant difference was detected between copepods and the controls (ANOVA, LD A: $F_{1,16} = 0.50$, $p = 0.49$; LD B: $F_{1,18} = 0.13$, $p = 0.7$; LD C: $F_{1,20} = 0.44$, $p = 0.51$). DON (Fig. 3d, h, l) showed significant differences between the treatment and control in LD A and LD C (ANOVA, LD A: $F_{1,20} = 8.99$, $p = 0.007$; LD C: $F_{1,20} = 29.0$, $p < 0.0001$), and this difference was associated with the increment in concentration at T1 in LD A and T0 in LD C in the treatment with copepods. At LD B non-significant differences were detected between treatments and controls through the time (ANOVA, $p > 0.05$).

Phosphate in LD A showed a higher concentration during the first hour of incubation, followed by a sharp decrease (from 0.17 to 0.05, approximately; Fig. 4a) in the treatment with copepods as well as control. Despite significant differences in time (ANOVA, $F_{1,20} = 17.7$, $p < 0.0001$), treatment versus control differences were not significant (ANOVA, $F_{1,20} = 4.5$, $p = 0.06$). Also, no significant differences in phosphate distribution through time and between treatments were found in LD B and LD C experiments (Fig. 4c, e; ANOVA, LD B: $F_{1,18} = 0.13$, $p = 0.7$; LD C: $F_{1,20} = 0.44$, $p = 0.51$). DOP concentrations (Fig. 4b, d, f) did not vary significantly through time in the three experiments (ANOVA, LD A: $F_{1,20} = 0.3$, $p = 0.87$; LD B: $F_{1,20} = 0.5$, $p = 0.43$; LD C: $F_{1,20} = 0.70$, $p = 0.60$), but significant differences were only observed between the treatment with copepods and control in LD B and LD C (ANOVA, LD A: $F_{1,20} = 0.43$, $p = 0.52$; LD B: $F_{1,20} = 8.92$, $p = 0.008$; LD C: $F_{1,20} = 7.94$, $p = 0.01$).

During the experiments, the resulting DIN, DIP and DON: DOP ratios are shown in Fig. 5. In the LD A experiment the DIN: DIP ratios (Fig. 5a) were lower than Redfield ratios during the first hour of incubation followed by a ratio increment from 12.5 to 32.4 at the end of incubation, only in the treatment with copepods, whereas a decrease in DIN: DIP ratio was observed at the end of incubation in the controls. In the same experiments in LD A, DON: DOP ratio decreased from 25 to 16.4 at the end of the experiment (Fig. 5b). In LD B experiment (Fig. 5c), lower DIN: DIP than Redfield ratio was found through the experiment, suggesting a N deficiency relative to P, except at 1 h of incubation when a higher DIN: DIP ratio was observed (2.6 times higher than Redfield) in the treatment with copepods, linked to a nitrate increment (see Fig. 3f). The DON: DOP ratios were higher than Redfield ratio in the treatment with copepods and control (Fig. 5d). In LD C the DON: DOP ratio (Fig. 5f) indicated a substantial accumulation of N with respect to P from the first hour of the incubation in the treatment with copepods, showing an important deviation from the Redfield ratio ($> 16:1$).

3.3 Changes in bacterioplankton abundance during the experiment evolution

Changes in bacterioplankton abundance are shown in the Fig. 6. Initial experimental conditions showed that bacterioplankton were more abundant in LD B, followed by LD C and LD A experiments. In LD A experiment (Fig. 6a) bacterioplankton

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presented an increase through time towards the end of the incubation, from 100×10^3 to 200×10^3 cell mL⁻¹, showing significant statistical differences between the sampling times (ANOVA, F_{1,20}: 13.5, p<0.001) but not between treatments (ANOVA, F_{1,20}: 0.004, p=0.95). In contrast, in LD B experiment (Fig. 6b), a decrease in bacterioplankton abundance (around 300×10^3 cell mL⁻¹) was observed between 0.5 to 1 h and between 1 h and 2 h of incubation, in copepod and control treatments, respectively, giving significant differences both between sampling times (F_{1,16}: 33.8, p<0.000) and treatments (copepods and control, ANOVA, F_{1,16}: 7.69, p=0.014). In LD C (Fig. 6c), a strong difference (> 200×10^3 cell mL⁻¹) was observed between sampling times (ANOVA, F_{1,20}: 9.93, p<0.000) and between treatments and control (ANOVA, F_{1,20}: 6.22, p=0.02).

3.4 Active bacterial composition and their response during the incubations

The alpha diversity derived from normalized 16S rDNA and rRNA libraries is shown in the Fig. 7. The bacterial taxonomic richness determined (OTU number) and expected on the Chao1 index was higher in DNA in situ compared to the cDNA in situ and the samples related to the experiment. In accordance, Shannon diversity index showed that the bacterial community obtained in situ from 16S rDNA reached higher values compared with the incubated community. The alpha diversity showed slight differences between the treatments with copepods in the three experiments. These differences were characterized by higher values at the beginning of the incubation in the LD A and LD B stations (T1 and T2, respectively; Fig. 7a and 7c) and by higher diversity values at the end of the experiments (T4) in the treatment with copepods in LD C station (Fig. 7b).

The total, in situ bacterial community composition (Fig. 8a) was characterized by a higher contribution of Alphaproteobacteria, mainly by SAR 11, dominating slightly more in LD C, followed by LD B and LD A, respectively, reaching 49.8%, 41.1% and 31.1% of libraries derived from DNA (Fig. 8b). Cyanobacteria were also present in our libraries with a higher contribution in LD C than in LD A and LD B. In addition, a high number of abundant phyla were observed in LD A compared to the other stations. Additionally, the active in situ bacterial composition (Fig. 8c) 16S rRNA was consistent with 16S rDNA, with the predominance of Alphaproteobacteria and SAR11 class followed by the same trend (Fig. 8b and 8c), with higher contribution in LD C, followed by LD B and LD A stations.

In general, clustering analyses showed that the active bacterial community composition varied significantly during the incubation compared to the in situ community in the three experiments (Fig. 9). During LD A incubation, the bacteria community composition was characterized by an increase of Gammaproteobacteria from 39% to 68% in T0 and T4 in the copepods treatments (Fig. 9a). Also, a decrease in Cyanobacteria, Chloroflexi and other low frequent taxa was found. During the experiment at LD B, smaller differences in taxa contribution were observed through the incubation. Gammaproteobacteria, increased their contribution through the incubation from 28% at T0 to 32% at T4 (Fig. 9b). In contrast to the other experiments, the incubation at LD C (Fig. 9c) presented a decrease in Gammaproteobacteria contribution and an increase in Alphaproteobacteria. The cluster analysis (Fig. 9) associated with bacterial orders contribution changes during the experiments indicate that the variability was mainly associated to the incubation time, T0, T1 and T2 versus T3 and T4, in LD A and LD B

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experiments, respectively. However, in LD C experiment the bacterial orders were mainly grouped by treatments without effect of incubation time (with and without copepods; Fig. 9c).

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In addition, the greatest dissimilarities, based on SIMPER analysis (Fig. 10; Table S3), between copepod treatments and control in LD A (Fig. 10a) was mainly due to an increase in Alteromonadales and Oceanospirillales in the treatment with copepods, whereas SAR11 and Rhodobacterales increase their relative abundance in controls. At LD B experiment (Fig. 10b), smaller dissimilarities between treatment and control (9.5%) were observed and mainly due to a successive decrease of Alteromonadales contribution and increase of SAR11 contribution in the treatment with copepods. In contrast in the LD C station (Fig. 10c), the dissimilarities were mainly due to a larger contribution in Alteromonadales, replaced by SAR 11 at the end of the incubation in the treatment with copepods. The specific order contribution for the three experiments are shown in Fig. S2, S3 and S4, as abundant (>0.5%), semirare (0.5%-0.1%) and rare (<0.1%; Pedrós-Alió, 2012).

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PCO analysis with Orders contribution and environmental parameters indicates that in LD A experiment the initial sampling points (T0, T1 and T2) were associated with inorganic compounds (NH_4^+ , NO_3^- , NO_2^- and PO_4^{3-} ; Fig. 11a) for the treatment with copepods and control. Meanwhile, nitrite concentrations were associated with the last time-points in LD B (Fig. 11b). In LD C experiment (Fig. 11c), no visual association was observed between environmental parameters and the order contribution. In addition, the PERMANOVA main test revealed significant differences among all samples regarding the different experiments (pseudo-F=12.7, p=0.001). However, differences between treatments with copepods and control, and between times not show significant differences (PERMANOVA, pseudo-F=2.1-1.3, p=0.12-0.25).

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20 4 Discussion

The two oceanographic regions in which our experiments were performed presented contrasting trophic conditions. The MA region was characterized by a gradient of nutrient conditions, with the highest and the lowest nitrate concentrations in LD A and LD B, respectively. Both stations were characterized by the presence of *Trichodesmium* spp bloom, with higher N_2 fixation rates (Bonnet et al., 2017; Caffin et al., this issue). According, the satellite data, De Verneil et al., (this issue) suggest that the low nutrient concentration found in LD B was the consequence of a two months old diazotrophs bloom. In contrast to the MA region, the SG region is characterized by lower nitrate concentrations, higher phosphate concentrations, resulting in lower DIN: DIP ratio. These conditions were reflected in the abundance and composition of zooplankton, and also in the composition of bacterioplankton. In the case of zooplankton, the average abundance in the MA region was 1,212 ind. m^{-3} (sd=334 ind. m^{-3} , highest value 2,017 ind. m^{-3}), whereas in the SG region the average abundance was 665 ind. m^{-3} (sd=213 ind. m^{-3} , highest value 409 ind. m^{-3} ; Carlotti et al., this issue). On the other hand, the bacterioplankton community composition revealed a higher diversity in the MA region compared to SG, with a higher dominance of Alphaproteobacteria in both regions, however, SAR 11 increased their contribution in the ultra-oligotrophic area (SG).

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4.1 Biogeochemical changes in response to the dissolved compounds released by copepods

During our study, we evidenced that copepods exert an influence on biogeochemical composition during the incubations at contrasting trophic conditions including ultra-oligotrophic (SG) versus oligotrophic conditions (MA), showing significant increase of ammonium and DON accumulations. In addition, also copepods influence DON recycling but differentially at the different stations. In the experiment with copepods carried out in the LD A station, higher DON concentrations were observed at initial times (between 0 and 1 h). This DON was actively consumed between 1 and 2 hours of incubation. However, this higher DON consumption ($3.62 \mu\text{mol L}^{-1} \text{h}^{-1}$; Table S4) was not regenerated, since ammonium did not accumulate with time. In addition, at the same time and until the end of incubation an increase in bacterioplankton abundance was observed ($22.8 \times 10^3 \text{ cell mL}^{-1}$). Although this increase was non-statistically significant, it has been reported that zooplankton can release highly labile compounds through their metabolism and these compounds can be rapidly assimilated by microbial communities, as heterotrophic bacteria in a variety of marine environments (Aristegui et al., 2014; Vargas et al., 2007). On the other hand, the second experiment carried out in the MA region (at LD B) evidenced a potential nitrification associated with copepods excretion. This because, at the initial time, a decrease in ammonium concentration (between 0.5 and 1 h of incubation) related with nitrate accumulation (highly variable between the triplicates) was observed in the treatment with copepods, but not observed in the control. Nitrifying and heterotrophic communities can rapidly respond to DON and ammonium pulses excreted by copepods in the upwelling area off central Chile, revealed a tight coupling between excretion and the functional microbial groups involved in ammonia oxidation (Valdes et al., 2017a).

In the SG region (at LD C station), the experiment with copepods presented the highest evidence of DON remineralization/ammonification based on the differences in ammonium concentrations between copepods treatments and control up to $0.3 \mu\text{mol L}^{-1}$, several times higher than in situ concentration (Table 1). DON consumption was observed through the first 2 h of incubation in the treatment with copepods (Fig. 3; Table S4). In addition, as in LD B, nitrate accumulation was observed at the end of incubation in the treatment with copepods compared to the control. Our results suggest presence of two processes which could occur in the ultra-oligotrophic station in the SG: the release of DON by copepods stimulating ammonium regeneration by bacteria, and the increment in nitrate concentration strongly linked to the nitrification process. Thus, DON remineralization could provide additional substrate for microbial and phytoplankton growth in LD C station. In this sense, Raimbault and Garcia (2008) demonstrate that in the very low productive area of South Pacific Gyre, most of the primary production is sustained by active regeneration process, being nitrification active in surface layer and often balances the biological demand for nitrate. These results support the idea that DON excretion by copepods in oligotrophic and ultra-oligotrophic water could play a major role in nitrogen cycling.

On the other hand, DOP showed significantly higher concentrations in the treatment with copepods compared to the control in the three experiments, although not over the whole incubation period but over shorter durations. Our results suggest that

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copepods could potentially contribute with substantial amount of DOP and provide an alternative source of phosphorus for phytoplankton and microbial loop in this study area. During LD B station, a bloom of *Trichodesmium* spp was observed, and the satellite data suggested that the bloom was already two months old by the sampling time at station LD B (de Vernejl et al., this issue), which is in agreement with our lower nutrients concentrations obtained in situ in this station (Table 1) and through our incubation in this station. The contribution of DOP by copepods could contribute to maintain the longer bloom in this region (MA), providing an additional source of phosphorus for biomass growth. *Trichodesmium* spp are capable to hydrolyze DOP compounds (Mulholland et al., 2002), thus zooplankton metabolism could potentially supply a significant portion of the cellular phosphorus necessary for their growth.

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In this study, the increase in the concentration of the different compounds cannot be attributed only to the excretion process. Firstly, the leaching is one of the possible pathways through which zooplankton can release dissolved compounds, along with the sloppy feeding and excretion. However, it has been demonstrated that the contribution provided by leaching is insignificant compared to excretion, less than 6% in the case of the release of DON, whereas ammonium was undetected (Saba et al., 2011; Steinberg et al., 2000). We did not monitor leaching from fecal pellets produced during our experiments, considering leaching from fecal pellets as negligible, and then DON accumulation could account for up to $1.91 \mu\text{mol L}^{-1} \text{h}^{-1}$ excretion in the LD A experiment. However, the experimental copepod densities were several orders of magnitude higher than those expected in situ (10 copepods for 0.5 L volume, i.e. $20,000 \text{ ind. m}^{-3}$). Consequently, our results might be overestimated, because copepods concentration in our bottles did not reflect the in situ abundance found at each LD station (MA: $1,500 \text{ ind. m}^{-3}$ and SG: 500 ind. m^{-3} ; Carlotti et al., this issue). However, such concentration was strictly necessary through the experiments to obtain a signal in ammonium concentrations, which were very low in the study area (Table 1). In addition, we cannot estimate excretion rates for all compounds since they appear later in the incubation probably modulated by microbial metabolism. Moreover, running an experiment without microbial influences as control was impossible since most antimicrobial approaches (e.g., autoclaving, adding antibiotics, filtrating, etc) could have negative effects on copepods microbiome and water chemistry affecting also our results.

4.2 Changes in bacterial community composition induced by dissolved compounds released by copepods

During our experiments, we determined the response of microbial community in terms of changes in the abundance and active bacterial composition. In our experiments, the bacterioplankton abundance followed the same patterns of variation in the experiments with copepods and in the controls, for each of the three experiments. We found significant differences between treatments (with and without copepods) and between sampling times, except for LD A, in the bacterioplankton community structure (mainly by Gammaproteobacteria and Alphaproteobacteria). A faster response in Alteromonadales (Gammaproteobacteria) is frequently observed in microcosm studies during incubations, due to their high growth rate and their ability to exploit DOM rapidly when is available (Alonso-Saez and Gasol, 2007; Fuchs et al., 2000; Landa et al., 2013), which

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is in according with our results. However, the changes in structure associated with the release of dissolved compounds by copepods compared to the controls were not accompanied by an evident increase in bacterioplankton abundance. Several studies have suggested that enrichment experiments tend to enhance the abundance of organism rarely found in nature, but that have opportunistic and copiotrophic qualities that allow them to rapidly adapt to changes in environmental conditions, outcompeting abundant groups in the field (Nelson and Wear, 2014; Pedler et al., 2014; Logue et al., 2016). In contrast, in the experiment carried out in LD B station, a substantial decrease in Alteromonadales was observed in the treatment with copepods compared to the control. The higher contribution through the time was due Alphaproteobacteria, in which SAR11 increased their relative abundance (~8%) at 1 hour of incubation. SAR 11 was the major constituent of 16 rDNA (in situ) and also was active in according to our 16 rRNA (in situ) reads. SAR11 is the most abundant bacterioplankton in the ocean, is well adapted to low concentration of nutrients and specialized to oxidizing many labile and low-molecular-weight compounds produced by other plankton (Giovannoni, 2017). During our incubations, SAR11 increased their contribution only in LD B experiment which coincided with the station with the lowest nutrients concentrations. On the other hand, in the LD C experiment, an increase in Alteromonadales and Vibrionales was observed. Alteromonadales decreased their contribution in the treatment with copepods at the end of the incubation, while increasing their contribution in control. The reverse pattern was observed for SAR11 which increased their contribution at the end of incubation. Based in our experiments, Alteromonadales increase is in coincidence with DON available from copepods excretion during the first 2 h of incubation, afterwards at the end of the experiment nutrients were diminished, and such conditions potentially favoured the versatile SAR 11. Peduzzi and Herndl (1992) observed high monomeric carbohydrate concentration and bacterial activity in experiments where copepods were included. Furthermore, these authors observed that bacterial communities living in oligotrophic areas can be efficient to utilize the newly available substrate source, in according with our results.

Bacterial remineralization of DOM derived from copepods metabolism could be a highly efficient mechanism to maintain the nutrients in the upper layer supporting the phytoplankton and microbial growth. In this sense, the response associated with the different regions of our study suggests that copepods in the MA region could provide DOP as an alternative substrate for phytoplankton and bacterial growth, while the enhanced remineralization by copepods could be more important in the ultra-oligotrophic area of the SG region. Furthermore, the biogeochemical impact of copepods metabolism may not be limited to the upper layer, as zooplankton can move through the water column by diel vertical migration, promoting the export of dissolved compounds through their metabolism at deeper layers. We conclude that copepod metabolism can provide substantial amounts of nitrogen and phosphorus (NH₄⁺, DON, DOP) which microbial communities can directly use in a short period of time enhancing the bacterioplankton remineralization.

Acknowledgement. This is a contribution of the OUTPACE (Oligotrophy from Ultra-oligoTrophyPACific Experiment) project funded by the French research national agency (ANR-14-CE01-0007-01), the LEFE-CyBER program (CNRS-INSU), the

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Bajado [1]: Our results suggest that copepods can provide substantial amounts of nitrogen and phosphorus (NH₄⁺, DON, DOP) which microbial communities can directly use in a short period of time enhancing the bacterioplankton remineralization in the WTSP.

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GOPS program (IRD) and the CNES (BC T23, ZBC 4500048836) and by European FEDER Fund under project 1166-39417. The OUTPACE cruise was managed by T. Moutin and S. Bonnet from the MIO (Mediterranean Institute of Oceanography). The authors thank to the crew of the R/V L'Atalante for outstanding shipboard operation. Dr. M. Pujo-Pay (Observatoire Océanologique de Banyuls sur mer), O. Grosso (MIO) and S. Helias (MIO) are thanked for their assistance during the analysis of nutrients. We thank C. Dupouy, T. Moutin and S. Helias for their support in providing Chlorophyll-a and DON-DOP data. This work was supported by the Comisión Nacional de Investigación Científicas y Tecnológicas (CONICYT) through Grants FONDECYT No 1130511 and 1150891 and Instituto Milenio de Oceanografía (IMO) Grant IC120019. Additional support has been provided by FONDAP grants IDEAL and INCAR (15110027 and 15150003, respectively), and CONICYT PIA PFB-31 COPAS Sur austral program. The work was developed in the frame of LIA MORFUN. Valdés and Donoso were funded by CONICYT Scholarship

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Table 1: Deep chlorophyll-a maximum (DCM) depth, temperature, chlorophyll-a, ammonium (NH₄⁺), nitrate (NO₃⁻), nitrite (NO₂⁻), phosphate (PO₄³⁻) concentration, DIN: DIP ratio, DON and DOP concentrations in the DCM at each sampling site. DON, DOP concentrations was extracted from Moutin et al, (this issue).

	LD A	LD B	LD C
DCM (m)	80	34	140
<u>Temperature (°C)</u>	<u>25.6</u>	<u>27.5</u>	<u>22.9</u>
<u>Chlorophyll-a (µg L⁻¹)</u>	<u>0.29</u>	<u>0.79</u>	<u>0.57</u>
NH ₄ ⁺ [µmol L ⁻¹]	0.025 ± 0.001	0.01 ± 0.00	0.02 ± 0.000
NO ₂ ⁻ [µmol L ⁻¹]	0.06 ± 0.002	0.02 ± 0.02	0.05 ± 0.004
NO ₃ ⁻ [µmol L ⁻¹]	1.53 ± 0.008	0.05 ± 0.021	0.25 ± 0.012
PO ₄ ³⁻ [µmol L ⁻¹]	0.16 ± 0.012	0.03 ± 0.025	0.24 ± 0.008
<u>DIN: DIP</u>	<u>9.97 ± 0.70</u>	<u>4.52 ± 4.07</u>	<u>1.21 ± 0.11</u>
<u>DON [µmol L⁻¹]</u>	<u>4.78</u>	<u>5.88</u>	<u>4.49</u>
<u>DOP [µmol L⁻¹]</u>	<u>0.12</u>	<u>0.18</u>	<u>0.11</u>

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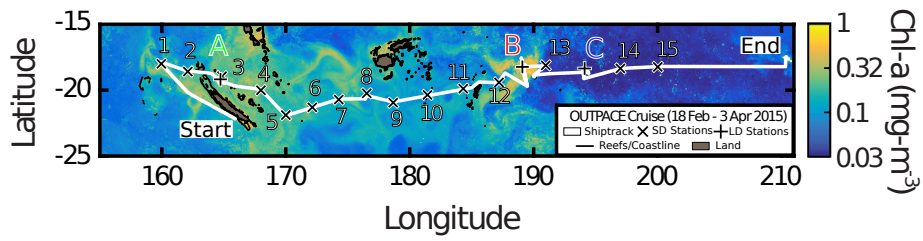
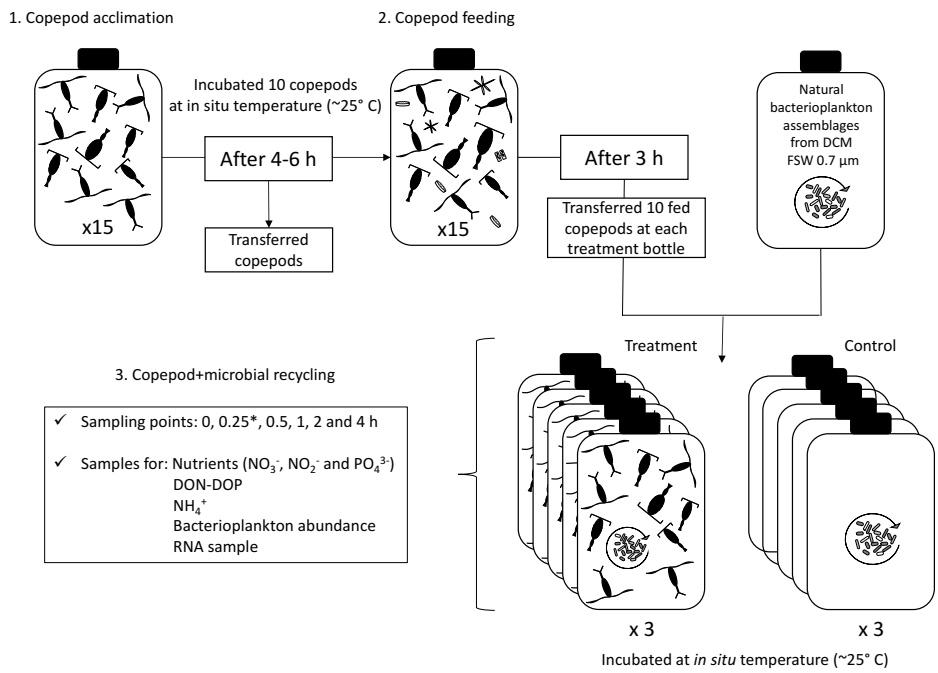


Figure 1: Quasi-Lagrangian Surface Chlorophyll-a concentration (mg m^{-3}) during the OUTPACE cruise. The satellite data are weighted in time by each pixel's distance from the ship's position for the entire cruise. The white line shows the vessel route (data from the hull-mounted ADCP positioning system). Coral reefs and coastlines are shown in black, land is grey, and areas of no data are left white. The positions of the short (long) duration stations are shown by cross (plus) symbols. Experiments were performed at each long duration station (Figure courtesy of A. De Verneil (02/06/2017)).

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Figure 2: Experimental design. The design consisted of three phases done sequentially. Each bottle with copepods was acclimated for at least 6 hours (phase 1), followed by feeding (phase 2) with the Deep Chlorophyll-a Maximum (DCM) water for 4 hours. Copepod+microbial recycling (phase 3) was studied by adding natural bacterial assemblages from DCM filtered seawater (DCM FSW) to the 15 bottles with fed copepod, and maintaining bottles with natural bacterial assemblages and without copepods (controls). Three bottles with copepods and three control bottles were sacrificed at each of the 5-sampling time. An additional time (0.25 h*) was added for ammonium determination in LD B and LD C.

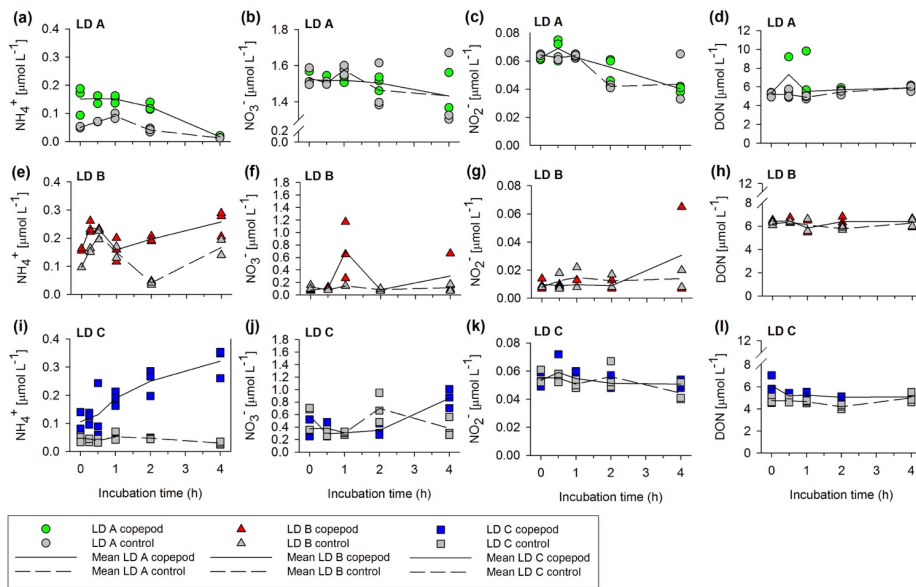


Figure 3: (a, e, i) Ammonium (NH_4^+), (b, f, j) nitrate (NO_3^-), (c, g, k) nitrite (NO_2^-) and (d, h, l) dissolved organic nitrogen (DON) variability through the incubation for treatments with copepods and control (without copepods) at each LD experiment.

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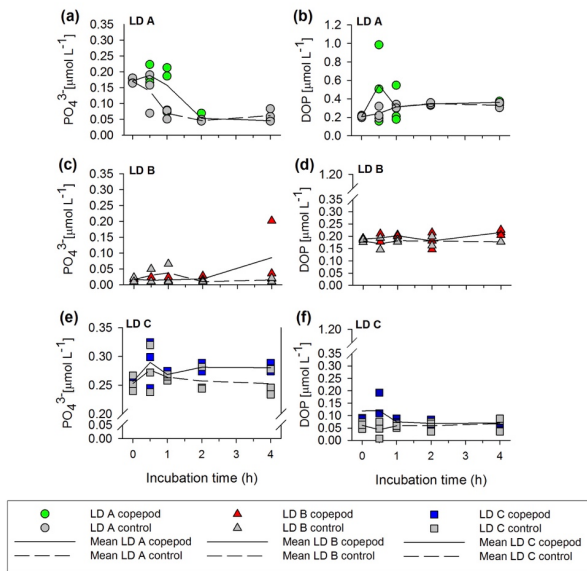


Figure 4: (a, c, e) Phosphate (PO_4^{3-}) and (b, d, f) dissolved organic phosphorus (DOP) variability through the incubation for treatments with copepods and control (without copepods) at each LD experiment.

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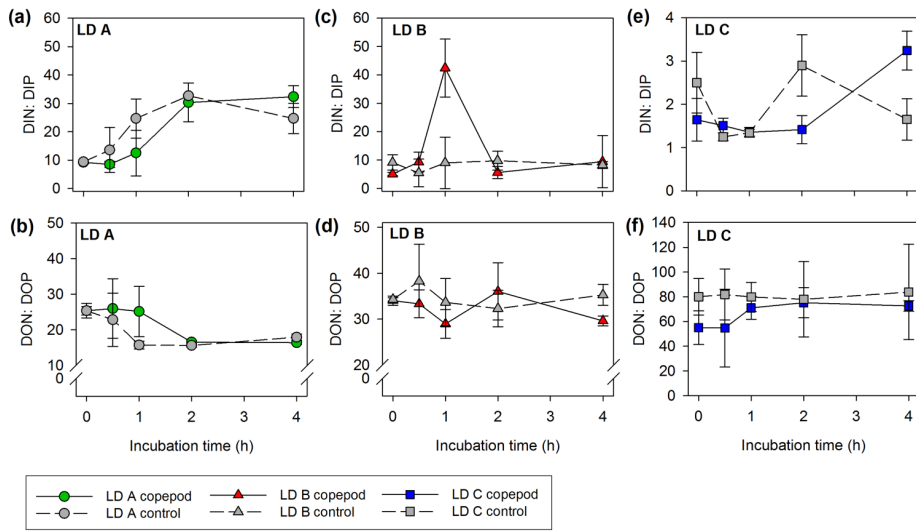


Figure 5: (a, c, e) N: P ratios of the inorganic (DIN: DIP) and (b, d, f) organic (DON: DOP) nutrients through the incubation for treatments with copepods and control (without copepods) at each LD experiment.

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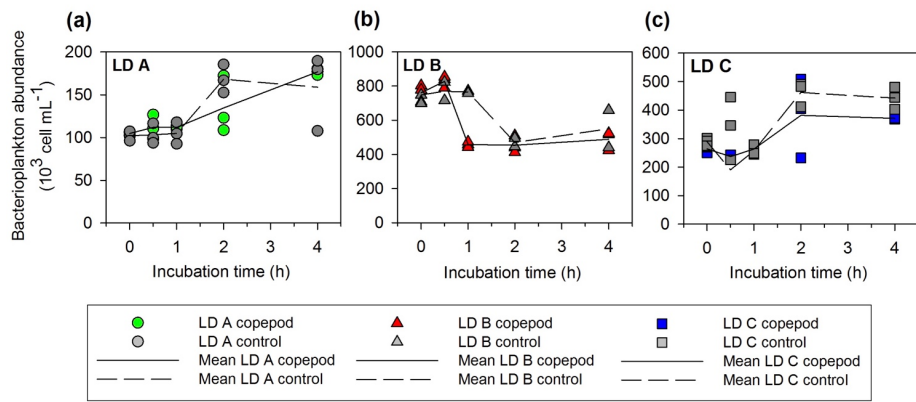


Figure 6: Bacterioplankton abundance through the incubation for treatments with copepods and control (without copepods) at each LD experiment. **(a)** LD A, **(b)** LD B and **(c)** LD C.

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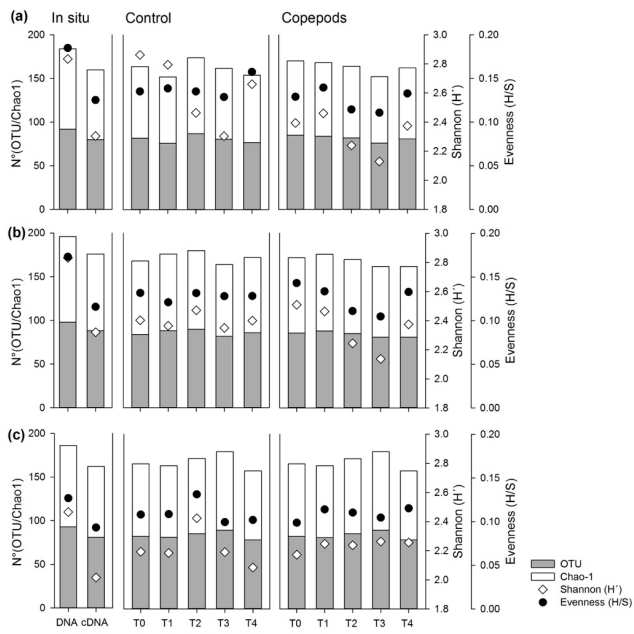


Figure 7: Richness, Chao1, Diversity (Shannon H') and Evenness from the total and active in situ bacterial community and through the incubation for the active bacterial community at each LD experiment: (a) LD A, (b) LD B and (c) LD C.

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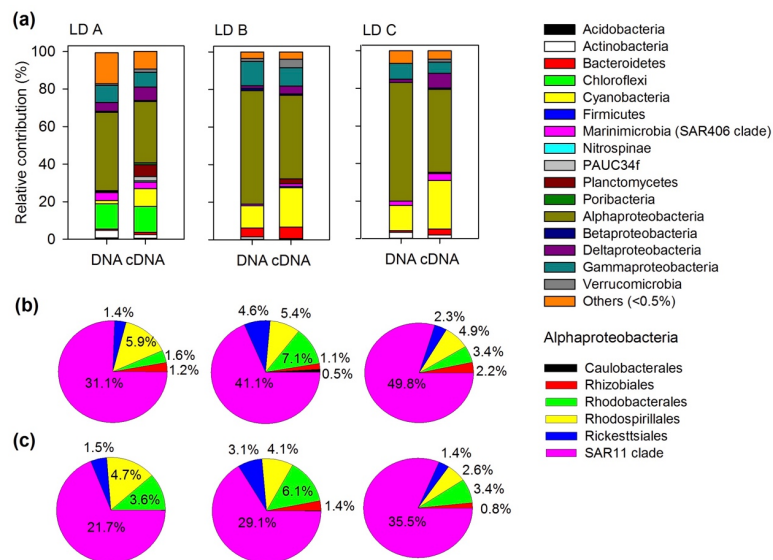


Figure 8: (a) Relative abundance of major bacteria phyla and Proteobacteria class at each LD station, with a zoom in Alphaproteobacteria order at initial in situ conditions for the three-sampling site: (b) total Alphaproteobacteria (DNA) and (c) active Alphaproteobacteria community (cDNA).

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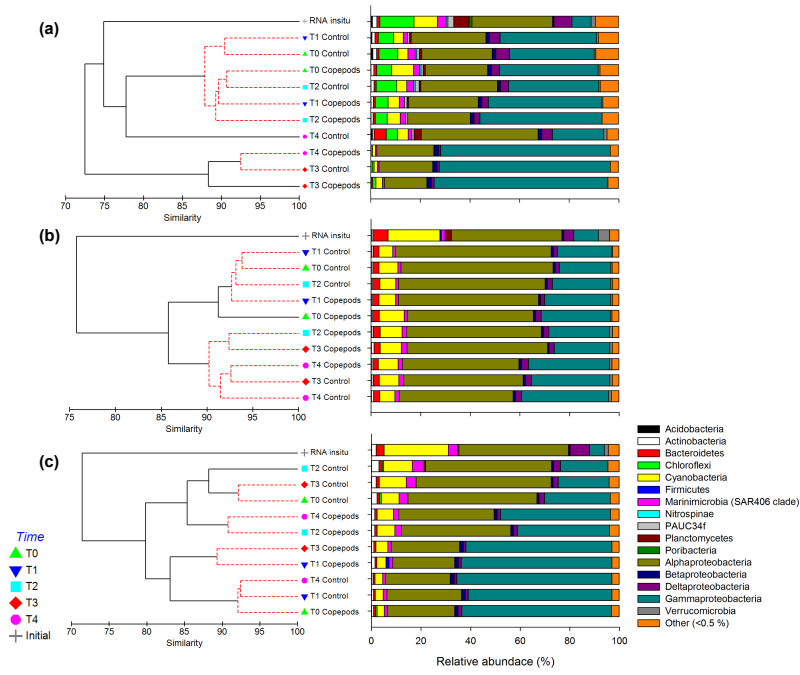


Figure 9: Similarity dendrograms based on the 16S rRNA and relative contribution of the abundant bacteria phyla and Proteobacteria subclasses for the initial in situ community, treatments with copepods and controls (left panel) through the incubation for the three experiments (a) LD A, (b) LD B and (c) LD C. Clustering in one the basis of a distance matrix computed using the Bray-Curtis index of similarity. The dendrogram was inferred with the unweighted pair-group average algorithm (UPGMA). Bacterial communities in the samples connected with red branch lines are not significantly different (SIMPROF test, $p < 0.05$).

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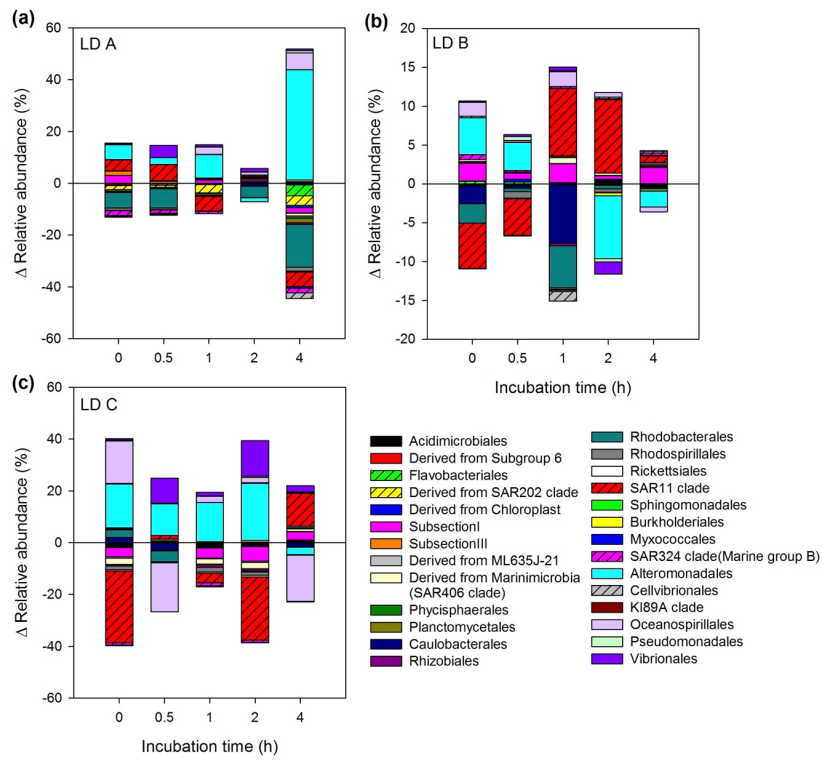


Figure 10: Contribution of active Orders through the incubation based on the SIMPER results. List of the OTUs explaining 50% of the dissimilarity observed through the experiment between treatment with copepods (top) and control (bottom) for each LD experiment: (a) LD A, (b) LD B and (c) LD C.

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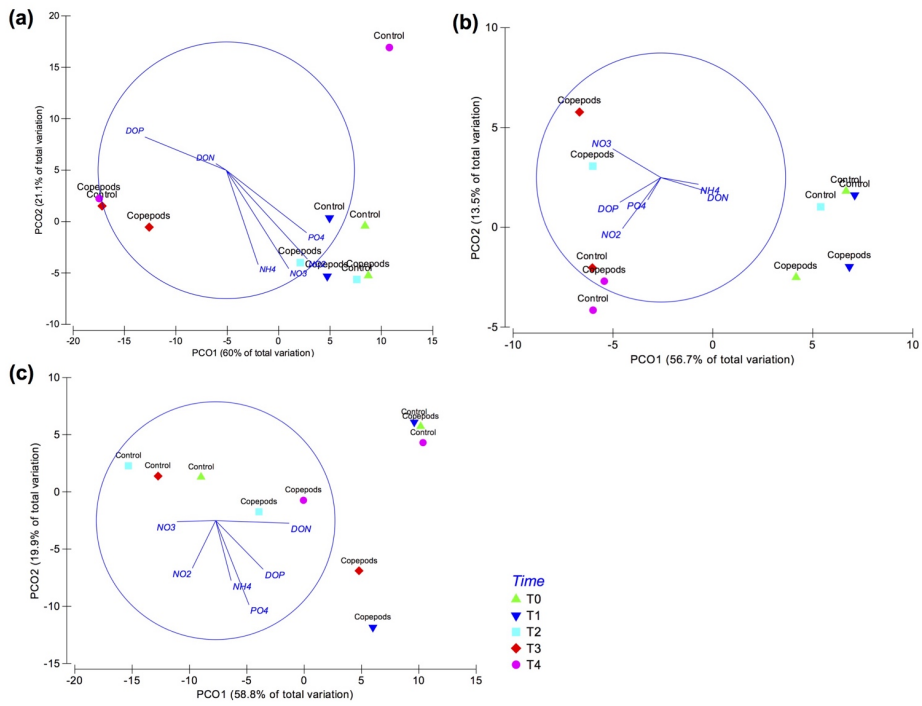


Figure 11: Principal component ordination based on Bray-Curtis similarity at order taxonomic level, of treatment with copepods and control (a) LD A, (b) LD B and (c) LD C. Vectors indicate the best environmental variables (normalized transformed) correlated with ordinations and vector lengths correspond with the correlation values.

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Página 11: [5] Eliminado **Valentina Valdes Castro** **17/4/18 11:07:00**

Página 11: [6] Eliminado **Valentina Valdes Castro** **16/3/18 12:28:00**

Página 11: [7] Eliminado **Valentina Valdes Castro** **16/3/18 16:50:00**

In addition, the highest differences in DON concentration between the treatment with copepods and the control (without copepods) were found at initial time points, mainly in LD A and LD C experiments. Zooplankton can excrete DON in highly concentrated spurts lasting up to an hour, unlike ammonium which is excreted continuously by crustacean (Gardner and Paffenhöfer, 1982; Steinberg et al., 2002). In this study, we cannot attribute the increase in the concentration of the different compounds only to the excretion process since we did

not monitor possible leaching from fecal pellets produced during the experiments. The leaching is also one of the possible pathways through which zooplankton can release dissolved compounds, along with the sloppy feeding and excretion. However, it has been demonstrated that the contribution provided by leaching is insignificant compared to excretion, less than 6% in the case of the release of DON, whereas ammonium was undetected (Saba et al., 2011; Steinberg et al., 2000). Thus, if we consider leaching from fecal pellets as negligible, then DON accumulation could account for up to $1.91 \mu\text{mol L}^{-1} \text{h}^{-1}$ excretion in the LD A experiment. However, the experimental copepod densities were several orders of magnitude higher than those expected in situ (10 copepods for 0.5 L volume, i.e. $20,000 \text{ ind. m}^{-3}$), so that our results might be overestimated because copepods concentration in our bottles did not reflect the in situ abundance (MA: $1,500 \text{ ind. m}^{-3}$ and SG: 500 ind. m^{-3} ; Carlotti et al., this issue). However, such concentration was strictly necessary through the experiments to obtain a signal in ammonium concentrations, which were very low in the study area (Table 1). In addition, we cannot estimate excretion rates for all compounds since they appear later in the incubation probably modulated by microbial metabolism.

Página 11: [8] Eliminado **Valentina Valdes Castro** **16/3/18 16:58:00**

In LD A experiments, phosphate was found in higher concentrations in the treatment with copepods compared to the control between 0 and 1 h of incubation. In LD B and LD C experiments, concentrations in the treatment with copepods versus control were significantly higher at the beginning and at the end of the incubation for LD B, also at the end of incubation for LD C.

Página 12: [9] Eliminado **Valentina Valdes Castro** **16/4/18 12:51:00**

, even if the abundance was slightly increased in the experiments with copepods. In our LD B and LD C experiments, this impact on bacterial community structure was significantly different with additions of copepods compared to the control.

Through the incubations, the bacterioplankton community was characterized by changes

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Página 13: [11] Eliminado **Valentina Valdes Castro** **17/4/18 12:05:00**

Página 13: [12] Eliminado **Valentina Valdes Castro** **14/3/18 17:30:00**

seems to play a critical role in nutrient recycling by fuelling microbial and phytoplankton growth in the vast oceanic regions of the world ocean.

Página 18: [13] Eliminado **Valentina Valdes Castro** **17/4/18 12:17:00**

1. Copepod acclimation

2. Copepod feeding

