Author comments

Answer to Referee #1

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.

We are very grateful to anonymous referee #1 for the positive evaluation of our work and the constructive suggestions and comments, which have certainly contributed to improve the manuscript. We have followed all her/his indications and have made the suggested modifications in the manuscript.

General comments:

- 1. The paper presents data from an experiment performed in two stages. First, natural water was bubbled in present day and future CO_2 conditions, and this water was using in the second stage of the experiment were a mix of this water, 1.2 μm filtered water with bacteria and 0.2 μm filtered water (without bacteria). The first stage of the experiment produced non-acidified organic matter (NO) and acidified organic matter (AO). The second phase used the NO and AO treatments and exposed them to the same CO_2 conditions used in the first phase termed low carbon (LC) and high carbon (HC). All combinations were used, i.e. there were four treatments LC_NO, LC_AC, HC_NO and HC_AO, each with three replicates.

It took me some time to figure out what actually had been done in the experimental set-up, but Fig 2 is very good in outlining this. It is an interesting set up with the two stages which were used to see what the potential carry over effects of water grown under different CO_2 concentration may have on the bacterial production.

One possible bias is that the water produced in the first phase of the experiment was frozen until the start of the second phase of the experiment. Freezing the water might affect the DOM pool. This was the same for all treatments so I do not see this as a major flaw, but you could consider taking this aspect up in the discussion.

Some information about the possible effects and rationale of freezing the samples between phase 1 and 2 has been included in the text to address this methodological issue with more detail, as proposed by the referee. (Lines 363-372)

We agree with the referee that some alterations of the dissolved organic matter pool can occur during the freezing process but we think they should not be significant for the objectives of this study (see explanation below). In addition, this methodological approach was the best for the objectives of our study due to the following reasons: 1) Freezing the samples was necessary to preserve the acidified and non acidified organic matter for later use and to avoid the excessive consumption of the organic matter between phase 1 and 2 in our experiment. DOC released by marine phytoplankton is highly labile to bacterial utilization and can be degraded significantly within hours (Chen & Wangersky 1996). 2) Freezing the samples instead keeping them refrigerated or filtered decreased significantly the survival rate of the microbial community from phase 1 (Postgate & Hunter 1961) and allowed to keep most of the organic matter, including both, the particulate and 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 (Piontek et al. 2010).

Regarding DOC preservation methods, unfortunately there is not too much available information for marine samples. Some of them include the acidification with clorhydric or phosphoric acid, or the poisoning of the samples with mercuric chloride solution, which are obviously not appropriate for our experimental design (Chen & Wangersky 1996, Griffith & Raymond 2011, Calleja et al. 2013). However, results from peatland waters show that changes in DOC concentration by freeze/thaw cycles are small (<5%) and do not show a clear pattern of increase or decrease (Peacock et al. 2015). In any case, such changes are in the range of typical precision for DOC analysis and, as indicated by the referee, all samples were processed similarly in our experiment, so we think that the freezing and thawing process was not a major problem for achieving the objectives of the study.

- 2. For the statistical test, why not use a two way ANOVA, you have two factors NO/AO and LC/HC? Also for the BR, BP I would suggest to do a regression of the development, e.g. on the cumulative respiration/production, then the whole time series could be taken into account. This way you could compare the cumulative results to the single point results.

A new approach using a two way ANOVA statistical test has been done and has been included in the present version of the manuscript, as proposed by the referee. A detailed explanation of this topic has been included in the Statistical analysis section (Lines 222-229).

Regarding the second suggestion proposed by the referee about using regressions of the cumulative values, both referees (Referee#1 and Referee#2) showed the importance of using a cumulative approach to improve the understanding of the results. We also agree that this approach improve the quality of the manuscript helping to understand the results. In this sense, we decided to use the integral of the cumulative values, as proposed by referee#2, instead the regression because it calculates the exact value under the cumulative response curve. In the new version of the manuscript we have included some text showing the results of the cumulative responses and statistical analysis (Lines 279, 299, 304 and 315) for the bacterial production, bacterial respiration, bacterial abundance, bacterial carbon demand and the bacterial growth efficiency 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 reading and understanding of the results much easier.

- 3. I would also like to see a deeper analysis of what happens with the physiological variables over time. You found a higher respiration initially in the HC treatment, but this shifted towards highest respiration in the LC_NO at the end. Why is that and what are the different processes that could be involved? Similar with BP, it increases initially in all treatments, but in the end there is a clear difference between treatments. I know you take up some aspects e.g. the effect of pH on enzymatic activity, but there could be other aspects e.g. intracellular pH regulation, and the literature points to different directions.

Additional information regarding other possible mechanisms recently shown by the literature (i.e. Bunse et al. (2016)) has been included as proposed by the referee (Line 415).

Overall the manuscript is well written and easy to follow. The figures are of sufficient quality and I have no problem supporting the publication after taking my suggestions above into consideration.

Minor comments:

4. Please add the actual p value throughout the results chapter where statistical tests were conducted, also where there is no statistical difference, i.e. $p < 0.05$ is not acceptable; the limit should be at $p < 0.001$ or $p < 0.0001$, so less than 0.1% or 0.01% probability for a type II error.

All the exact p-values, together with the n value and the statistical test used, have been included in the new version of the manuscript as proposed by the referee.

- 5. In line 475 you write: On one hand (should be: on the one hand), and it is only in line 490 as a start of a new paragraph where you have the follow up: on the other hand.... Please rephrase, these two points should come right after each other if you want to keep them in the 'one the one hand', 'on the other hand' form.

The text has been corrected as proposed.

- 6. y-axis in Figs 5 and 9 has ',' as decimal separator.

All figures have been revised and corrected.

- 7. Please add the axis title, for example Respiration to Fig 5c with units in parenthesis. It makes it much easier to see what data is presented.

Axis titles have been included for improving clarity as indicated by the referee.

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.

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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.

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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

Eliminado: concomitant

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

Eliminado: concomitant

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 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 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 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.

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).¶
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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:

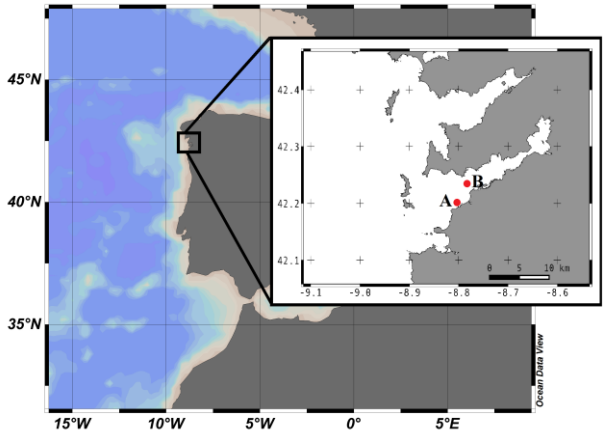

45°N
40°N
35°N
15°W
10°W
5°W
0°
5°E
42.4
42.3
42.2
42.1
-9.1
-9.0
-8.9
-8.8
-8.7
-8.6
B
A
0 5 10 km
Ocean Data View

FIGURE 2:

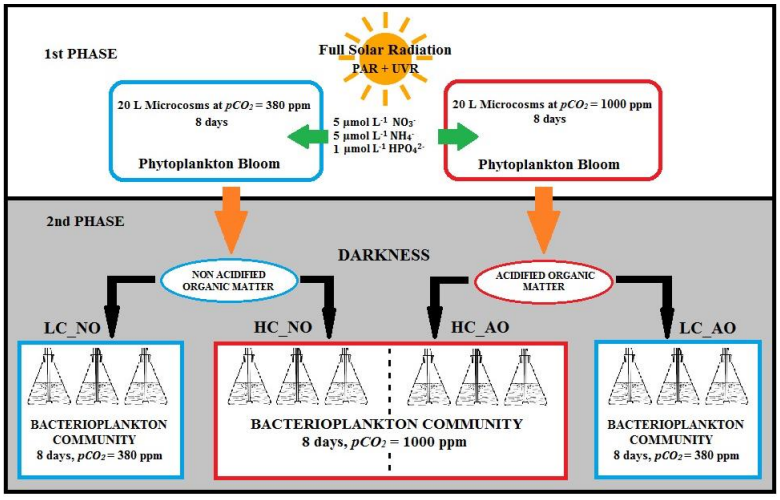

1st PHASE
Full Solar Radiation
PAR + UVR
20 L Microcosms at pCO_2 = 380 ppm
8 days
Phytoplankton Bloom
5 µmol L-1 NO_3^-
5 µmol L-1 NH_4^-
1 µmol L-1 HPO_4^{2-}
20 L Microcosms at pCO_2 = 1000 ppm
8 days
Phytoplankton Bloom
2nd PHASE
DARKNESS
NON ACIDIFIED ORGANIC MATTER
ACIDIFIED ORGANIC MATTER
LC_NO
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:

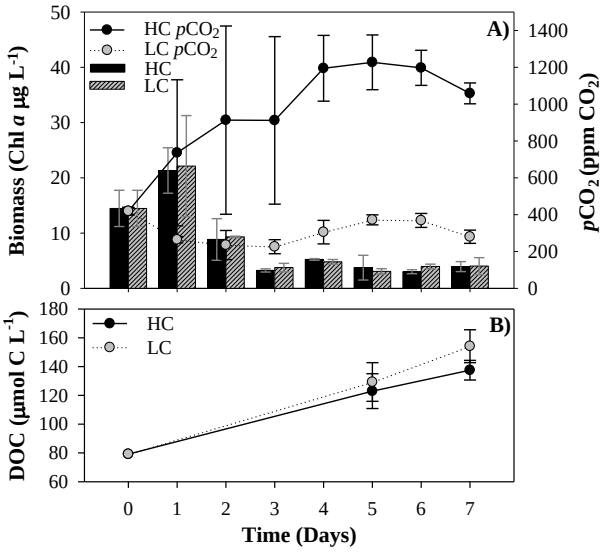

A)
HC pCO_2
LC pCO_2
HC
LC
Biomass (Chl *a* µg L^{-1})
pCO_2 (ppm CO_2)
B)
HC
LC
DOC (µmol C L^{-1})
Time (Days)

FIGURE 4:

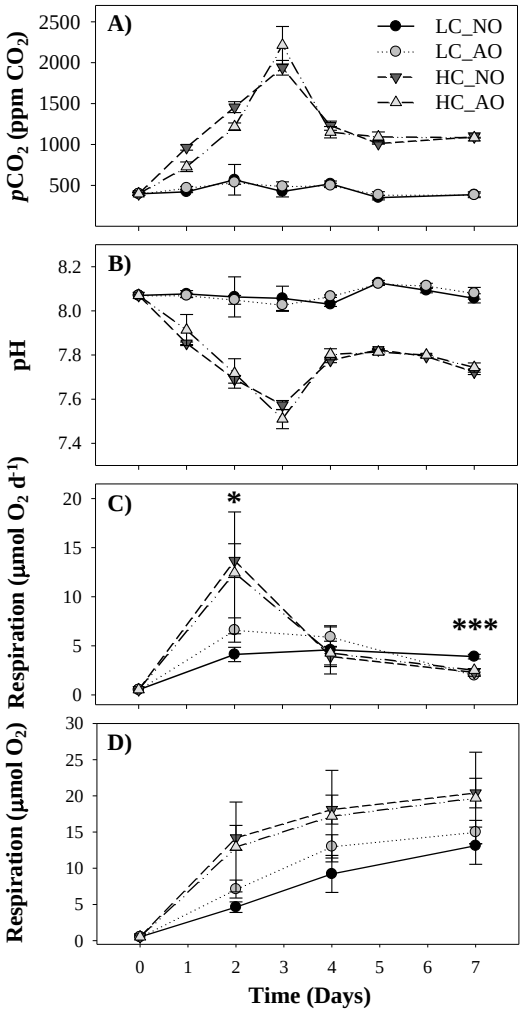

A)
B)
C)
D)
LC_NO
LC_AO
HC_NO
HC_AO
pCO_2 (ppm CO_2)
pH
Respiration (µmol O_2 d^{-1})
Respiration (µmol O_2)
*
***
Time (Days)

FIGURE 5:

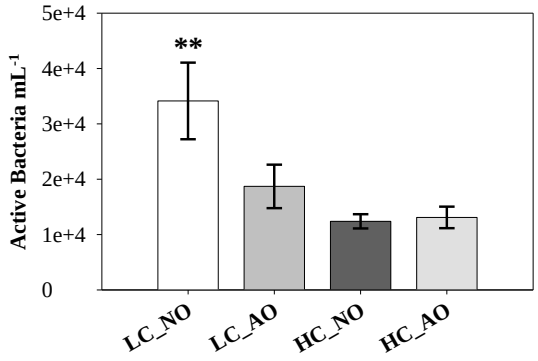


FIGURE 6:

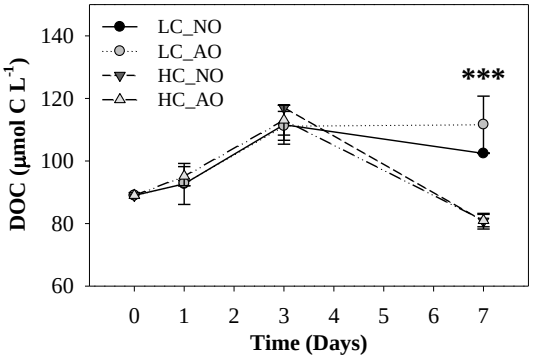

LC_NO
LC_AO
HC_NO
HC_AO
***
DOC (µmol C L-1)
140
120
100
80
60
0
1
2
3
4
5
6
7
Time (Days)

FIGURE 7:

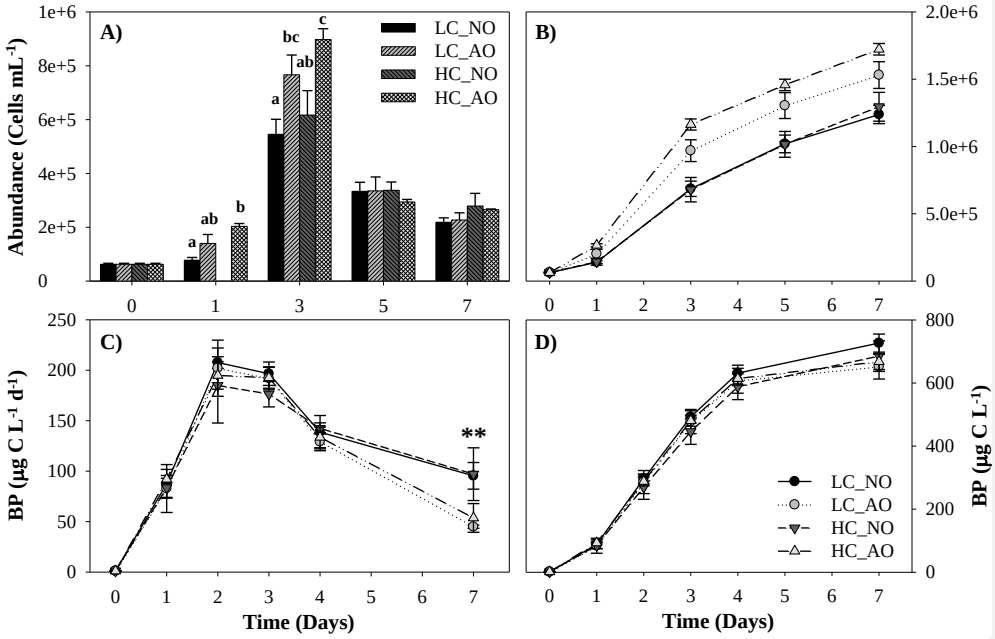
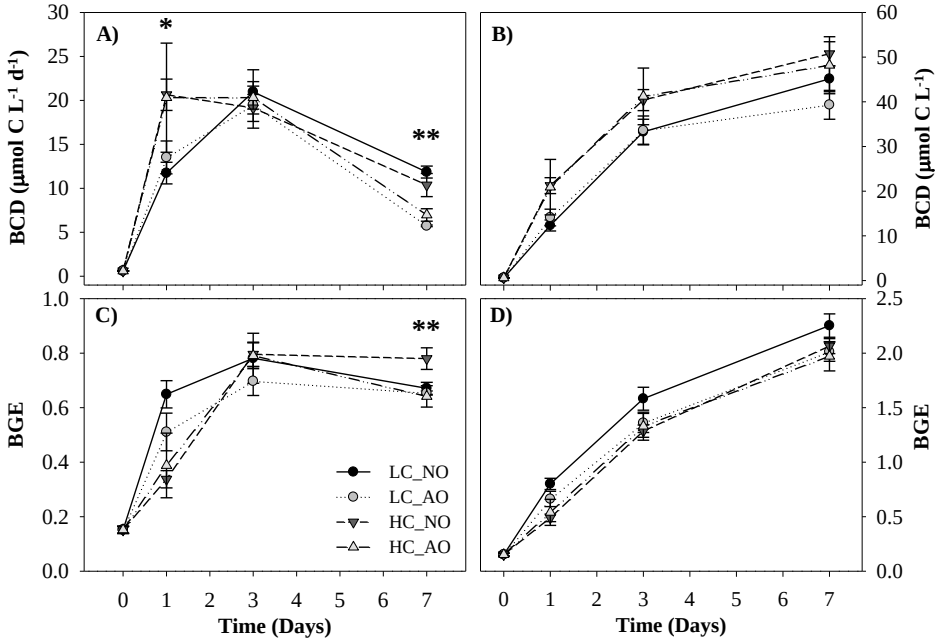

A)
B)
C)
D)
LC_NO
LC_AO
HC_NO
HC_AO
Abundance (Cells mL-1)
BP (µg C L-1 d-1)
BP (µg C L-1)
Time (Days)
Time (Days)
a
ab
b
a
bc
ab
c
**

FIGURE 8:

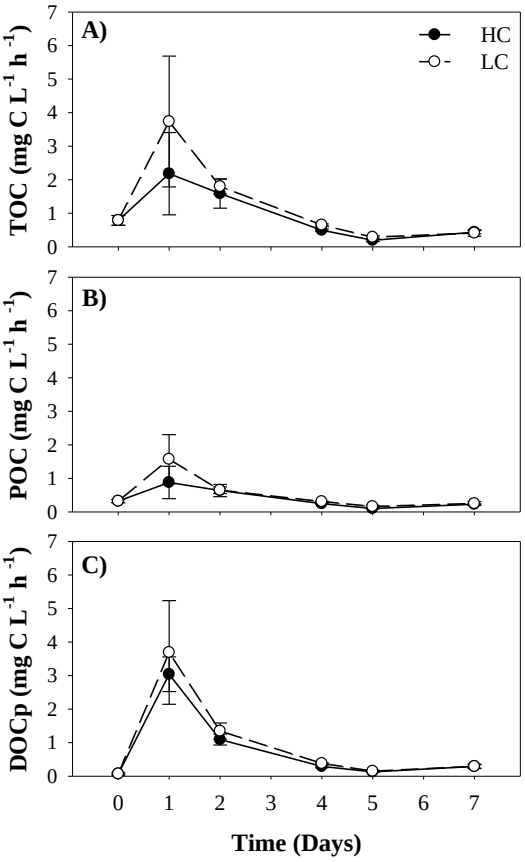

A)
B)
C)
D)
*
**
**
BCD (µmol C L-1 d-1)
BCD (µmol C L-1)
BGE
BGE
LC_NO
LC_AO
HC_NO
HC_AO
Time (Days)
Time (Days)

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