Author comments

Answer to Referee #2

Interactive comment on "Interaction between elevated CO_2 and phytoplankton-derived organic matter under solar radiation on bacterial metabolism from coastal waters" by Antonio Fuentes-Lema et al.

Authors want to thank anonymous referee #2 for her/his deep review and helpful suggestions on the manuscript. We have followed all her/his indications and have made the suggested modifications in the manuscript. Each comment has been addressed point by point as shown in the text below.

General comments:

- 1. The paper by Fuentes-Lema and co-workers addresses a topic of interest in marine biogeochemistry. The experimental design is appropriate although the results are far from concluding and I feel the authors have overexploited a bit their findings. Some of the conclusions do not hold or do so only for one of the sampling points, which diminishes the overall relevance of their contribution. I think they should be much more cautious in some statements. Another problem that complicates the interpretation of the dataset is that not all variables were sampled at the same time (e.g. bacterial abundance data are lacking on days 2, 4 and 6 and respiration is lacking on days 1, 3, 5 and 6). This makes the assessment of the effect of high CO_2 concentrations on DOM-heterotrophic bacteria interactions very difficult. I suggest an alternative approach. Rather than focusing on the analysis of specific sampling times I would like to see the analysis of integrated values of bacterial biomass, production and respiration over the course of the 7 days of the incubation of the second phase. Maybe the conclusions will change but they will be more reliable than in the current version. In general, the paper is well-written although there are a number of instances in which English usage and grammar needs to be improved.

We agree with the referee that the analysis of the data can be improved if a more holistic approach is used in addition to analyzing the days where the responses showed statistical differences, which aimed to explain changes in the physiological variables over time. Following his/her suggestions, in the new version of the manuscript we have included new results from the statistical analysis of the integral values obtained from the cumulative responses (Lines 279, 299, 304 and 315) and the corresponding figures (Fig. 4D, 7B, 7D, 8B and 8D). The results from this methodological approach

do not change the conclusions shown in the previous version of the manuscript but they do certainly help to make the understanding of the results much easier.

We have also carefully revised all the statements that might be controversial to avoid misunderstandings and the English usage and grammar has been corrected, as suggested by the referee.

Specific comments:

- 2. An important concern is related to the authors' point about the lability of DOC. By examining their Figure 7 one cannot really say anything about DOC lability since only in the HC treatments there was a net, although very slight, decrease in DOC concentration, presumably due to bacterial uptake. How can the authors explain the general pattern of increase rather than decrease in DOC for most of the experiment?

The experimental approach used for the study aimed to keep both fractions of the organic matter: the particulate and the dissolved organic matter fractions. We thought that keeping both, the particulate and dissolved organic matter fractions was a more realistic experimental approach since the particulate fraction could potentially be degraded during the second phase of the experiment due to the elevated CO_2 concentrations or the enzymatic activity (Piontek et al. 2010). Enzymatic activity on POC depends on the type of substrate and relates to the metabolic capabilities of the microbial community but can act on the temporal interval of our experiment (Arnosti et al. 2005). Therefore, it is likely that at the start of the bacterial incubation, production of DOC exceeded its consumption by heterotrophic bacteria, leading to a net accumulation of DOC in the microcosms. Counterintuitively, the high metabolic activity observed after adding the organic matter might have also contributed to the net accumulation of DOC since it has been recently demonstrated that microheterotrophs can release part of the substrate as new DOM, with a production efficiency of 11 ± 1%, 18 ± 2% and 17±2% for DOC, DON and DOP, respectively (Lønborg et al. 2009). Information regarding possible explanations for the increase in net DOC has been included in the text (Lines 353-363).

- 3. The manuscript implicitly assumes that UV played a distinct role in the amount and quality of DOC produced by phytoplankton (DOCp) but there is no way of distinguishing the effect of UV from the effect of PAR in their experimental design.

The fact that UVR plays an important role on DOC produced by phytoplankton (DOCp) has been already demonstrated in previous papers cited in the text (i.e. Carrillo et al. 2002, Helbling et al. 2013, Fuentes-Lema et al. 2015). Taking this into account we decided to expose the phytoplankton

community during the first phase incubation to the whole solar spectra, including UVR. Since most of the experimental studies do not usually use UVR transparent materials, or do not take into account the effect of UVR at all, we though that it was important to recall this fact along the text, when it was relevant. However, to study the effect of UVR on phytoplankton DOC was not among the objectives of the study. In order to avoid confusion regarding the role of UVR as an additional experimental factor in our study, the reference to solar radiation shown in the title has been deleted. In addition the information related to the effect of UVR on DOM production from phytoplankton has been deleted from the Introduction. The new version of the manuscript only shows a brief comment regarding the importance of UVR on DOM production from phytoplankton later in the Discussion section (Lines 323-326).

- 4. By incubating the samples in the dark the authors are introducing a potential source of error in their results. I fully concur with them that solar radiation plays an important role in DOM-microbial plankton interactions but then, why stop the normal diel cycle of light and darkness during 8 days? The authors should be aware of the possible role of photoheterotrophy in bacterioplankton communities (e.g. Ruiz-González et al. 2013 Frontiers in Microbiology) and their response to the two types of DOM. Moroever, the DOM enriched seawater could also be subject to further transformations caused purely by sunlight that are not accounted for in their setup.

We agree with the referee that the effect of UVR on DOM-microbial plankton interactions is a relevant issue to be studied. However, we rather focused our attention on the other two factors (i.e. CO_2 and organic matter addition) that have been much less studied and therefore could bring more original information. Exposing the bacterioplankton samples to solar radiation, without testing the effect of UVR as an additional experimental factor, would have complicated significantly the interpretation of the results, and adding a third factor to the second phase incubation of our experimental design was logistically impossible. A third experimental factor with three replicates per treatment would have duplicated our experimental design from 12 to 24 experimental units, which was unavoidable by the authors. Just as an example, an experimental approach with 24 experimental units used in a microcosm study aimed to test the effect of CO_2 x Irradiance x nutrients addition on the microbial plankton responses was successfully carried out during the Group of Aquatic Productivity Workshop in Málaga in 2012 thanks to the collaboration of 23 researchers (ej. Sobrino et al. 2014, Mercado et al. 2014). The new version of the manuscript include a brief comment about the concern of using a third experimental factor (Lines 148-151).

- 4. Since no attempt was made to estimate empirical leucine-to-carbon conversion factors for calculating bacterial production, known to change dramatically in different environmental conditions (see for instance Teira et al. 2015 Applied and Environmental Microbiology, and references therein), presumably met

during their second phase incubations, the uncertainties of this variable (BP) and that of bacterial carbon demand (BCD) are very high.

The determination of the leucine-to-carbon conversion factors for calculating bacterial production under different CO_2 scenarios and organic matter additions is certainly an interesting study to be carried out. Unfortunately most of the researchers use a theoretical factor since it is sometimes unapproachable to determine them for each experimental condition, time and taxonomical composition, more especially when working with incubations of natural samples. In our experimental design we used a conversion factor, that despite being theoretical, it had been previously determined in an experiment aimed to study the differential responses of phytoplankton and heterotrophic bacteria to organic and inorganic nutrient additions in Ría de Vigo (Martínez-García et al. 2010). In this paper empirical Leu to C conversion factors for natural plankton communities from two different sampling sites of the Ría de Vigo, characterized by having different CO_2 concentrations (Ría and shelf) (Gago et al. 2003), was determined. The inner station (Ría) is the same sampling site than the location chosen for our study. Martínez-García et al. (2010) shows that no significant differences in conversion factors were found between both sampling locations (t-test, $p > 0.05$) or between the control and the treatment with organic matter additions. This lack of differences could imply an absence of significant effect of CO_2 concentration or organic matter addition on the conversion factor in our experiment. In addition, the conversion factor used in our study has been previously used in a paper aimed to analyze the effects of different pCO_2 concentrations on marine bacteria (Teira et al. 2012), which allows comparison with previously published results. However due to the lack of further empirical information supporting or rejecting the suitability of the conversion factors used in the manuscript, the authors have decided to delete most of the information related to the analysis of the bacterial carbon demand and bacterial growth efficiency, following the referee´s suggestion. The information has been deleted in the Abstract and in the Discussion sections, and it has been significantly decreased in the Results section. Information about the rationale for choosing the Leu to carbon conversion factor used in our study and some text about the uncertainties raised up by the referee related to the conversion factor, including new references from Martínez-García et al. (2010) and Teira et al. (2015), have been included in the text (Lines 179-185 and 460-469).

- 5. Information about phytoplankton cell counts of two idly defined groups (Region 1 and Region 2 in Table 1 for which we do not even know their sizes) in flow cytometry analysis, assuming that the huge initial (Day 0-1) increase in chlorophyll a concentration was mostly due to large cells not detected by flow cytometry make this section virtually irrelevant. Also, I guess that *Synechococcus* cyanobacteria were surely present at least in Day 2, with abundances much higher than 1000-10000 cells mL-1. The authors should ellaborate more on these results or simply delete them.

The purpose of including these flow cytometry results in the manuscript was to show that the phytoplankton composition in both CO_2 treatments during the first phase incubation, aimed to obtain the organic matter that was going to be used for the second phase incubation, was similar. These findings, in accordance with the lack of differences between treatments for primary production, are related to the absence of significant differences in DOM concentration at the end of the first incubation. In any case, since this information is more straightforward shown by the PP and DOM concentration, and the flow cytometry results do not represent the whole phytoplankton community, the table has been deleted as proposed by the referee.

Technical:

- 6. The title is very confusing. The interaction is established between DOM and bacteria, not between elevated CO2 and phytoplankton-derived DOM. Also, what does it mean "Interaction: : : on bacterial metabolism"? The expression "Under solar radiation" is not necessary to be included in the title. "bacterial metabolism from coastal waters" also reads awkwardly. Please change to a more informative, correct title.

A new more concise title has been written following the suggestions raised by the referee (Line 1).

Minor comments:

- 7. L. 54-55. "Phytoplankton" and "heterotrophic counterparts" are not logical choices. Please refer to autotrophic and heterotrophic microbes or something similar.

The text has been modified for clarification (line 53).

- 8. L. 60. What do the authors refer by "The other way round"? Please explain.

That was a misunderstanding. The sentence has been rephrased (line 59).

- 9. L. 64-65. Provide more detail about "the abiotic AND biotic factors".

The text has been modified for clarification (line 63).

- 10. L. 67. “Adaptation towards a fast acclimation” sounds odd. The underlying mechanisms are different, please rephrase.

The sentence has been modified to show the referee’s suggestion (line 67).

- 11. L. 103. “subjected… concentrations” can be safely eliminated here.

The text indicated by the referee has been eliminated (line 97).

- 12. L. 142. Surely there were other phytoplanktonic taxa/groups present along with “mainly diatoms”.

Due to the lack of data to corroborate this statement and that the sentence does not provide highly relevant information, the sentence has been deleted following the referee´s concern.

- 13. L. 194. The R2 value does not inform about the significance of this difference. Did the authors performed a t-test/one-way ANOVA to support their statement?

Information about the results from the one-way ANOVA statistical test has been included in Appendix 1 (Line 732). The analysis of the correlation between TOC and POC+DOC showed no significant differences (ANOVA, p=0.637, n=34) as indicated in the previous version of the manuscript.

- 14. L. 206. Duplicate Winkler bottles seem too few for oxygen changes measurements. Usually a minimum of 4-5 bottles are used.

Results for the analysis of bacterial respiration in our study come from the mean ± SD of 3 replicates (i.e. 12 L PC bottles) for each of the 4 treatments (LC_NO, LC_AO, HC_NO, HC_AO). For each replicate 4 Winkler bottles were used: Two (2) bottles to assess the O_2 concentration at the start of

the incubation and another 2 to quantify the concentration at the end of the incubation. This makes a total of 12 bottles per treatment. Regarding variability within each technical replicate (i.e. duplicates from each 12 L PC bottles), the error was very low, with an average of 1.7% (max: 8.6%, min: 0.06) and 1.3% (max: 5.6%, min: 0.01) for the analysis of the O_2 concentration at the start and at the end of the incubation, respectively.

The original text has been modified to clarify the point raised by the referee about the number of Winkler bottles used in each treatment (Line 188).

- 15. L. 249. "to compare non-parametric paired samples" is an odd phrasing.

The sentence has been deleted from the Statistical analysis section.

- 16. L. 293. It does not seem so obvious to me.

The sentence has been rephrased to improve clarity (Lines 268-270).

- 17. L. 302 and L. 310. Why using RMANOVA for comparing differences at one single sampling point?**.** I do not follow the rationale for using the two statistical tests here. There is some confusion about statistics throughout the manuscript. The authors should clearly state which tests they used and why or try a different analysis (see my general comment) with changes integrated over the course of the incubation of phase 2.

The statistical analysis has been revised throughout the whole manuscript. A new approach using a two-way ANOVA statistical test has been done in the present version of the manuscript. A detailed explanation of this topic has been included in the Statistical analysis section (Lines 222-229).

- 18. L. 328. "biased" is probably not the best word here.

The term "biased" has been replaced by the more appropriate term (Line 310).

- 19. L. 329. Replace "on" by "of".

The correction has been done as indicated.

- 20. L. 341. “there have been…simulate” reads awkward. Please rephrase.

The sentence has been rephrased to improve clarity as suggested by the referee (Line 327).

- 21. L. 345. Are the authors sure of this statement?

The original statement has been modified in accordance with the information from the cited study (Line 330).

- 22. L. 370-371. This is not true in view of the different sampling points and the data shown in the corresponding figures.

The new approach proposed by the referee using the integral of the cumulative responses along the incubation (see answer to general comment 1) helps to obtain a global response of the obtained results and reinforce the author’s findings from the previous version. The sentence has been corrected taking into account the referee´s comment (Line 377)

- 23. L. 396-397. “having…production rates” is not correct English usage.

The sentence has been corrected (Line 402).

- 24. L. 398-400. Please see my previous comment about lability.

Please, see answer to specific comment above (Question #2).

- 25. L. 432. Do the authors imply that their water samples collected on June 27th were "cold"? Maybe there was a strong upwelling on that day but this information is not provided and ca. 15_C is not exactly cold.

No, we were not referring to the temperature of the natural waters but to the possibility of having active extracellular enzymes coming from the first incubation after freezing the sample, as happened in the reference cited in the manuscript (i.e. Steen & Arnosti 2011). The sentence has been modified for improving the understanding on this issue (Line 452).

- 26. L. 454. Respiration rate and organic matter are not independent.

The text has been modified taking into account the referee´s point (Lines 470-472).

- 27. L. 455. These results are far from "demonstrating" that claim.

The sentence has been deleted.

- 28. I am not conviced that "acidified organic matter" and "non acidified organic matter" are the best terms for their treatments, did they check that the resultant DOM was of lower pH in the former treatment? Fig. 5B simply shows that the sample water had a lower pH but not that the DOM was indeed of lower pH.

The text has been changed throughout all the manuscript to clarify that the pH was measured in the water sample that contains the organic matter and to avoid possible misunderstandings related to the DOM pH. We have also explained in the new text that the treatment tittles (i.e. "acidified organic matter" and "non acidified organic matter") are only a brief way of naming the treatments using the key words for each experimental factor, but they do not imply that the DOM was acidic after bubbling the sample with 1000 ppm CO_2. (Lines 140-144)

- 29. µ_M is not the appropriate SI unit, please correct it to _mol L-1.

Axis nomenclature has been corrected in the text and in figures 3-B and 6.

- 30. Fig. 4. Please replace the "P" in the Y-axes by TOC, POC and DOC. This is not a very relevant figure and can be eliminated or moved to supplementary information.

Axis nomenclature has been corrected in figure 4. Following the referee´s suggestion the figure has been moved to supplementary material (Appendix 1).

- 31. The authors use total abundance of bacteria but probably data about the contribution of low and high nucleic acid content (LNA and HNA) cells are available, as well as some indication of changes in cell size that would provide a good estimate of bacterial biomass that could be compared with changes in BP, even if they were assuming data from the literature to convert from leucine incorporation rates to carbon units.

Unfortunately we are not able to provide the information requested by the referee since Dr. Luis Lubian, that was the coauthor in charge of the flow cytometry results, passed away recently and did not leave any data related to the information requested by the referee.

- 32. Dubbing cells able to reduce CTC as "viable" is not the best term. Most authors, including the cited references, refer to them as cells actively respiring or showing active respiration but the number of viable cells in their incubations was likely much higher, just by comparing the cell abundance numbers of Figs. 6 and 8. It is uncommon to show CTC positive cells before total bacterial abundance. Also, why not showing the dynamics of CTC positive bacteria for the entire experiment rather than only at day 7?

The term "viable", when naming the cells that were able to reduce CTC, has been replaced by cells that were actively respiring, as proposed by the referee.

The reason why the CTC positive bacteria test was only performed at day 7 is that this analysis was not included in the preliminary experimental planning. It was proposed after seeing the significant respiration responses at the beginning of the incubation.

Regarding the reason for showing the CTC results before the bacterial abundance is that they were more closely related to the respiration rates, which at the same time, were related to the pH and CO_2 responses, normally shown at the beginning of the Results section.

- 33. Fig. 9B. BGE is given either as a percentage or as a ratio, what does “r.u.” mean? Also, given the use of a very high and constant leucine-to-carbon conversion factor of 3.1 kg C mol Leu-1, if the values are close to 80% BGE, they are extremely high, very difficult to reconcile with almost no net uptake of DOC (Fig. 7).

The units in the axis titles have been corrected in both, figure 9-A and 9-B.

Since no net uptake can be quantified based on the experimental design of our study (please see answer to question #2) we cannot really correlate DOM results to BGE. BGE values shown in our study are similar and within the range of the values obtained from previous studies carried out with samples of the Ría de Vigo with additions of organic matter (Martínez-García et al. 2010).

A NEW VERSION OF THE MANUSCRIPT, INCLUDING ALL THE SUGGESTIONS RAISED BY THE REVIEWER, IS INCLUDED AT THE END OF THE REFEREE´S RESPONSE AS A POSSIBLE NEW VERSION OF THE MANUSCRIPT.

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EFFECTS OF ELEVATED CO_2 AND PHYTOPLANKTON-DERIVED ORGANIC MATTER ON THE METABOLISM OF BACTERIAL COMMUNITIES FROM COASTAL WATERS

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Running head: Ocean acidification and DOM on bacteria

Abstract. Microcosm experiments to assess bacterioplankton response to phytoplankton-derived organic matter obtained under current and future-ocean CO_2 levels were performed. Surface seawater enriched with inorganic nutrients was bubbled for 8 days with air (current CO_2 scenario) or with a 1000 ppm CO_2–air mixture (future CO_2 scenario) under solar radiation. The organic matter produced under the current and future CO_2 scenarios was subsequently used as inoculum. Triplicate 12 L flasks filled

with 1.2 µm-filtered natural seawater enriched with the organic matter inocula were incubated in the dark for 8 days under CO_2 conditions simulating current and future CO_2 scenarios to study the bacterial response. The acidification of the media increased bacterial respiration at the beginning of the experiment while the addition of the organic matter produced under future levels of CO_2 was related to changes in bacterial production and abundance. This resulted in 67% increase in the integrated bacterial respiration under future CO_2 conditions compared to present CO_2 conditions and 41% higher integrated bacterial abundance with the addition of the acidified organic matter compared to samples with the addition of non acidified organic matter. This study demonstrates that the increase in atmospheric CO_2 levels can affect bacterioplankton directly by changes in the respiration rate and indirectly by changes on the organic matter with concomitant effects on bacterial production and abundance.

KEY WORDS: bacterioplankton, phytoplankton, organic matter, ocean acidification.

Eliminado: The balance between both, respiration and production, made that the bacteria grown under future CO_2 levels with an addition of non-acidified organic matter showed the best growth efficiency at the end of the incubation. However, cells grown under future scenarios with high CO_2 levels and acidified organic matter additions did not perform differently than those grown under present CO_2 conditions, independently of the addition of acidified or non-acidified organic matter.

1 Introduction:

The increase in fossil fuel burning, cement production and deforestation together with changes in land use have resulted in an accumulation of atmospheric CO_2 at levels never seen before the last two million years (Caldeira & Wickettt 2008, Le Quere et al. 2015). Atmospheric gases can freely diffuse into the ocean surface, which has already absorbed about 30% of the emitted anthropogenic CO_2, perturbing the carbonate system and decreasing ocean pH in a process known as ocean acidification (Sabine et al. 2004, Burke et al. 2014).

The latest IPCC report shows that the pH of surface ocean waters has already decreased by 0.1, corresponding to a 26% increase in acidity. If mitigation strategies for global change are not adopted and CO_2 emissions continue as usual, ocean pH values will drop about 0.3-0.7 units by the end of the 21st century (Burke et al. 2014). The decrease in seawater pH has strong effects on the ecosystem, the aquatic organisms and the interactions among them. Studies about its consequences in the surface ocean have been primarily focused on calcifying organisms such as corals or coralline algae because they participate in the formation of habitats and human services (Langdon et al. 2003, Fabry et al. 2008). Recent meta-analysis studies also revealed decreased survival, growth, development and abundance of a broad range of marine organisms, although the magnitude of these responses varies between taxonomic groups, including variation within similar species (Kroeker et al. 2013). Additionally, other authors have demonstrated that ocean acidification can increase growth, primary production and N_2 fixation rates in some phytoplankton species (Barcelos e Ramos et al. 2007, Fu et al. 2007, Levitan et al. 2007, Iglesias-Rodriguez et al. 2008). In contrast with the abundant information about phytoplankton, very little is known about the response of bacterioplankton.

Heterotrophic bacteria play an important role in the planktonic community since they are responsible for the majority of the organic matter remineralization (Cole et al. 1988, Azam et al. 1998, Nagata et al. 2000) allowing the primary producers to make use of the recycled inorganic nutrients. They also return dissolved organic carbon (DOC) to the marine food web via its incorporation into bacterial biomass through what it is called the microbial loop (Azam et al. 1983). However, the microbial response can change depending on phytoplankton taxonomic composition and the nutrient levels, and therefore productivity, of the water (Teira et al. 2012, Bunse et al. 2016, Sala et al. 2016). Despite this important role of bacterioplankton in the marine food web and biogeochemical cycles, only few studies have been designed to elucidate the effects of ocean acidification on bacteria metabolism or its interaction with the abiotic (i.e. temperature, ultraviolet radiation, mixing, etc.) and biotic factors (i.e. algal derived organic matter, microbial community composition, trophic interactions, etc.). Some of these studies suggest an absence of significant metabolic responses in experiments where CO_2 levels were manipulated (Rochelle-Newall et al. 2004, Allgaier et al. 2008, Newbold et al. 2012). Fast acclimation of the metabolic machinery to low pH values might have occurred in those experiments since occasionally bacteria and other heterotrophic organisms, suffer lower pH values than those predicted by ocean acidification scenarios (Joint et al. 2011). On the contrary, other authors have reported that a decrease in seawater pH can potentially influence bacterial metabolism by changes in bacterial production and growth rates in natural communities, although the results show different responses depending on the study (Coffin et al. 2004, Grossart et al. 2006, Motegi et al. 2013, Spilling et al. 2016). More recently, results from a phytoplankton bloom mesocosm study have demonstrated, through metatranscriptome

analysis, that acidification can enhance the expression of genes encoding proton pumps to maintain homeostasis under high CO_2 conditions (Bunse et al. 2016).

An interesting point is that the experimental design in most of the published CO_2 studies did not allow distinguishing between direct effects on the bacteria *per se* and indirect effects, due for example to changes in phytoplankton community composition or to changes in organic matter. Therefore, indirect pathways such as those affecting the availability of organic matter in terms of quantity, because of an increase in phytoplankton primary production (Hein & Sand-Jensen 1997, Riebesell et al. 2007), or in terms of quality, because of changes in the composition of phytoplankton-derived organic matter (Engel et al. 2014), should be also studied to determine the effect of ocean acidification on bacterioplankton. For example, recent results have demonstrated that pH values predicted for future ocean acidification scenarios were close to the optimum values for extracellular enzyme activity (Grossart et al. 2006, Piontek et al. 2010, Yamada & Suzumura 2010). Higher enzymatic activity resulted in a higher rate of organic matter transformation and increases in bacteria performance. It has been also demonstrated that decreases in pH might increase the rate of extracellular dissolved organic carbon production from phytoplankton (DOCp) and the formation of transparent exopolymer particles (TEPs) (Engel 2002, Engel et al. 2004), although the contrary has also been observed and there is not a clear response to this matter (Sobrino et al. 2014). In addition, a higher CO_2 concentration in seawater could modify the C:N:P ratios of particulate organic matter, which may substantially affect the activity of bacteria and the carbon fluxes in the future ocean (Riebesell et al. 2007, Engel et al. 2014).

The main goal of our study was to investigate the direct and indirect effects of ocean acidification on the interaction between phytoplankton derived organic matter and bacterial metabolism. We analyzed

Eliminado: Environmental drivers such as the ultraviolet radiation (UV: 280-400 nm) should be also taken into account when studying the biological and chemical responses of the planktonic assemblages and the environment, since it plays a crucial role on the physiology of plankton communities and on the ecology of the aquatic ecosystems. UVR induces photomineralization of coloured dissolved organic matter (CDOM) increasing the biological availability of the resulting DOM (Moran & Zepp 1997). UV radiation can also increase DOCp production in surface waters (Carrillo et al. 2002, Helbling et al. 2013, Fuentes-Lema et al. 2015).¶

the changes in bacterial abundance, production and respiration in a coastal plankton community from an upwelling system.

Eliminado: and viability

Eliminado: subjected to current and future CO_2 concentrations

2 Materials and Methods:

2.1 Experimental Setup

The experiment to determine the response of bacterioplankton communities to phytoplankton-derived organic matter produced under current and future CO_2 scenarios were performed in two phases at the Toralla Marine Science Station, from now on ECIMAT (Estación de Ciencias Mariñas de Toralla, University of Vigo (ecimat.uvigo.es)) (Fig. 1). The first phase consisted in 8 days incubation under full solar radiation (UV radiation included) of natural phytoplankton communities enriched with inorganic nutrients under current and future CO_2 conditions. In the second phase, the organic matter obtained from the previous incubation was added to a natural bacterioplankton community to assess the interactions between organic matter amendments and acidification on bacterial metabolism for 8 days (Fig. 2).

Water from 5 m depth was collected with a Niskin bottle on board of the R/V Mytilus from a fixed central station at the Ría de Vigo (42.23 N; Long: 8.78 W. Fig. 1) the 27th of June 2013 and immediately transported to the ECIMAT (approx. 30 min). The Ría de Vigo is a highly productive and dynamic embayment located in the Northwestern Iberian Peninsula, characterized by the intermittent upwelling of cold and inorganic nutrient-rich Eastern North Atlantic Central water (Fraga 1981). Upwelled water in the Iberian system also brings high CO_2 concentrations, so annual pCO_2 can oscillate between maximum values of 750 ppm CO_2 during the upwelling events and minimum values of 270

ppm CO_2 during the downwelling season (Alvarez et al. 1999, Gago et al. 2003). The sample was pre-screened using a 200 µm sieve to avoid zooplankton grazing and distributed in six, aged and acid washed, UVR transparent 20 L cubitainers (NalgeneR I-Chem Certified Series™ 300 LDPE Cubitainers). The cubitainers were submerged in two 1500 L tanks located outdoors in an open area free of shadows. The tanks were connected to a continuous seawater pumping system and covered by a neutral density screen (75% Transmittance) to assure cooling at the *in situ* temperature and to avoid photoinhibition by the highly damaging summer irradiances under the static conditions of the cubitainers, respectively. Nitrate, ammonium and phosphate were added the first and 5th day of experiment to maintain saturating nutrient conditions (5 µmol L^{-1} nitrate (NO_3^-), 5 µmol L^{-1} ammonium (NH_4^+) and 1 µmol L^{-1} phosphate (HPO_4^{-2}) final concentrations). Triplicate samples were bubbled with regular atmospheric air (Low Carbon treatment: LC, aprox. 380 ppm CO_2) or with a mixture of the atmospheric air and CO_2 from a gas tank (Air Liquide Spain) (High Carbon treatment: HC, 1000 ppm CO_2). At the end of the incubation, the samples grown under present and future levels of CO_2 were stored frozen at -20 ˚C until the start of the second phase, ten days later, to be used as a naturally-derived organic matter inocula. The inocula included both, dissolved and particulate organic matter, as observed in nature, but since bacteria preferentially use the labile dissolved organic matter pool for growth (Nagata et al. 2000, Lechtenfeld et al. 2015) we focused our measurements of the organic matter on the dissolved fraction.

For the second phase, water collection was similar to the previous one. Once at ECIMAT, seawater was filtered through 1.2 µm pore size glass fibber filters (GF/C Whatman Filters) to separate the bacterioplankton community from the other plankton cells. The bacterioplankton sample was distributed

Eliminado: , mainly diatoms for this time of the year (Tilstone et al. 2000, Fuentes-Lema & Sobrino 2010)

in 12 acid washed polycarbonate NALGENE 12 L bottles together with the phytoplankton derived organic matter inoculum and 0.2 µm filtered seawater (FSW) following a 3:1:6 bacterioplankton: organic matter: FSW volume ratio, respectively. This proportion aimed to reach 10 µmol L^{-1} of organic carbon, which represent around 25-30% of the mean excess of organic carbon observed in the surface layer of the middle Ría de Vigo as compared with the bottom waters (Doval et al. 1997, Nieto-Cid et al. 2005). Half of the bottles were inoculated with the organic matter produced under current CO_2 conditions in the previous incubation (from herein named as Non acidified Organic matter, NO, n=6) and the other half with the organic matter produced under future CO_2 conditions by acidifying the media as explained above (from herein named as Acidified Organic matter, AO, n=6). In each case, three replicates were aerated with ambient CO_2 levels (Low Carbon treatment (LC): LC_NO, LC_AO) or air with 1000 ppm CO_2 (High Carbon treatment (HC): HC_NO, HC_AO). This experimental setup produced 4 different treatments from the less modified sample (LC_NO) to the most altered sample (HC_AO) (Fig. 2). The bottles were located in a walk-in growth chamber under dark conditions at 15 ºC, similar to the *in situ* temperature. The dark incubation for the bacterioplankton samples was aimed to focus the experiment on the two factors of the study, CO_2 and organic matter addition, avoiding potential effects of solar radiation on bacterioplankton and organic matter that might complicate the interpretation of the results.

2.2 DIC, pH and CO_2 analysis

Triplicate 30 mL samples were filtered daily through 0.2 µm size pore nitrocellulose filters. The filtrate was encapsulated without air bubbles in 10 mL serum vials and stored at 4 °C and dark conditions until

analysis immediately after the incubation ended. Dissolved Inorganic Carbon (DIC) analysis was carried out through acidification with 10% HCl using a N_2 bubbler connected to an infrared gas analyzer (LICOR 7000) and calibration was performed using a Na_2CO_3 standard curve. pH and temperature were daily measured with a Crison pH 25 pH meter and salinity with a thermosalinometer Pioneer 30. The pH meter was calibrated to the total hydrogen ion concentration pH scale with a 2-amino-2-hydroxymethyl-1,3-propanediol (tris) buffer prepared in synthetic seawater (Dickson & Goyet 1994). The partial pressure of CO_2 (pCO_2) in the samples was calculated from salinity, temperature, pH and DIC using the software csys.m from Zeebe & Wolf-Gladrow (2001).

2.3 Chlorophyll a concentration

Seawater samples for Chl *a* analysis were taken every day during the first incubation. A volume of 150 mL from each cubitainer was gently filtered through GF/F filters under dim light and immediately stored at -20 ˚C until further analysis. For Chl *a* extraction, the filters were kept at 4 °C overnight in acetone 90%. Chl *a* concentration was estimated with a Turner Design Fluorometer TD-700 and a pure Chl *a* standard solution was used for calibration.

2.4 Bacterial production and respiration

Bacterial production (BP) was measured following the [^{3}H] leucine incorporation method (Smith & Azam 1992). Three replicates and 1 killed control were sampled (1 mL) from each experimental unit on days 0, 1, 2, 3, 4 and 7 of the second incubation. Samples were spiked with 40 µL Leucine (47 µCi mL^{-1} specific activity stock solution), and incubated for 80 min in the same chamber and growth conditions

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2.4 Primary production¶

Incubations were performed at noon and lasted 3 to 3.5 hours. Fifteen mL samples of each microcosm were inoculated with $H^{14}CO_3^-$ (approximately 1 µCi mL^{-1} final concentration) and incubated in UVR transparent Teflon-FEP bottles under full solar radiation exposures in a refrigerated tank contiguous to the experimental microcosms. The teflon bottles were tied on top of a UVR transparent acrylic tray, keeping all bottles under flat and constant position. Tray was wrapped with 2 layers of neutral density screen to obtain saturating but non-photoinhibitory solar exposures. For analysis of the fraction of the fixed carbon incorporated into particulate (POC) and dissolved (DOC) organic carbon, 5 mL samples were filtered through 0.2 µm PC filters (25 mm diameter) under low pressure (50 mm Hg) after the light incubation period, using 2 manifolds simultaneously (10 positions per manifold). POC was retained on the filter while the filtrate was directly collected in scintillation vials to assess ^{14}C activity in the dissolved fraction (DOC). Simultaneously, the total amount of organic carbon incorporated in the cells (TOC) was measured independently of the DOC-POC filtration by processing 5 mL of the incubated samples. Non-assimilated ^{14}C was released by exposing the filters (POC) to acid fumes (50% HCl) or by adding 200 µl of 10% HCl to the liquid samples (DOC & TOC, respectively) and shaking overnight. The radioactivity of each sample was measured using a Wallac WinSpectral 1414 scintillation counter (EG&G Company, Finland). There was no significant difference between measurements of TOC compared to the sum of the particulate and dissolved fractions. Data analysis of the Log-Log relationship between TOC and POC+DOC determined that both results were significant correlated (y= 1.05x (±0.07) + 0.05 (±0.03), R^2=0.85, ANOVA, p=0.637, n=34).¶

than the bacterioplankton assemblages. Processed samples were analyzed in a Wallac Win-Spectral 1414 scintillation counter and the BP was calculated from the Leucine uptake rates employing a theoretical leucine to carbon conversion factor of 3.1 kg C mol Leu^{-1} (Teira et al. 2012). This conversion factor was obtained from natural bacterioplankton communities sampled at two different sampling sites of the Ría de Vigo, characterized by having different CO_2 concentrations (Ría and shelf) (Gago et al. 2003), and subjected to additions of organic and organic nutrients (Martínez et al. 2010). The inner station (Ría) is the same sampling site than the location chosen for our study. No significant differences in conversion factors were found between both sampling locations or between the control and the treatment with organic matter additions (Martínez et al. 2010).

Samples for bacterial respiration (BR) were taken on days 0, 2, 4 and 7 of the second incubation. BR in each sample was determined from the difference of the dissolved oxygen concentration consumed between the end and the start of a 24 h dark incubation using 50 mL Winkler bottles in duplicate for each incubation time and replicate (i.e. 12 Winkler bottles per treatment). The 24 h incubation was carried out at the same temperature than the experiment. Dissolved oxygen concentrations were determined by automated high-precision Winkler titration, using a potentiometric end point detector, Metrohm 721 DMS Titrino, as described in Serret et al. (1999). Bacterial carbon demand (BCD (µmol C L^{-1} d^{-1})) was calculated as the sum of BP and BR. Bacterial growth efficiency (BGE) was obtained from the proportion of the BCD that was used for bacterial production (BGE= BCD/BP).

2.5 Flow cytometry analysis

Eliminado: For phytoplankton (i.e. first incubation), samples were collected, fixed with P+G (1% paraformaldehyde + 0.05% glutaraldehyde) and analyzed with a FACScalibur flow cytometer (Becton–Dickinson). Measurements of the different photodetectors were made with a logarithmic amplification for each signal, and the trigger was set on red fluorescence (FL3). Phytoplankton counts were obtained at a high flow rate (1.05 µL s^{-1}) during 10 min. Two size groups of cells (R1 and R2) were discriminated on the bivariate plots of side light scatter (SSC) *vs.* FL3.

For bacterioplankton counts (i.e. second incubation), samples were stained with 2.5 µM of SYTO-13 (Molecular Probes) dissolved in dimethyl sulfoxide. The samples were incubated during 10 min at room temperature in the dark, followed by the addition of 10 µL of a microspheres solution (FluoSpheres carboxylated-modified microspheres (1.0 µm nominal diameter). ThermoFisher Scientific) as internal standard for instrument performance. Samples were then immediately analyzed. The threshold was set on the green fluorescence (FL1). Stained bacteria were discriminated and counted in a bivariate plot of SSC *vs.* FL1.

Viability of bacterioplankton community was measured on day 7 using the 5-Cyano-2,3,-ditolyl tetrazolium chloride (CTC) dye (Sieracki et al. 1999; Gasol and Arístegui 2007). The CTC can freely diffuse into the cells where it is reduced by healthy respiring bacteria, producing a precipitated colored red/orange formazan product. This product is detectable and quantifiable by flow cytometry (Rodriguez et al. 1992). Samples were stained with 45 mM of CTC during 60 minutes and then analyzed. Threshold was set on the FL3 and bacteria showing active respiration were counted in a bivariate plot of SSC *vs.* FL3.

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All data were acquired and analyzed with the software CellQuest (Becton– Dickinson) as Flow Cytometry Standard files.

2.6 DOC concentration

Dissolved organic carbon samples were taken in 250 mL acid-washed all-glass flasks and were gently filtered through acid rinsed 0.2 µm Pall-Supor filters. All this process was done in an acid-cleaned all-glass device under low N_2 flow pressure. About 10 mL of the filtrates were distributed in pre-combusted

(450 ˚C for 24 h) glass ampoules acidified with 50 µL of 25% H_3PO_4. The ampoules were heat sealed immediately and stored at 4 ˚C until analysis with a Shimadzu TOC-VCS analyzer following the high temperature catalytic oxidation method (Álvarez-Salgado & Miller 1998).

2.7 Statistical analysis

Data followed a normal distribution and homoscedasticity, tested by Jarque-Bera test and Bartlett test, respectively. Thus, a parametric one-way ANOVA or two-way ANOVA statistical tests were used to determine differences between one or two experimental factors, respectively (i.e. CO_2 in the first incubation, CO_2 and organic matter addition in the second incubation). If the interaction between the two factors was significant a multiple comparison post-test (MCP-test) was carried out. The confidence level was established at the 95%. Statistical analysis was performed using the software package MatLab R2012b.

Eliminado: or *t*-Test were employed to determine differences among the mean of several or paired samples, respectively. In the case that data did not follow a normal distribution, a non-parametric repeated-measures ANOVA (RMANOVA) and a Tukey-Kramer multiple comparison post-test were chosen. The Wilcoxon signed rank test was used to compare non-parametric paired samples.

3 Results:

During the first incubation, aimed to obtain the organic matter inocula under current and future CO_2 conditions, the LC treatment pCO_2 values were close to the atmospheric equilibrium, with values ranging between 419 ± 21 ppm CO_2 on day 0 and 226 ± 38 ppm CO_2 on day 3 (mean and SD, n=3) (Fig. 3A). In the HC treatment pCO_2 values increased since the beginning of the incubation until reaching values around 1200 ppm the last four days. Maximum values were observed at day 5 with 1227 ± 149 ppm CO_2. Chl *a* used as an indicator of the phytoplankton biomass showed similar trends in the two treatments. It increased with the initial addition of inorganic nutrients showing an early bloom

on day 1 with Chl *a* values of 21 ± 4 µg L^{-1} and 22 ± 9 µg L^{-1} for LC and HC treatments, respectively. Chl *a* concentration decreased after the bloom, keeping values close to the lowest concentrations on day 6 for the HC treatment with 3.0 ± 0.4 µg L^{-1} and on day 5 for the LC treatment, 3.1 ± 0.5 µg L^{-1} (Fig. 3A). Analysis of phytoplankton composition by flow cytometry did not show significant differences in community structure between LC and HC treatments (data not shown).

Primary production rates followed the Chl *a* pattern with a marked peak blooming the first incubation day followed by a decline afterwards in all the treatments (Figure 1 in Appendix 1). The increase in total carbon fixation during the bloom was due to an increase in both, POC and DOCp production, but it was mainly due to POC assimilation. The percentage of extracellular release of dissolved carbon (PER=DOCp/(POC+DOCp)) ranged between 18% and 77%. DOCp, POC, and therefore TOC, were higher during the bloom under the LC treatment but not significantly different than the rates observed in the HC treatment (1-way ANOVA, TOC: p=0.307, POC: p=0.242, DOCp: p=0.527, n=6). Differences in the production rates between both treatments became negligible after the second incubation day (Figure 1 in Appendix 1).

Bulk DOC concentration increased from day 0 to maximum values on day 7, and similar to Chl *a* concentration and production rates, there were not significant differences between the two CO_2 treatments at the end of the incubation (1-way ANOVA, p=0.096, n=6) (Fig. 3B). Parallel analysis of DOM fluorescence (*i.e.* protein-like and humic-like substances) also supported the later results (data not shown).

In the second incubation, aimed to assess the effects of CO_2 and the organic matter additions on bacterioplankton, pCO_2 and pH were similar within the same CO_2 treatment (*i.e.* LC_NO and LC_AO

Eliminado: a succession of two different phytoplankton populations along the incubation characterized by differences in cell size and Chl content (Data not shownTable 1). However, differences in the

Eliminado: both treatments, larger cells with higher Chl *a* fluorescence and cell complexity (Region 1, Table 4.2.1), dominated the phytoplankton community at the beginning of the incubation. The abundance of these large cells decreased, especially in the HC treatment, and at the end of the incubation smaller cells, with lower Chl content became more abundant (Region 2, Table 4.2.1). At day 7 the small size fraction population was dominant and the analyzed phytoplankton community was similar in both, HC and LC treatments.

vs. HC_NO and HC_AO), but significantly different between LC and HC treatments (Fig. 4A and B). pCO_2 in the LC ranged between 350 ± 28 ppm and 568 ± 187 ppm CO_2 (mean and SD, n=3) on day 5 and 2, respectively. In contrast, HC treatments increased from 397 ± 18 ppm on day 0 to a maximum value of 2213 ± 229 ppm on day 3, significantly different that the expected 1000 ppm pCO_2 in the HC treatments. The maximum was followed by a pronounced decrease on day 4, and subsequently, the values were similar to the bubbled air concentrations (1011 ± 75 ppm on day 5) (Fig. 4A). As expected from the pCO_2, pH values in the LC treatments were fairly constant with a mean value of 8.07 but decreased markedly from 8.03 on day 0 to 7.51 on day 3 in the HC treatment. After this minimum, pH increased to values around 7.8 until the end of the experiment (Fig. 4B).

The changes in CO_2 concentration and pH were accompanied with an increase in BR in the HC treatments (Fig. 4C). BR was also fairly constant in the LC treatments, but showed a pronounced increase from day 0 to day 2 being significantly different in the HC treatments (2-way ANOVA, p=0.048, n=12). After reaching the maximum, bacterial respiration dropped to similar values than those observed in the LC treatments (Fig. 4C). Among the LC treatments, the LC_NO treatment showed the lowest variability in the respiration rates and the highest values at the end of the incubation (1-way ANOVA and MCP-test, p<0.001, n=6). Flow cytometry analysis of the CTC-positive bacteria, which have been previously related to actively respiring bacteria, also showed that the LC_NO treatment had significantly higher values than the other treatments on day 7 (1-way ANOVA and MCP-test, p=0.007, n=6). In contrast, the other three treatments did not show significant differences among them (Fig. 5). Similar to pCO_2 and pH, statistical differences between samples with organic matter inocula produced under current and future CO_2 scenarios within each CO_2 treatment were not significant

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Eliminado: Similar to pCO_2 and pH, statistical differences between samples with inocula produced under current and future CO_2 scenarios within each CO_2 treatment were not significant regarding respiration rates.

regarding respiration rates. The analysis of the cumulative responses, measured as the integrated bacterial respiration during the whole incubation, showed 67% higher values under HC than under LC conditions (2-way ANOVA, p=0.010, n=6) (Fig. 4D).

The initial concentration of dissolved organic carbon (DOC, µmol C L^{-1}) in the samples was quite similar among the four treatments until the peak in respiration (Fig. 6). DOC before the addition of the organic matter inocula was 89 µmol C L^{-1} and increased in all the treatments to maximum values of 111 ± 5 µmol C $L^{-1}$and 117 ± n.d. µmol C L^{-1} in treatments LC_AO and HC_NO on day 3, respectively. Afterwards DOC in the LC treatments kept approximately constant but decreased 27% in the HC treatments compared to the LC treatments (81 ± 3 µmol C L^{-1} and 81 ± 2 µmol C L^{-1} in HC_NO and HC_AO, respectively). Statistical analysis showed significant differences between both pCO_2 treatments on day 7 (2-way ANOVA, p<0.001, n=12) (Fig. 6).

Unlike pCO_2, pH, respiration and DOC, bacterial abundance and production showed differences regarding the origin of the organic matter. The addition of the organic matter inocula produced a fast increase in production and abundance from day 0 to day 3 in all the treatments. Cell abundance increased from 6.2 ± 0.4 x 10^4 bacteria mL^{-1} before the addition of the organic matter inocula to a maximum of 9.0 ± 0.4 x 10^5 bacteria mL^1 in the HC_AO treatment (Fig. 7A). Differences for the bacterial abundance started to be significant in day 1 (1-way ANOVA and MCP-test, p=0.012, n=6) but they were more clearly observed during the maximum at day 3 (1-way ANOVA and MCP-test, p<0.001, n=6). Bacterial abundances were 29% and 31% higher in samples where the acidified organic matter was added than in those with the addition of organic matter produced under the current CO_2 scenario, in the LC and HC treatments, respectively (Fig. 7A). This resulted in 41% higher integrated

bacterial abundance with the addition of the acidified organic matter than in samples with the addition of non acidified organic matter (2-way ANOVA, p<0.001, n=12) (Fig. 7B).

Additionally, bacterial production increased from a minimum value of 1.1 ± 0.1 µg C L^{-1} d^{-1} on day 0 to maximum values on day 2 for the four treatments, ranging between 185 ± 37 µg C L^{-1} d^{-1} and 208 ± 4 µg C L^{-1} d^{-1} for HC_NO and LC_NO, respectively. However, the analysis of the integrated response did not show significant differences among treatments (Fig 7.D). Only punctually, the treatments with the addition of acidified organic matter (LC_AO and HC_AO) showed a higher decrease in the production rates than those with the addition of the non-acidified organic matter, resulting in significant differences later on (2-way ANOVA, p=0.001, n=12). On day 7 treatments LC_NO and HC_NO produced 53% and 45% more than treatments LC_AO and HC_AO, respectively (Fig. 7C).

The BCD was affected by the respiratory activity at the beginning of the incubation (day 1, 2-way ANOVA, p=0.041, n=12) and by the production at the end (day 7, 2-way ANOVA, p=0.002, n=12) (Fig 8A). In consequence, the BGE was higher for the LC treatments, at the beginning of the incubation during the activity peak, but decreased at the end. At day 7 the HC treatment with the addition of non-acidified organic matter (HC_NO) showed significantly higher efficiency than the other treatments (1-way ANOVA and MCP-test, p=0.002, n=6) (Fig 8C). Overall, integrated BCD resulted 24% higher under HC conditions while the BGE was 11% lower in the same conditions, compared to LC conditions (2-way ANOVA, p=0.011, p=0.019, respectively, n=6) (Fig 8B, D).

4 Discussion:

Eliminado: Conversely, the HC_AO treatment expected for future scenarios of global change did not show significant differences with neither of the LC treatments.¶

Eliminado: Except in the case of the bacterial respiration the significant differences founded in particular days were reinforced after analyzing the cumulative response of the two factors employed along the whole 2nd incubation. The integrated area of the bacterial abundance increased significantly a 40.5% with the addition of the acidified organic matter (2-way ANOVA, p<0.001, n=12). However, the integrated area of bacterial respiration BCD and BGE showed significant differences related to the pCO_2 being 66.6% and 23.6% higher in BR and BCD under the HC treatment

The main goal of the current study was to distinguish between the direct and indirect effects of ocean acidification on natural bacterial assemblages. To achieve this objective we performed a 2×2 experimental design combining the acidification of seawater and the addition of phytoplankton-derived organic matter produced under current and future CO_2 conditions and natural solar exposures. UVR induces photomineralization of coloured dissolved organic matter (CDOM) increasing the biological availability of the resulting DOM (Moran & Zepp 1997) and can also increase DOCp production in surface waters (Carrillo et al. 2002, Helbling et al. 2013, Fuentes-Lema et al. 2015).

Although there have been described different methodologies to modify the seawater pH to simulate an ocean acidification scenario, the continuous bubbling of the natural plankton communities with a target CO_2 concentration of 1000 ppm CO_2 was chosen in the present study to simulate the pCO_2 and pH conditions expected for the end of the century. This method simulates the natural variations of sea surface pCO_2 driven by differences between atmospheric and sea water CO_2 concentration providing quite realistic responses of the organisms to this environmental factor (Rost et al. 2008). It also allows that changes in the biological activity of the samples enable the modification of the pCO_2 values if, for example, the photosynthetic or respiratory rates become faster than the rates needed to achieve the CO_2 chemical equilibrium in seawater. Changes in pCO_2 values due to microbial activity are usually observed in natural waters during bloom events in surface waters or in areas with high amount of organic matter (Joint et al. 2011). pCO_2 also increases with depth due to the increase in heterotrophic activity compared to the autotrophic activity in surface (Pukate & Rim-Rukeh 2008, Dore et al. 2009) and can change due to upwelling events, for example in the same area where the samples were

Eliminado: This method ensures quite realistic responses in terms of acclimation rates

collected, reaching values close to those observed in the present work (Alvarez et al. 1999, Gago et al. 2003).

In our study, the CO_2 enrichment did not produce a significant effect on phytoplankton production or biomass, measured as ^{14}C incorporation or Chl *a* concentration, respectively. Phytoplankton community composition changed from bigger to smaller phytoplankton cells, as has been observed in similar microcosm studies (Reul et al. 2014, Grear et al. 2017), but differences between present and future CO_2 treatments were not observed. DOCp production increased during the bloom evolved at the beginning of the incubation but bulk DOC concentration showed similar values between CO_2 treatments, as expected based on the lack of differences observed in the biological and metabolic variables. Despite several studies indicate an increase in phytoplankton carbon production and biomass under future scenarios of CO_2 (Kroeker et al. 2013), in our study exposure of the cells to natural conditions including solar UVR might have counteracted the stimulatory effect of the high CO_2, since it increases the sensitivity of phytoplankton to photoinhibition (Sobrino et al. 2008, 2009, Gao et al. 2012).

The addition of the organic matter and the start of the aeration in the second incubation produced a burst in the metabolic activity of the bacteria. However this increase in bacterial activity was not followed by a significant decrease in DOC. Taking into account the experimental design of the study keeping both, the particulate and the dissolved fractions of the organic matter from the first phase incubation, it is likely that production of DOC exceeded its consumption by heterotrophic bacteria, leading to a net accumulation of DOC in the microcosms. The particulate fraction could potentially have been degraded during the second phase of the experiment due to the elevated CO_2 concentrations or the enzymatic activity (Piontek et al. 2010). Enzymatic activity on POC depends on the type of substrate and relates to

the metabolic capabilities of the microbial community but can act on the temporal interval of our experiment (Arnosti et al. 2005). Counterintuitively, the high metabolic activity observed after adding the organic matter might have also contributed to the net accumulation of DOC since it has been recently demonstrated that microheterotrophs can release part of the substrate as new DOM (Lønborg et al. 2009). Freezing the sample with the organic matter produced in the first incubation might have also contributed to the DOC production during the second incubation. Freezing was necessary to avoid an excessive consumption of the acidified and non acidified organic matter for later use, since DOC released by marine phytoplankton is highly labile to bacterial utilization and can be degraded significantly within hours (Chen & Wangersky 1996). However, results from Peacock et al. (2015) suggest that changes in DOC concentrations by freeze/thaw cycles in samples stored no longer than six months are less than 5% and do not show a clear pattern of increase or decrease (Peacock et al. 2015). Such changes are within the range of typical precision for DOC analysis and should not be significantly different among the experimental treatments since all the samples were processed in a similar way.

Eliminado: produce alterations in the concentration and spectroscopic properties of dissolved organic matter (DOM) (Thieme et al. 2016)

Eliminado: , nevertheless, the freezing process as preservative methodological approach

Bacterioplankton growing in the HC treatments showed higher rates of respiration the first two days. This response was opposite to that described for some bacterial cultures (Teira et al. 2012) and seems to be related to an acclimation to the new pH values, somehow similar to observed for phytoplankton (Sobrino et al. 2008). Consequently, pCO_2 in the HC treatments increased more than expected due to the biological activity carried out by bacterioplankton. The increase in respiration was also parallel to an increase in bacterial abundance and production. However, the results indicate that while changes in respiration were related to pCO_2 values, changes in bacterial abundance were mainly related to the origin of the organic matter amendments. The bacterial abundance was stimulated by the presence of

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505 organic matter from a phytoplankton community grown under high CO_2 conditions compared to the addition of organic matter values grown under current CO_2 conditions. On the other hand, differences in bacterial production were only significant at the end of the experiment (day 7).

Possible explanations for the preference of the organic matter produced under future CO_2 conditions by bacteria are changes in the composition and quality of this organic matter, changes in the capability of 510 bacteria to use this organic matter via extracellular enzymatic activity or a combination of both. Several mesocosm studies have also shown that the rate of extracellular release of phytoplankton dissolved organic matter production and the formation of transparent exopolymer particles (TEPs) is higher under high CO_2 conditions (Engel 2002, Liu et al. 2010, Endres et al. 2014). TEPs are mostly carbon rich, can be easily consumed by heterotrophic bacteria and can aggregate and coagulate in larger gel-like 515 structures that provide nutrients and attachment surface to bacteria, finally acting as hotspots where bacteria can grow and develop (Azam & Malfatti 2007). However, our results from the initial incubation aimed to obtain phytoplankton-derived organic matter inocula for bacteria do not suggest differences on DOC concentration under current and future CO_2 conditions (Fig. 3B), and neither composition, since phytoplankton community, productivity and fluorescence DOM properties were 520 similar in both treatments at the end of the experiment (Figure 1 in Appendix 1). Recent results from mesocosm experiments showing no significant differences in DOM concentration and composition between current and future CO_2 levels also corroborate these findings (Zark et al. 2015). Other than this, very little was found in the literature about the direct impact of ocean acidification on the DOM pool, particularly on its molecular composition and long-term reactivity.

525 Despite the significant effect on bacterial abundance during the activity peak, bacterial production only

showed significant differences among treatments later during the incubation, resulting in lower production rates in those bacteria inoculated with the organic matter produced under high CO_2 conditions than those observed in the bacteria inoculated with the organic matter produced under low CO_2 conditions. These results might be due to a higher bioavailability of the organic matter produced under high CO_2 conditions to bacteria earlier during the incubation, increasing bacterial abundance, and decreasing production later at the end of the experiment. The lower DOC values in the HC treatments at the end of the experiment compared to the LC treatments partially supports this contention in the HC_AO treatment. However, they mainly indicate a clear effect of the pH, independently of the origin of the organic matter inocula. The lower DOC in the HC treatments compared to the LC treatments at the end of the experiment could be an indirect consequence of the higher respiration rates, which lowered the pH values (i.e. pCO_2 values above the expected 1000 ppm) in these treatments during the activity peak. It has been recently described from metatranscriptome analyses that marine bacteria maintain pH homeostasis by enhancing the expression of genes encoding proton pumps, such as respiration complexes, proteorhodopsin and membrane transporters, that might affect the cellular energy demand (Bunse et al. 2016). Between the two incubation performed for this study, the significant effect of the pH on the DOC content might have been only observed in the second incubation because it reached almost double de pCO_2 than the first one (i.e. min pH= 7.48 for the bacterioplankton incubation *vs.* min pH= 7.71 for the phytoplankton incubation). Following this contention, if acidification increases the degradation rate and bioavailability of the organic matter, that would explain why respiration in the control treatment (LC_NO) was only significantly higher at the end of the experiment, where neither the organic carbon added nor the environment were modified by the acidification with CO_2. Flow

Eliminado: having

Eliminado: lower production rates

Eliminado: We hypothesized that low pH, directly or indirectly, could be responsible for a faster and more efficient degradation of the most recalcitrant organic carbon, also in the HC_NO, at the end of the experiment.

cytometry results confirmed the highest rates of respiration in the LC_NO treatment since the CTC dye is reduced to a quantifiable fluorescent CTC-formazan inside cells when the electron transport system is active (Sieracki et al. 1999, Gasol & Arístegui 2007).

Furthermore, previous studies have confirmed that many enzymes involved in the hydrolysis of organic matter are sensitive to pH variations. It has been reported that important enzymes for bacterial metabolism such as leucine aminopeptidase or the α- and β-glucosidase enhance their activity and transformation rates with small decreases in pH, similar to the values measured in this study (Grossart et al. 2006, Piontek et al. 2010, 2013). The effects of ocean acidification on enzyme activity is not constant and could vary depending on enzyme type and geographical location (Yamada & Suzumura 2010). Despite our results show that the increase in bacterial abundance is mainly related to the presence of organic matter produced under future CO_2 conditions and not so much to the decrease in pH, a trend that supports a synergistic effect of both, acidified organic matter and pH, can be observed. This trend shows the highest effects under the most extreme HC_AO treatment and the lowest effects under the less aggressive LC_NO treatment (i.e. for bacterial abundance the 3rd day of incubation and partially for production and viability on day 7). It can be also possible that enhanced activity of the extracellular enzymes produced by the microbial community under HC conditions during the first incubation, aimed to obtain the organic matter inocula, came with the frozen acidified organic matter since there is evidence that β-glucosidase, leucine aminopeptidase, and phosphatase enzymes are stable in cold waters for weeks (Steen & Arnosti 2011).

The uncoupling between pH and organic matter effects on bacterial respiration and production made that the carbon demand was biased by the most significant effect along the incubation. Nonetheless,

Eliminado: ¶
the high metabolic activity observed after adding the organic matter might have also contributed to the net accumulation of DOC since it has been recently demonstrated that microheterotrophs can release part of the substrate as new DOM (Lønborg et al. 2009).¶
¶
most bacteria maintain an intracellular pH between 7.4 to 7.8 (Padan et al. 2005), which might result in energy savings when extracellular pH approaches to the predicted values for future scenarios of ocean acidification. Additionally, results from bacteria cultures evidenced that the response to elevated CO2 depends on the species, stimulating the high CO2 concentrations the growth efficiency in Flavobacteria but without significant effects on Rhodobacteria (Teira et al. 2012).¶
¶

Eliminado: Therefore the least perturbed treatment with cells grown under present CO_2 conditions and non acidified organic mater (i.e. LC_NO) showed the highest growth efficiency after the organic matter addition, during the activity peak, because of the lack of pH changes and lower respiration rates. However, after 7 days an intermediate treatment with high CO_2 levels but non-acidified organic matter addition (i.e. HC_NO) had the highest growth efficiency due to the higher production rates and the relatively lower respiration rates compared to the treatment grown under current conditions but addition of non-acidified organic matter (LC_NO). Cells grown under future scenarios with high CO_2 levels and acidified organic matter additions did not perform differently than those grown under present CO_2 conditions, independently of the addition of acidified or non-acidified organic matter. BGE and bacterial abundance did agree at the end of the experiment showing the HC_NO treatment the highest cell abundance, although without significant differences among treatments.

further studies are needed to disentangle the lack of agreement among bacterial production, abundance and growth efficiency, more specially during the peaks of activity since they seem to show the biggest uncoupling between the measured parameters (del Giorgio & Cole 1998). Lack of total agreement regarding the statistical analysis might be related to differences in methodological sensitivity and variability. Moreover, leucine (or thymidine) to carbon ratios can change dramatically in different environmental conditions (Teira et al. 2015) and bacteria cultures under controlled conditions in the lab have shown that CO_2 fixation can increase under high carbon conditions, representing 8 to 9% of the bacterial production (Teira et al. 2012). All these factors can affect the bacterial production calculation. Despite our work used a conversion factor that had being empirically tested for natural samples collected from the same area without significant differences between stations with different CO_2 concentrations (Martínez-García et al. 2010), other factors such as the bacterial composition or the nature of organic matter additions might be affecting the final production rates. An attempt to empirically estimate the leucine-to-carbon conversion factors should be addressed in order to decrease uncertainties related to the bacterial production and the BCD and finally enhance our understanding in this topic.

In summary, the results from this investigation show that ocean acidification can significantly affect bacterioplankton metabolism directly by changes in the respiration rate and indirectly on bacterial abundance by changes in the organic matter.

Eliminado: with concomitant effects on bacterial production and abundance

Eliminado: They demonstrate imply that future scenarios of global change, with higher acidification might not result in a higher turnover of organic matter by bacteria.

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6 Figure legends:

Figure 1. Geographical location of Ría de Vigo in the NW Iberian Peninsula. The insert shows a more detailed map of the Ría de Vigo and the locations of A) ECIMAT and B) sampling station.

Figure 2. Experimental design of the study.

Figure 3. A) Phytoplankton biomass measured as Chl *a* concentration and pCO_2 evolution along the first incubation period aimed to obtain the organic matter inocula under current and future CO_2 conditions. Black and striped vertical bars correspond to the Chl *a* mean ± SD (n=3) (µg L^{-1}) obtained under high and low carbon (HC and LC) treatments, respectively. Black and grey circles correspond to the pCO_2 mean ± SD (n=3) (ppm CO_2) in HC and LC treatments, respectively. B) Temporal evolution of the dissolved organic carbon (DOC) concentration (µmol C L^{-1})) during this first incubation. The black and grey dots indicate the mean ± SD (n=3) of DOC from HC and LC treatments, respectively.

Figure 4. A) pCO_2 (ppm CO_2) and B) pH values in the four treatments of the second incubation period, respectively. Mean ± SD (n=3). C) Temporal evolution of bacterial respiration (µmol O_2 d^{-1}) and D) cumulative bacterial respiration (µmol O_2) during the second incubation period in the four treatments, mean ± SD (n=3). In all figures, black and grey triangles represent HC_NO and HC_AO treatments, respectively. Black and grey circles correspond to LC_NO and LC_AO treatments. The asterisk indicate

significant differences between LC *vs.* HC treatments with p-value < 0.05. The three asterisks indicate a significant difference between LC_NO with respect to the other treatments with p-value < 0.001.

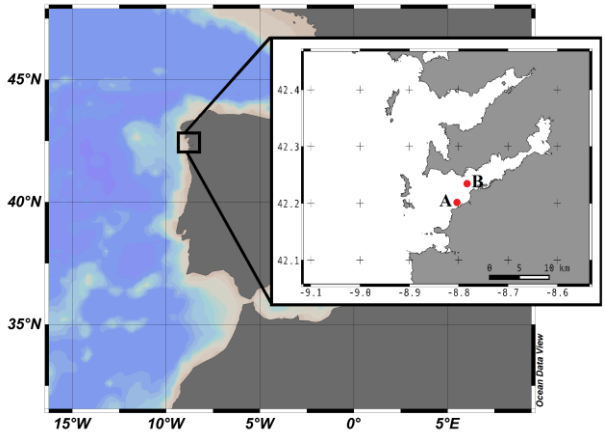
Figure 5. Bacterial viability (Active bacterial mL^{-1}) measured with the CTC dye on day 7, mean ± SD (n=3). The two asterisks indicate mean significant difference with the other treatments with p-value < 0.01.

Figure 6. Temporal evolution of the dissolved organic carbon (DOC) concentration (µmol C L^{-1}) during the second incubation period, mean ± SD (n=3). Black and grey triangles represent HC_NO and HC_AO treatments, respectively. Black and grey circles correspond to LC_NO and LC_AO treatments, respectively. The three asterisks indicate significant differences between LC *vs.* HC treatments with p-value < 0.001.

Figure 7. A) Histogram of bacterial abundance evolution (Cells mL^{-1}), treatments with different letter indicate significant differences (p-value < 0.01), and B) cumulative response of bacterial abundance (Cells mL^{-1}) in the four treatments during the course of the second incubation period, mean ± SD (n=3).C) Bacterial production (µg C L^{-1} d^{-1}) and D) cumulative bacterial production (µg C L^{-1}) from the four treatments during the second incubation period, mean ± SD (n=3). The two asterisks indicate significant differences between NO *vs.* AO treatments with p-value < 0.01. Black and grey triangles represent HC_NO and HC_AO treatments, respectively. Black and grey circles correspond to LC_NO and LC_AO treatments, respectively.

Figure 8. Temporal evolution of A) bacterial carbon demand (BCD (µmol C L^{-1} d^{-1})), B) cumulative BCD (µmol C L^{-1}), C) bacterial growth efficiency (BGE) and D) cumulative BGE during the second incubation calculated from the bacterial production and respiration rates obtained from this study. One asterisk indicate significant differences with p-value < 0.05 between HC *vs.* LC treatments. Two asterisks indicate significant differences with p-value < 0.01 between NO *vs* AO treatments, for the BCD and between HC_NO *vs.* the rest of the treatments (LC_NO, LC_AO and HC_AO) for the BGE. Black and grey triangles represent HC_NO and HC_AO treatments, respectively. Black and grey circles correspond to LC_NO and LC_AO treatments, respectively.

FIGURE 1:


45°N
40°N
35°N
15°W
10°W
5°W
0°
5°E
42.4
42.3
42.2
42.1
-9.1
-9.0
-8.9
-8.8
-8.7
-8.6
B
A
0 5 10 km
Ocean Data View

890

FIGURE 2:

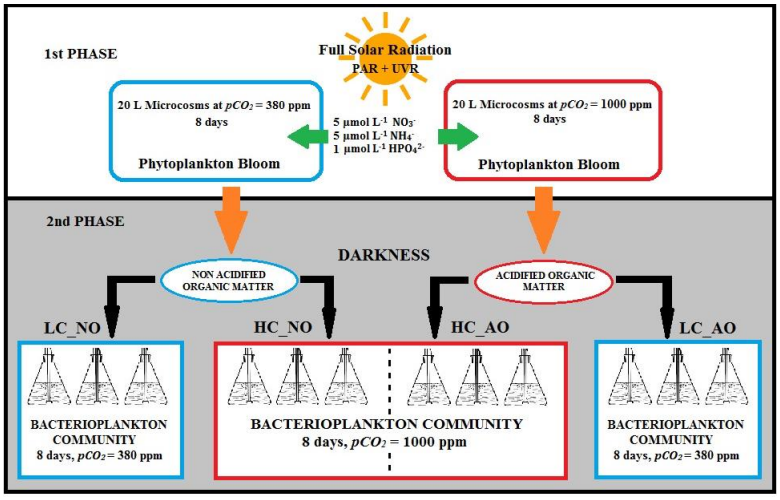

1st PHASE
Full Solar Radiation
PAR + UVR
20 L Microcosms at pCO_2 = 380 ppm
8 days
Phytoplankton Bloom
5 µmol L-1 NO3-
5 µmol L-1 NH4-
1 µmol L-1 HPO4 2-
20 L Microcosms at pCO_2 = 1000 ppm
8 days
Phytoplankton Bloom
2nd PHASE
DARKNESS
NON ACIDIFIED ORGANIC MATTER
ACIDIFIED ORGANIC MATTER
LC_NO
HC_NO
HC_AO
LC_AO
BACTERIOPLANKTON COMMUNITY
8 days, pCO_2 = 380 ppm
BACTERIOPLANKTON COMMUNITY
8 days, pCO_2 = 1000 ppm
BACTERIOPLANKTON COMMUNITY
8 days, pCO_2 = 380 ppm

FIGURE 3:

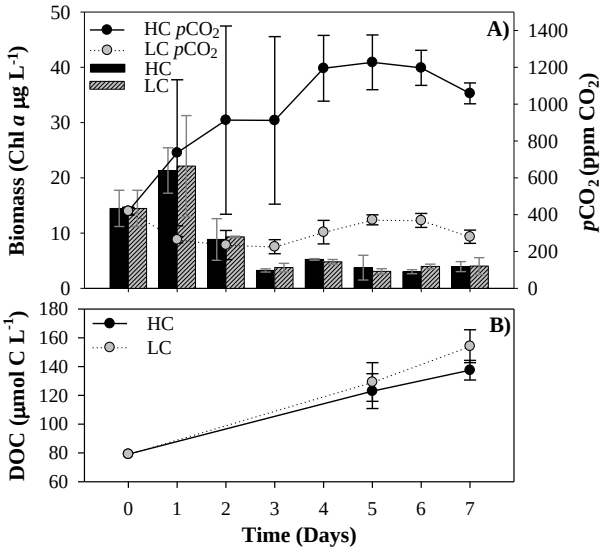

A)
HC pCO_2
LC pCO_2
HC
LC
Biomass (Chl *a* µg L^{-1})
pCO_2 (ppm CO_2)
B)
HC
LC
DOC (µmol C L^{-1})
Time (Days)

FIGURE 4:

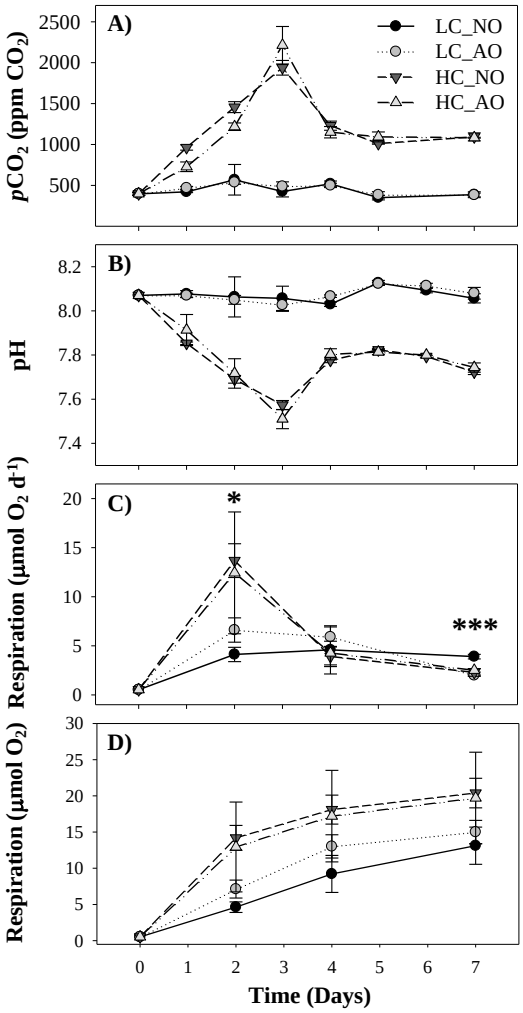

A)
LC_NO
LC_AO
HC_NO
HC_AO
pCO_2 (ppm CO_2)
2500
2000
1500
1000
500
B)
pH
8.2
8.0
7.8
7.6
7.4
C)
*
***
Respiration (µmol O_2 d^{-1})
20
15
10
5
0
D)
Respiration (µmol O_2)
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
Time (Days)

FIGURE 5:

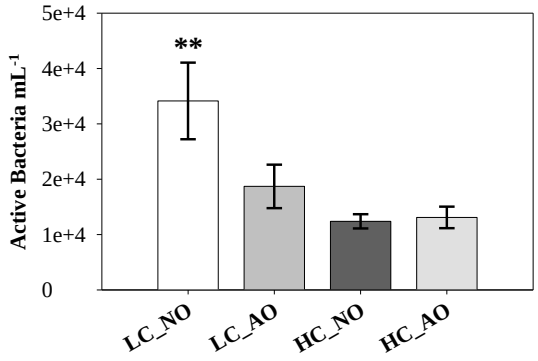


FIGURE 6:

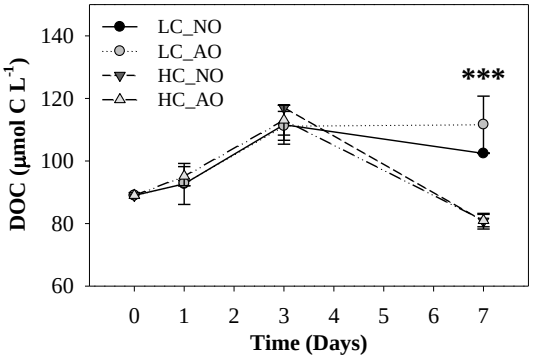

LC_NO
LC_AO
HC_NO
HC_AO
***
DOC (µmol C L-1)
140
120
100
80
60
0
1
2
3
4
5
6
7
Time (Days)

FIGURE 7:

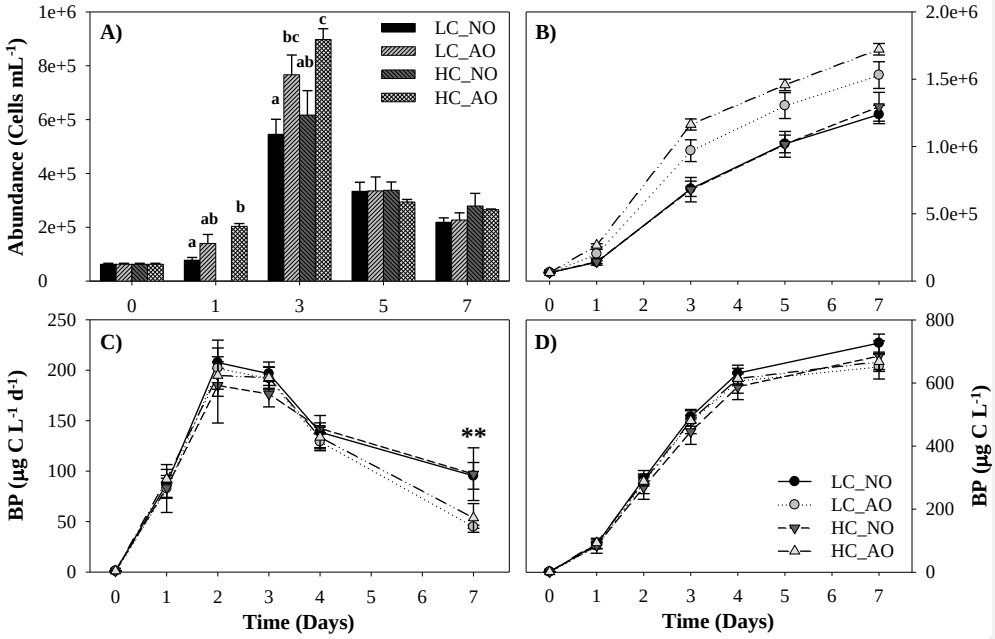
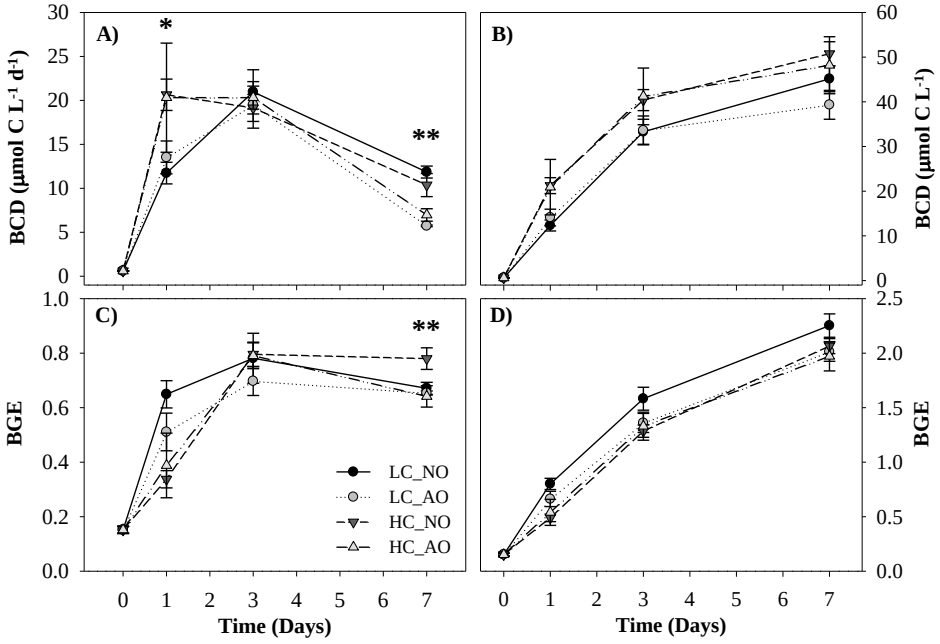

A)
B)
C)
D)
LC_NO
LC_AO
HC_NO
HC_AO
Abundance (Cells mL-1)
BP (µg C L-1 d-1)
BP (µg C L-1)
Time (Days)
Time (Days)
a
ab
b
a
bc
ab
c
**

FIGURE 8:

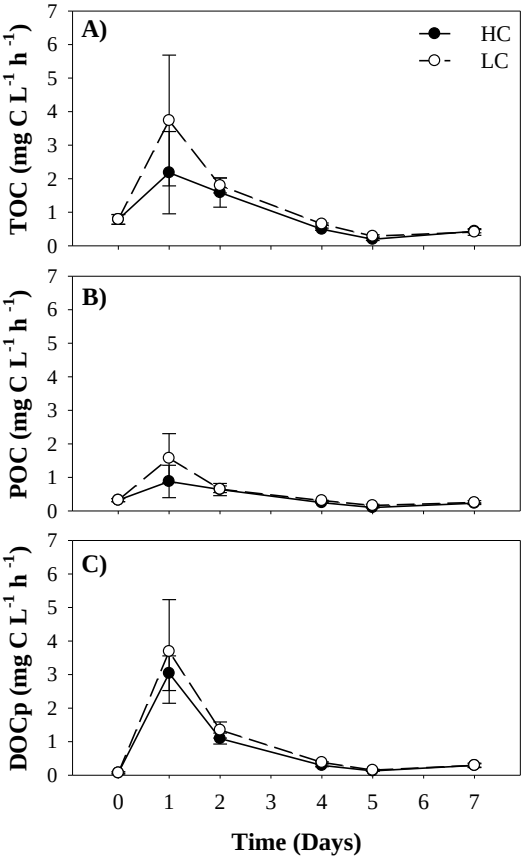

A)
B)
C)
D)
*
**
**
BCD (µmol C L-1 d-1)
BCD (µmol C L-1)
BGE
BGE
Time (Days)
Time (Days)
LC_NO
LC_AO
HC_NO
HC_AO

7. Appendix 1:

Primary production

Incubations were performed at noon and lasted 3 to 3.5 hours. Fifteen mL samples of each microcosm were inoculated with $H^{14}CO_3^-$ (approximately 1 µCi mL^{-1} final concentration) and incubated in UVR transparent Teflon-FEP bottles under full solar radiation exposures in a refrigerated tank contiguous to the experimental microcosms. The teflon bottles were tied on top of a UVR transparent acrylic tray, keeping all bottles under flat and constant position. Tray was wrapped with 2 layers of neutral density screen to obtain saturating but non-photoinhibitory solar exposures. For analysis of the fraction of the fixed carbon incorporated into particulate (POC) and dissolved (DOCp) organic carbon, 5 mL samples were filtered through 0.2 µm PC filters (25 mm diameter) under low pressure (50 mm Hg) after the light incubation period, using 2 manifolds simultaneously (10 positions per manifold). POC was retained on the filter while the filtrate was directly collected in scintillation vials to assess ^{14}C activity in the dissolved fraction (DOCp). Simultaneously, the total amount of organic carbon incorporated in the cells (TOC) was measured independently of the DOCp-POC filtration by processing 5 mL of the incubated samples. Non-assimilated ^{14}C was released by exposing the filters (POC) to acid fumes (50% HCl) or by adding 200 µl of 10% HCl to the liquid samples (DOCp & TOC, respectively) and shaking overnight. The radioactivity of each sample was measured using a Wallac WinSpectral 1414 scintillation counter (EG&G Company, Finland). Data analysis determined that both, TOC and POC+DOCp results, were significantly correlated ($y= 1.05x (\pm0.07) + 0.05 (\pm0.03)$, $R^2=0.85$, ANOVA, $p=0.637$, $n=34$).

Figure 1. Primary production rates measured as the incorporation of ^{14}C into organic compounds during the first incubation period aimed to obtain the organic matter inocula under current and future CO_2 conditions A) Total organic carbon fixation rates obtained from a different sample than the sample used for the determination of POC and DOCp fixation rates (TOC) B) Particulate organic carbon fixation rates (POC) C) Rates of dissolved organic carbon production from phytoplankton origin (DOCp). Mean ± SD (n=3).


A)
B)
C)
HC
LC
TOC (mg C L-1 h-1)
POC (mg C L-1 h-1)
DOCp (mg C L-1 h-1)
Time (Days)
0
1
2
3
4
5
6
7