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Effect of CO_2, nutrients and light on coastal plankton. II. Metabolic rates

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ABSTRACT: We conducted a microcosm experiment aimed at studying the interactive effects of high CO_2, nutrient loading and irradiance on the metabolism of a planktonic community sampled in the Western Mediterranean near the coast of Málaga. Changes in the metabolism of phytoplankton and bacterioplankton were observed for 7 d under 8 treatment conditions, representing the full factorial combinations of 2 levels each of CO_2, nutrient concentration and solar radiation exposure. The initial plankton sample was collected at the surface from a stratified water column, indicating that phytoplankton were naturally acclimated to high irradiance and low nutrient concentrations. Nutrient addition combined with high irradiance resulted in a significant increase in primary production. Nitrate uptake by phytoplankton was also stimulated under high nutrient conditions. High nutrients, high irradiance and the combination of low CO_2 and high irradiance positively affected bacterial production. Light was the main factor affecting the respiration rates of the community, which were higher at the high light level. After 7 d of incubation, nutrient loading was the only factor that significantly affected the amount of particulate organic carbon (POC) accumulated in the microcosms. Therefore, the changes in metabolic rates produced at high CO_2 had no effect on net production of particulate organic matter. If these results are extrapolated to the natural environment, it could be hypothesized that high levels of CO_2 will have a limited impact on biological pump activity in the northern Alboran Sea since it is assumed that POC export towards deeper layers determines the potential for carbon sequestration.

KEY WORDS: Acidification · Bacterioplankton · Nutrients · Phytoplankton · Primary productivity · Respiration · UVR

INTRODUCTION

The ocean plays an important role in counteracting the accumulation of anthropogenic CO_2 in the atmosphere (Sabine et al. 2004). Carbon fixation into particulate organic carbon (POC) performed by marine photosynthetic organisms and the subsequent sinking of POC which is sequestered in deep waters (the so-called biological pump) are 2 of the main organism-mediated processes that are involved in this CO_2 sink effect. In pelagic ecosystems, a large proportion of the POC produced by the phytoplankton is re-

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mineralized by respiration in the surface layer and transferred back into the dissolved inorganic carbon (DIC) pool (Marañón et al. 2005). Phytoplankton also release some fixed carbon as dissolved organic carbon (DOC), most of which is remineralized by bacteria. Only a small part of the fixed carbon is exported towards deeper layers in the particulate form of zooplankton faecal pellets (Fowler & Knauer 1986), marine snow aggregates (Kiørboe 2001) and transparent exopolymer particles (TEP, formed by aggregation of dissolved polysaccharides; Passow 2002). The net balance between primary production and respiration in the upper layer of the ocean determines its potential for carbon sequestration (del Giorgio & Duarte 2002).

The Mediterranean Sea is an oligotrophic basin where primary production has been long recognised to be limited by phosphorus (Krom et al. 1991, 2004, Siokou-Frangou et al. 2010), although some more recent studies have qualified this paradigm (Tanaka et al. 2013). A large proportion of the organic carbon enters the DOC pool with limited contribution to the biological pump (Thingstad & Rassoulzadegan 1995). However, this overall productivity pattern is modified locally in areas affected by coastal upwelling and fronts caused by geostrophic currents where phytoplankton biomass and productivity rates are an order of magnitude higher than in the Mediterranean oligotrophic areas (D'Ortenzio & Ribera d'Alcalà 2009). One of these areas is the northwestern Alboran Sea (the westernmost basin in the Mediterranean Sea), where wind-induced upwelling injects CO_2 and nutrients into the euphotic zone triggering the growth of large-sized phytoplankton with high sinking rates (Goldman 1993, Mercado et al. 2007). The phytoplankton response to these nutrient enrichment episodes is quite rapid (24 to 48 h), and bloom decline occurs within 5 to 6 d after reaching the biomass peak. Ramírez et al. (2006) reported enhanced plankton respiratory activity during upwelling episodes, but this increase was comparatively lower than the primary production increase. Consequently, the upwelling significantly increases net production of exportable POC in the Alboran Sea (a common phenomenon in other upwelling areas; Martínez 1997).

Ocean acidification will potentially modulate the biological pump activity by means of changes in production and carbon consumption in the upper ocean layer (Riebesell & Tortell 2011, Passow & Carlson 2012). However, results of experiments where natural plankton assemblages were subject to elevated CO_2 concentrations indicate that the response to acidification could be community-specific. Primary productivity increased in most natural phytoplankton assemblages subjected to elevated CO_2 (Hein & Sand-Jensen 1997, Tortell et al. 2002, 2008, Engel et al. 2013), although some other communities showed no response (Tortell et al. 2000). Egge et al. (2009), Motegi et al. (2012) and Tanaka et al. (2013) found no effects of elevated CO_2 on respiration at the plankton community level. Therefore, the increase of primary production at elevated CO_2 could add synergistically to the phytoplankton production stimulation induced by nutrient enrichment, and the biological pump should then strengthen. Furthermore, Piontek et al. (2013) reported that both heterotrophic use of carbon and phytoplankton production increased at elevated CO_2, resulting in an increase of exportable POC.

In the present work, the hypothesis that stimulation of POC production following a nutrient enrichment episode is increased at elevated CO_2 was tested in experiments carried out with natural coastal plankton assemblages sampled at the northern Alboran Sea. For this objective, the short-term metabolic responses of bacterioplankton and phytoplankton were researched at present and at elevated (1000 ppmv) CO_2 concentrations. The experiments were done using surface plankton which were subjected to a combination of different treatments including 2 levels of nutrients and irradiance. The experimental set-up and levels of the tested factors, as well as the physical and chemical responses are described in detail in Neale et al. (2014, this Theme Section). The effects of the treatments on phytoplankton abundance, composition and size structure are discussed in Reul et al. (2014, this Theme Section). The changes in cell viability and DOC excretion are discussed in Sobrino et al. (2014, this Theme Section). In the present study, the changes in net production of POC, bacterial production and respiration are presented and discussed.

MATERIALS AND METHODS

Experimental design

The plankton community was sampled in a coastal station located offshore of Fuengirola (southern Spain), in the northwestern Alboran Sea 36.54° N, 4.60° W). The experimental setup is described in detail by Neale et. al (2014). To remove mesozooplankton, the seawater samples were pre-screened through a 200 µm mesh (this procedure did not substantially affect the composition of the phytoplankton community as abundance of chain-forming and large diatoms in the initial sample was low; Neale et al. 2014). The effects of elevated CO_2 and nutrient addi-

tions on the metabolism of the planktonic community were tested under 2 levels of solar irradiance for 7 d. Different combinations of 3 factors were assayed: CO_2 (C), nutrient (N) and light (L). Two levels of each factor (referred as H and L for high and low level, respectively) were tested. The incubations at low (present) and high CO_2 (LC and HC) consisted of bubbling the cultures with normal air and CO_2-enriched air (1000 ppm), respectively. In the high-nutrient treatments (HN), additions of a combination of nitrate, phosphate and organic nutrients were made on the first (Day 0) and the third (Day 2) day of incubation. Prior to the C and N treatments, the samples were acclimated to high light (HL) and low light (LL) conditions for 24 h (Day −1). HL (61% of full solar irradiance) and LL (32%) were carried out using neutral density screen mesh. Samples were maintained in UVR-transparent 20 l LDPE cubitainers, so both UVR and photosynthetically active radiation (PAR) were included in the spectra. In total, 8 treatments were tested and 3 independent replicates of each treatment were performed.

Chlorophyll *a* (chl *a*) concentration and abundance of bacteria and autotrophic picoplankton were measured each day. Additionally, the abundance and taxonomic composition of >5 µm phytoplankton were analysed on Days 0, 2, 4 and 6 (final day); these data are presented and discussed in more detail in Reul et al. (2014). Primary production of the plankton size fractions <20 and >20 µm and bacterial production were estimated on Days 0, 2, 4 and 6, as well as respiration rates of the whole plankton community. Carbon and nitrogen contents of particulate organic matter (POM) and uptake rates of DIC and nitrate were estimated on Days 0 and 6.

Biomass calculations of different plankton fractions

Biomass of heterotrophic picoplankton (including bacterial and micro-zooplankton) as well as <20 and >20 µm autotrophs were estimated from the abundance data obtained by flow cytometry and microscopy techniques as described in Reul et al. (2014) and Neale et al. (2014). Heterotrophic picoplankton biomass was calculated by multiplying bacterial abundance by the cell carbon content calculated from bacterial biovolume according to Loferer-Krößbacher et al. (1998). Bacterial cell biovolume was estimated in each treatment from the inverted microscope measurements of cell size performed on at least 10 individuals of each bacterial morphotype (i.e. small cocci, small bacilli, large bacilli and vibrio).

Biovolumes of *Synechococcus*, *Prochlorococcus* and picoeukaryotes were calculated using the values given in Ribes et al. (1999) for samples collected in the northwestern Mediterranean Sea. Cell biovolumes corresponding to the most abundant species, genus and taxa of phytoplankton and heterotrophs identified by the inverted microscope method were calculated by using the appropriate formula according to their geometric shape (Sun & Liu 2003, Olenina et al. 2006, Vadrucci et al. 2007). Biovolume values were converted into biomass using the formulae proposed by Morel et al. (1993) for *Prochlorococcus*, Kana & Glibert (1987) for *Synechococcus*, Verity et al. (1992) for <15 µm eukaryotic phytoplankton and Menden-Deuer & Lessard (2000) for >15 µm dinoflagellates and diatoms.

Primary production and nitrate uptake rates

Rates of POC production were estimated by means of ^{14}C experiments. Methods of incubation, analysis of ^{14}C in the incubated samples and calculation of ^{14}C uptake rates are described in Sobrino et al. (2014).

For simultaneous determination of ^{13}C and ^{15}N uptake rates, 250 ml samples from each microcosm were incubated in 1 l polycarbonate transparent bottles under the same conditions of temperature and PAR as in the incubation tanks on Day 6. Before the measurements, solutions of sodium bicarbonate enriched in ^{13}C and potassium nitrate enriched in ^{15}N were added to reach isotope final concentrations of 150 µM and 0.05 µM, respectively, which resulted in increases of DIC by 7% and of nitrate by 1 to 55% (depending on the nitrate concentration in the initial sample). For each microcosm, 2 independent incubations plus a control (i.e. sample without addition of ^{15}N) were incubated for 4 to 5 h. The isotope enrichment and the concentration of the POC and particulate organic nitrogen (PON) were estimated at the beginning of the experiment by filtering 0.5 l of initial non-incubated seawater (i.e. without ^{13}C or ^{15}N added) through 25 mm glass microfibre filter (Whatman GF/F). After incubation, the samples were filtered through precombusted (450°C for 2 h) Whatman GF/F filters. The filters were frozen until they were exposed to HCl fumes overnight and then dried and pelleted for isotopic analysis. POC and PON were analyzed with a Carlo Erba Instrument C:N:H analyzer. Carbon and nitrogen stable isotope natural abundance measurements were made by continuous flow isotope-ratio mass spectrometry with a Delta plus Finnigan MAT mass spectrometer connected to the C:N:H analyzer.

The calculations of ^{13}C and nitrate uptake rates were performed according to Dugdale & Wilkerson (1986) and Knap et al. (1996), respectively. POC and PON concentration in non-incubated samples were used for the calculations, as they varied by less than 10% after incubation. For the samples coming from the LN treatments in which addition of ^{15}N increased the nitrate concentration above 10%, the uptake rates were corrected following the procedure proposed by MacIsaac & Dugdale (1972). Note that ^{13}C uptake measured by this method is not fully comparable with carbon uptake measured by ^{14}C in Sobrino et al. (2014) since the UVR transparency is lower in polycarbonate bottles (used for ^{13}C uptake experiments) than in Teflon-FEP flasks (used for ^{14}C uptake experiments).

Concentration of TEPs

The concentration of TEP was determined by the method of Claquin et al. (2008), which is a modification of the method of Passow & Alldredge (1995). In short, 100 ml samples were filtered through 0.4 µm polycarbonate filters at low vacuum pressure and then frozen at −20°C until analysis. Each filter containing cells and TEP was mixed with a 2 ml solution of 0.02% Alcian Blue (Sigma) dissolved in 0.06% acetic acid and immediately centrifuged (3200 × *g*, at 20°C for 30 min) to remove the excess dye. The pellet was rinsed with 1 ml of Milli-Q water and centrifuged at 3200 × *g* for 30 min. This procedure was repeated several times until the pellet was uncolored. The supernatant was removed after each centrifugation cycle. Then 4 ml of 80% H_2SO_4 was added to the pellet. After 2 h incubation, absorbance of the stained sample was measured spectrophotometrically at 787 nm. TEP concentrations in terms of xanthan gum equivalent weight (mg Xequival l^{-1}) were calculated by interpolating the absorbance values onto a standard regression line built with known concentrations of xanthan gum (Claquin et al. 2008).

Bacterial production

Heterotrophic bacterial production (BP) was determined by incorporating ^{3}H-thymidine (specific activity = 49.2 Ci $mmol^{-1}$, Perkin Elmer) into the bacterial DNA (Fuhrman & Azam 1982). ^{3}H-thymidine was added to each tube containing 1.5 ml of sample (3 replicates and 2 blanks for each experimental treatment) to 30 nM saturating final concentration. Vials were incubated at *in situ* temperature for 60 min in darkness. Extraction was carried out with 5% (final conc.) cold trichloroacetic acid (TCA). The tubes were centrifuged at 16 000 × *g*, rinsed twice with 5% TCA and measured in a scintillation counter equipped with autocalibration (Beckman LS 6000 TA). In all calculations, data were corrected by blanks (killed with 5% TCA before addition of the radiotracer). The factors used to convert incorporated tracers to BP in terms of cell carbon were 1×10^{18} cells mol^{-1} of thymidine (Bell 1993) and 2×10^{-14} g C $cell^{-1}$ (Lee & Fuhrman 1987).

Oxygen consumption rates

Oxygen consumption measurements in darkness were performed using a PreSens system consisting of sensor-spot optodes (SP-PSt3-NAU-D5-YOP) and an optic-fiber oxygen transmitter (Fibox 3) connected to a computer equipped with the software Oxyview 6.02 (Marchand et al. 2009). Before each measurement, a 2-point calibration of the electrodes was carried out with a sodium sulphite saturated seawater solution (0% oxygen saturation) and oxygen saturated sterilized seawater. Three independent subreplicates of each microcosm were measured at 21°C. A respiratory quotient equal to 1 was used to convert oxygen consumption rates to respiratory CO_2 production (Packard & Williams 1981).

Statistical analysis

Statistical significance of treatment effects was tested using repeated measures ANOVA. Calculations were performed using a generalised linear model (GLM) procedure with main effects, time (repeated measure) and interaction terms. For the variables that were measured on Day 6, ANOVAs for main effects were done. The relationships between some variables were tested by Pearson's correlation analyses. p-values for the correlation coefficients were calculated in order to determine their statistical significance. ANCOVA with a separate slope model was used in order to assess the difference in the slopes of linear regression models used to test the relationship between changes in POC and PON in the HN and LN treatments. Prior to conducting these statistical tests, the normality and homogeneity of variance of the variables were assessed. Normality was evaluated from the normal probability plot of the residuals of each variable. Homoscedasticity (by

Cochran's and Levene's tests) and correlation between means and standard deviations were checked in order to verify the assumptions of the repeated measures ANOVA. The assumptions of the parametric correlations and ANOVA were satisfied by all variables. The software package Statistica v.7.1 (Statsoft) was used for all statistical analyses.

RESULTS

Plankton biomass

Autotrophic plankton biomass increased significantly in all treatments in comparison with the initial biomass (Day 0) (Fig. 1a). Maximal autotrophic biomass was obtained by Day 4 in most of the treatments with the exception of LC LN HL and HC LN LL, in which maximal autotrophic biomasses were obtained by Days 2 and 6, respectively. In all 4 LC treatments biomass decreased by Day 6 in comparison with Day 4. Among the HC treatments, plankton biomass decreased by Day 6 only at high irradiance.

Bacteria accounted for more than 70% of heterotrophic biomass in all samples analysed (Fig. 1b) with the exception of the HC HN HL treatment on Day 4 (where bacteria accounted for 54% of biomass). Total heterotrophic biomass increased by Day 2 in comparison with Day 0 in HN treatments, and decreased in the LN treatments (with the exception of the HC LN LL treatment). In all treatments, biomass of heterotrophs decreased steeply by Day 4 and recovered afterwards, reaching values higher than those obtained on Day 0 with the exception of the LC LN LL treatment.

In terms of biomass, >20 µm autotrophic cells were the main plankton fraction in all the analysed samples, including the initial one (Day 0). Therefore, the variations in plankton biomass among the treatments and within each treatment (i.e. over the incubation time) were mainly caused by changes in the large (>20 µm) phytoplankton. Contribution of >20 µm phytoplankton to plankton total biomass increased steeply from Day 0 to Day 4, when about 80% of plankton biomass was attributable to this fraction. Contribution of >20 µm phytoplankton to plankton biomass was reduced at Day 6 because of both a decrease in the abundance of cells >20 µm and an increase in the small cell fraction (<20 µm).

The main effects of the experimental factors and their interactions on the biomass of each plankton fraction were not statistically significant, with the exception of bacterial biomass, which was significantly higher at HN (data not shown).

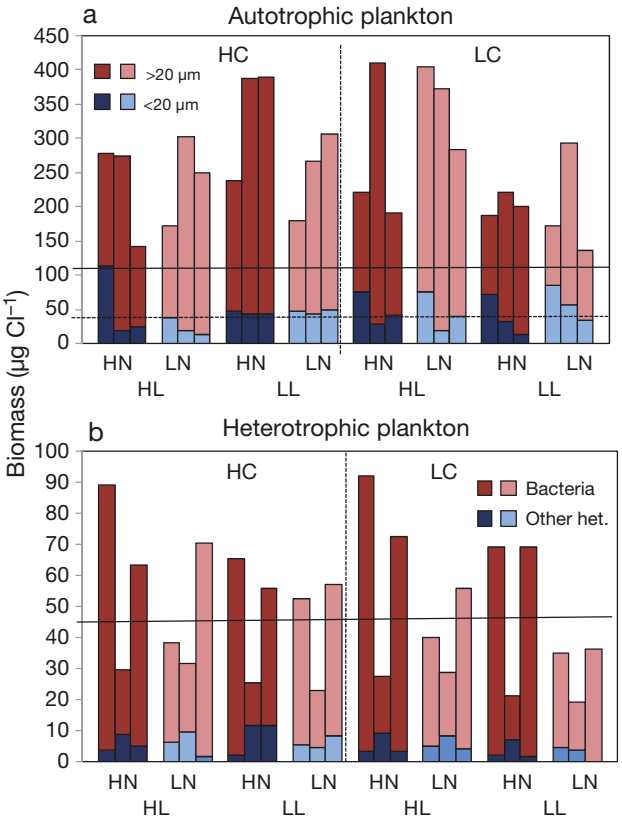

Fig. 1. Temporal evolution of biomass of (a) phytoplankton and (b) heterotrophic plankton under the different experimental conditions (high and low CO_2 concentrations, HC and LC; high and low nutrient concentrations, HN and LN; high and low irradiance, HL and LL). For each treatment, the 3 columns represent Day 2, 4 and 6 data. The horizontal lines in panel (a) indicate initial biomass (Day 0) of >20 µm cells (continuous line) and <20 µm cells (dashed line). In panel (b), the horizontal line indicates initial bacterial biomass (Day 0)

Primary production

Primary production (PP) increased in all the treatments by Day 2 in comparison with Day 0 (Fig. 2a) and continued increasing until Day 6. Contribution to PP of the 2 analysed phytoplankton size fractions varied considerably over the experiment. The >20 µm cells contributed 27 to 40% to the PP at Day 2; their contribution increased by 50 to 83% on Day 4 and was reduced at Day 6 (19 to 70%). The highest contribution of the <20 µm phytoplankton to PP occurred on Day 2. The linear regression between PP and biomass of cells >20 µm was statistically significant ($r = 0.66$, $p < 0.01$), indicating that changes in PP over time and its variability among treatments were mainly due to shifts in the biomass of larger size phytoplankton.

In all treatments, chl *a* specific PP (PP^B) increased by Day 2 in comparison with Day 0 (Fig. 2b). Afterwards, PP^B decreased markedly in the HC HN HL, LC HN HL and LC LN HL treatments. In contrast, PP^B increased in the LC and LL treatments. On Day 6, PP^B did not vary considerably with respect to Day 4 in the HC treatments. However, a reduction was produced in the treatments of LC and HN. PP^B by >20 µm phytoplankton was relatively constant throughout the experiment (Fig. 2c). In contrast, PP^B by <20 µm cells varied greatly over time and among treatments (Fig. 2d).

The main effects of the factors and their interactions on PP were not significant, with the exception of the interaction of N × L (Table 1). Interactions with time and the 2-way interaction of Time × L were also significant.

Bacterial production

BP increased by Day 2 in comparison with Day 0 in the HN treatments (Fig. 3a) and decreased in the LN treatments. BP decreased in all the treatments by Day 4, when the lowest values were obtained. Afterwards, BP increased in the HN treatments but did not change significantly in the LN treatments (apart from the HC LN LL treatment). Biomass specific BP (BP^B) changed significantly over time (Fig. 3b). The changes in BP^B were more pronounced at HN, with an increase from Days 2 to 4 and a decrease by Day 6. BP^B did not increase by Day 4 in the LN treatment, but decreased by Day 6.

The responses of both BP and BP^B were significant for N over the whole experiment and as an interaction with time (repeated measures ANOVA; Table 1). Responses of BP to L and the interaction of C × L were also significant (BP was significantly higher at HL, and both BP and BP^B increased at LC HL).

Respiration rates

Respiratory CO_2 production rates of the planktonic community calculated from oxy-

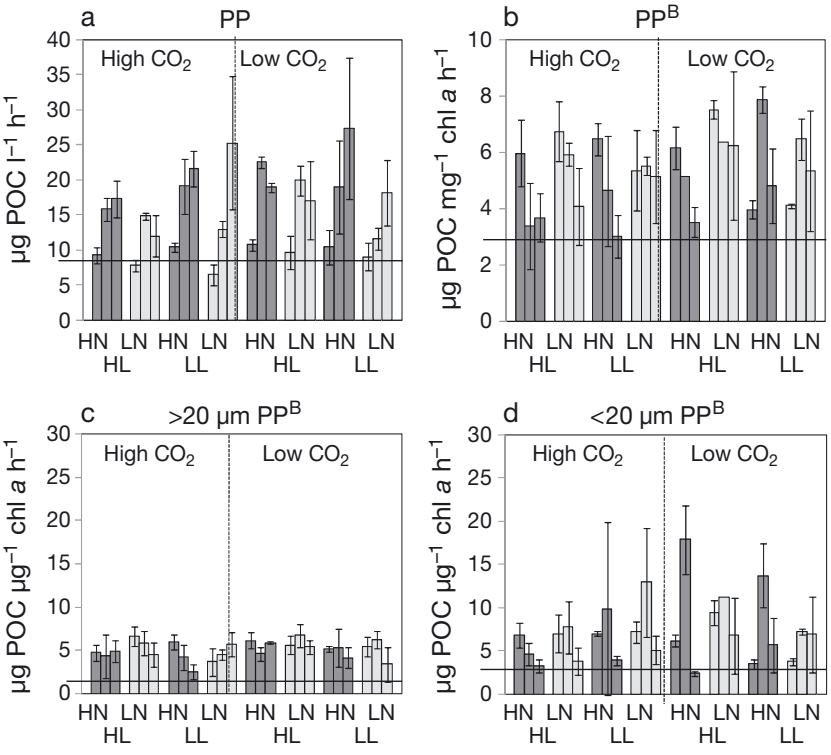

Fig. 2. Temporal evolution of (a) primary production (PP) by phytoplankton and (b) chl *a* specific PP (PP^B) under the different experimental conditions (treatment abbreviations as in Fig. 1). PP^B by phytoplankton (c) >20 µm and (d) <20 µm are also shown. For each treatment, the 3 columns represent Day 2, 4 and 6 data. In each panel, the horizontal line indicates the initial value (Day 0). Data are mean ± 1 SD of 3 microcosm replicates

Table 1. ANOVA for treatment effects on the response variables primary production (PP), bacterial production (BP), bacteria biomass specific BP (BP^B), respiration rates estimated from oxygen consumption (RR), plankton biomass specific respiration rate (RR^B), ratio of PP to RR and transparent exopolymer particle (TEP) concentration. The *F*-ratios for each treatment are shown. The results are for repeated measures ANOVA for main effects, time and interaction terms. Two-way interactions with time were not significant. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

Source of variation	PP	BP	BP^B	RR	RR^B	PP:RR	TEP
CO_2 (C)	0.30	0.46	2.09	0.16	0.04	0.10	2.29
Nutrient (N)	0.28	178***	80.9***	2.11	2.07	0.20	3.48
Light (L)	0.76	7.0*	4.64	19.4***	6.81*	1.53	0.19
C × N	0.002	0.15	0.92	6.03*	6.93*	1.14	0.31
C × L	4.30	5.62*	5.77*	0.10	2.26	1.34	3.87
N × L	8.41*	3.45	0.82	2.66	6.02*	2.67	0.35
C × N × L	2.00	0.21	0.01	0.87	0.47	0.04	1.09
Time	7.14**	113.0***	24.2***	5.43*	4.61*	0.87	7.43**
Time × C	0.76	2.10	1.64	8.06***	5.25*	1.58	1.25
Time × N	0.16	22.7***	11.4***	0.12	0.41	0.36	1.82
Time × L	3.88*	0.83	3.48*	0.23	0.27	0.36	1.45

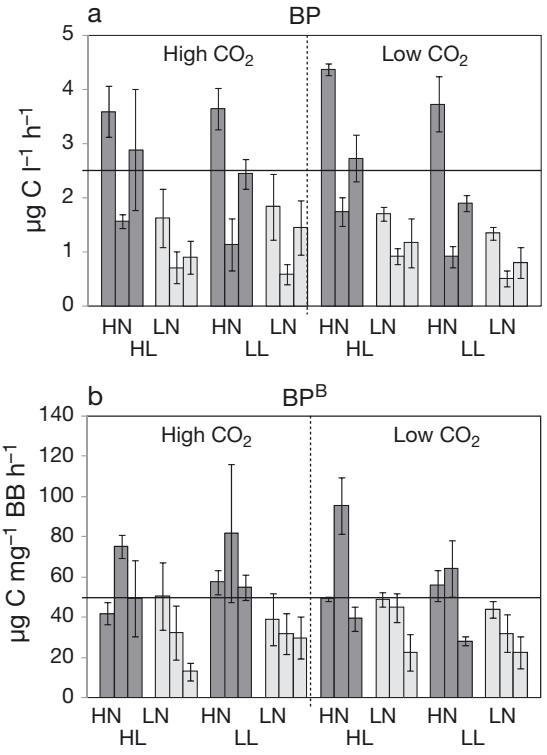

Fig. 3. Temporal evolution in (a) bacterial production (BP) and (b) biomass specific bacterial production (BP^B) under the different experimental conditions (treatment abbreviations as in Fig. 1). For each treatment, the 3 columns represent Day 2, 4 and 6 data. In each panel, the horizontal line indicates the initial value (Day 0). Data are mean ± 1 SD of 3 microcosm replicates

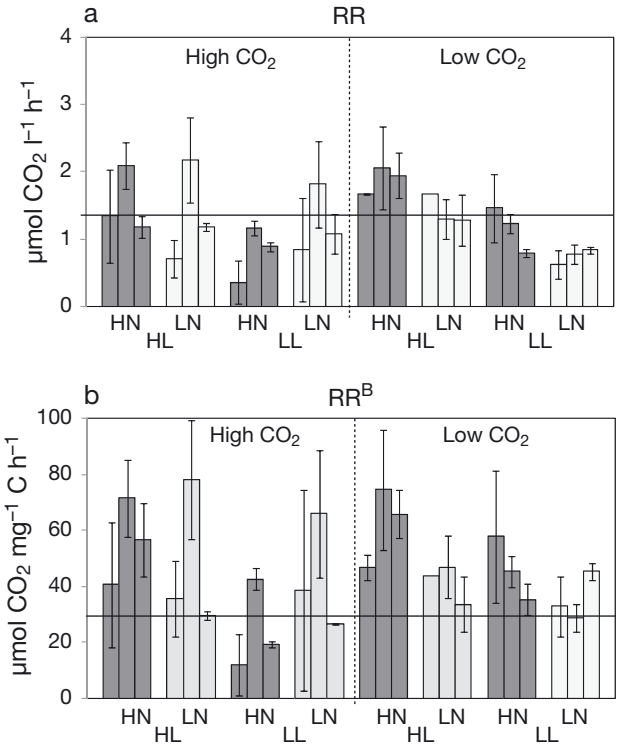

Fig. 4. Temporal evolution of (a) respiration rates estimated as oxygen consumption (RR) and (b) plankton biomass specific respiration (RR^B) under the different experimental conditions (treatment abbreviations as in Fig. 1). For each treatment, the 3 columns represent Day 2, 4 and 6 data. In each panel, the horizontal line indicates the initial value (Day 0). Data are mean ± 1 SD of 3 microcosm replicates

gen consumption rates (RR; Fig. 4a) did not vary significantly by Day 2 with respect to the initial value in the HC treatments. In these treatments, RR increased on Day 4 and decreased on Day 6. In the LC treatments, the time variability of RR was more reduced except in the LC HN LL treatment (for which RR decreased by Day 6), and in the LC LN LL treatment (for which RR was significantly lower than the initial value).

In the HC treatments, plankton biomass specific respiration rates (RR^B; Fig. 4b) did not change by Day 2 in comparison with Day 0 (except for HN LL), increased by Day 4 and decreased by Day 6. In contrast, RR^B was relatively constant in the LC treatments, excluding the LC HN HL condition.

The ratio of PP to RR increased in all treatments on Day 2 (Fig. 5). In the HC treatments, PP:RR reached values higher than 1 that day (apart from the HC HN HL treatment) whereas it was lower than 1 in the LC treatments. By Day 4, PP:RR decreased in the 4 treatments of HC but increased in the LC treatments. By the last incubation day (Day 6), PP:RR was higher than 1 in all treatments.

The results of the ANOVA indicate that both RR and RR^B decreased significantly over the whole experiment at LL in comparison with HL (Table 1). The responses of both variables were significant for the interaction of C × N. Interactions with time, and time × C were also significant. Furthermore, the response of RR^B to the interaction of N × L was significant. PP:RR was not significantly affected by the factors or the interaction between them (Table 1).

Balance of particulate carbon and nitrogen in the microcosms

In order to determine the effect of the treatments on net production of POM during the whole incubation period, the net balance of POC and PON (ΔPOC and ΔPON) was calculated in each treatment by subtracting the POC and PON concentrations on Day 0 from that of Day 6. For these calculations, the concentrations of organic matter estimated from the elemental analysis of particulates retained by 0.7 µm fil-

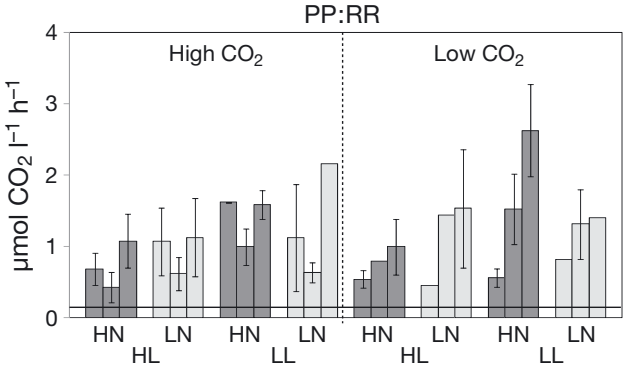

Fig. 5. Temporal evolution of the ratio of primary production (PP) to respiration rate (RR) under the different experimental conditions (treatment abbreviations as in Fig. 1). For each treatment, the 3 columns represent Day 2, 4 and 6 data. In each panel, the horizontal line indicates the initial value (Day 0). Data are mean ±1 SD of 3 microcosm replicates

ters were used (a more detailed analysis of variability of PON and PON is presented in Neale et al. 2014). There was high variability in ΔPOC and ΔPON among the 3 replications of each treatment. However, on average, ΔPOC and ΔPON were higher in the HN treatments. ANOVA results confirmed that the differences among the HN and LN treatments were statistically significant (Table 2). However, the effects of C, L and the interactions among C, N and L were not statistically significant (Table 2). The ratio of ΔPOC to ΔPON was significantly affected by N. Concordantly, the slopes of the regression line between ΔPOC and ΔPON (Fig. 6) calculated for HN and LN were different at $p < 0.001$. This result indicates that a comparatively lower increase of POC relative to PON was produced at HN.

Changes in TEP concentration over time did not follow a common variation pattern in the 8 treatments. As occurred with ΔPOC and ΔPON, TEP concentration was highly variable among the 3 replicates of each treatment (data not shown). The highest TEP concentration was obtained in the LC HN HL treatment on Day 2. On the other extreme, the lowest concentration was obtained in the LC LN HL treatment on Day 4. Reduction in TEP concentration by Day 4 with respect to Day 2 occurred in all treatments apart from HC LN LL and HC LN LL. The ANOVA results (Table 1) indicate that TEP concentration was not significantly affected by the treatments but there were significant differences between days.

Uptake rates of DIC (*R*DIC) and nitrate (RNO_3^-) normalized by POC and PON, respectively, by Day 6 are shown in Fig. 7. The results of the factorial ANOVA indicate that the 3 tested factors (C, N and L) significantly affected *R*DIC. Thus, HC, LN and LL produced a significant decrease in *R*DIC. In contrast, RNO_3^- was only affected by nutrients (Table 2). The ratio of *R*DIC to RNO_3^- varied significantly among the treatments (Fig. 8). The lowest values of this index were obtained at HC and HN. Consistently, the results of the factorial ANOVA demonstrate that the C level affected the ratio of *R*DIC to RNO_3^-. This index was reduced by half in the HC treatment in comparison with LC. Furthermore, the response of *R*DIC to RNO_3^- to the interaction of C × L was also significant.

DISCUSSION

Physiological performance of the initial plankton community

The hydrological conditions in the northern Alboran Sea during summer are normally characterised by strong stratification of the water column, which hampers the re-supply of new nutrients from the deep layer towards the euphotic zone (Ramírez et al. 2005, Mercado et al. 2012). Consequently, nitrate concentrations in the surface layer of the Alboran Sea coastal waters reported for the summer period are relatively low (ca. 0.3 µM; Mercado et al. 2012) and the dissolved inorganic nitrogen to phosphate molar ratio (ca. 3.0 according to Mercado et al. 2012) departs significantly from the Redfield ratio (16:1). Temperature and nutrient vertical profiles in the

Table 2. ANOVA for treatment effects by Day 6 on the response variables ΔPOC (net production of particulate organic carbon), ΔPON (net production of particulate organic nitrogen), ratio of ΔPOC to ΔPON, POC specific ^{13}C uptake rates (*R*DIC), PON specific nitrate uptake rates (RNO_3^-) and molar ratio of *R*DIC to RNO_3^-. The *F*-ratios for each treatment are shown. The results are for factorial ANOVA for main effects and interaction terms. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

Source of variation	ΔPOC	ΔPON	ΔPOC:ΔPON	*R*DIC	RNO_3^-	*R*DIC: RNO_3^-
C	4.02	4.2	0.27	11.09*	3.98	11.39***
N	9.75**	68.1***	33.86***	92.16***	44.8***	1.17
L	0.80	1.05	1.09	5.82*	2.36	4.39*
C × N	0.58	0.78	0.15	0.68	0.55	0.25
C × L	0.75	0.24	1.61	0.66	2.92	6.32*
N × L	0.14	0.16	1.47	4.75*	0.01	0.01
C × N × L	0.64	2.07	3.37	0.30	0.10	0.03

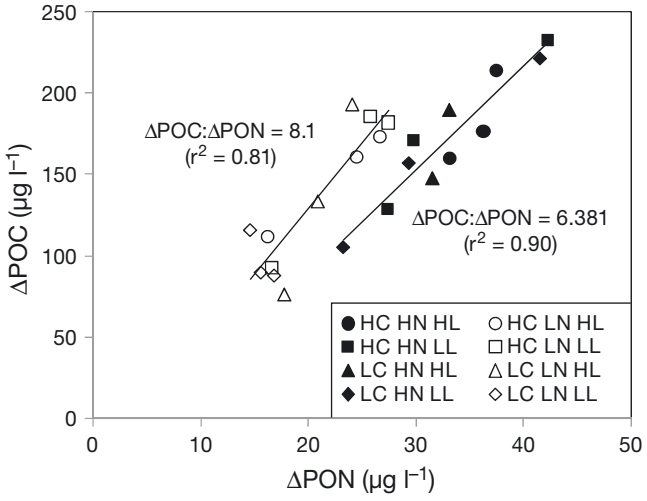

Fig. 6. Relationship between the net balances of particulate organic carbon and nitrogen (ΔPOC and ΔPON) measured by Day 6 under the different experimental conditions (treatment abbreviations as in Fig. 1). The results obtained for the 3 microcosm replicates are shown. The regression lines for the treatments of high (closed symbol) and low (open symbols) nutrients are shown. The slopes of the regression lines were significantly different at $p < 0.001$ according to the results of an ANCOVA with separate slope model

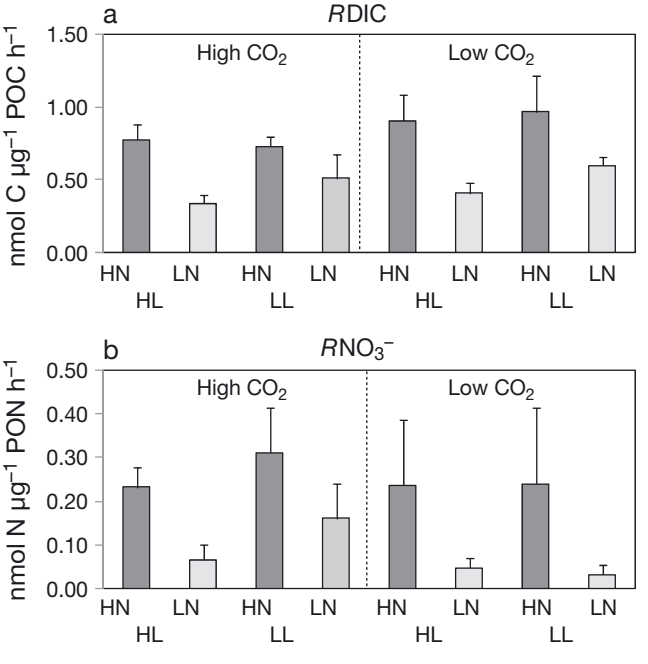

Fig. 7. Uptake rates of ^{13}C (*R*DIC) and nitrate (RNO_3^-) normalised by particulate organic carbon and nitrogen, respectively. The rates were measured on Day 6 under the different experimental conditions (treatment abbreviations as in Fig. 1). Results are mean ±1 SD of 3 microcosm replicates

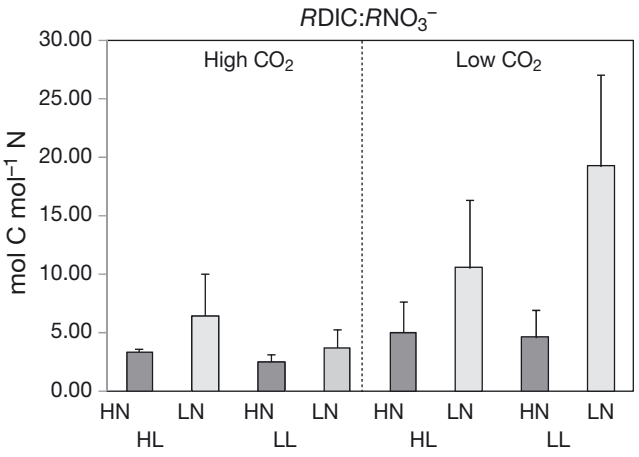

Fig. 8. Ratio of ^{13}C to nitrate uptake rates ($RDIC:RNO_3^-$) measured on Day 6 under the different experimental conditions (treatment abbreviations as in Fig. 1). Results are mean ±1 SD of 3 microcosm replicates

coastal station sampled for the GAP experiments (Neale et al. 2014) are consistent with those typical of summertime. The abundance and composition of phytoplankton in the initial sample (see Reul et al. 2014) also matches those described for summer samples collected by Reul et al. (2005) and Mercado et al. (2007), who reported abundance of diatoms and dinoflagellates of approximately 100 and 30 cell ml^{-1}, respectively. Consequently, it is reasonable to conclude that the phytoplankton community at the beginning of the experiment was fully acclimated to nitrate limitation and high irradiance. This hypothesis is also supported by the physiological features of the initial phytoplankton, with low chl *a* content per biomass (5.1 µg chl *a* mg^{-1} POC) which is even lower than that reported by Arín et al. (2002) for phytoplankton sampled in oligotrophic areas of the Alboran Sea. Another indicator of phytoplankton acclimation to low nitrate initial *in situ* conditions is the low rate of NO_3^- uptake, which is comparable with rates reported for Alboran phytoplankton isolated in nutrient limited areas (Mercado et al. 2014).

It could be expected that bacterioplankton in the initial sample were also fully acclimated to the *in situ* prevailing nutrient conditions. The bacterial abundance obtained in the initial sample (1.5×10^6 cell ml^{-1}) is within the range described by Fernández et al. (1994) and Van Wambeke et al. (2004) for the Alboran Sea but is higher than the values published for other oligotrophic areas of the Western Mediterranean Sea (0.7 to 0.9×10^6 cell ml^{-1}; Pedrós-Alió et al. 1999, Morán et al. 2001) and the Atlantic ocean (0.2 to 0.4×10^{-6} cell ml^{-1}; Mills et al. 2008).

Some authors have demonstrated that natural phytoplankton communities could actively take up thymidine when supplied at low concentrations (Mulholland et al. 2011). Consequently, thymidine incorporation rates could overestimate BP. In our experiments, the thymidine incorporation rates were significantly correlated with bacterial biomass (r^2 = 0.60; $p < 0.01$) but not with total plankton or phytoplankton biomass. Consequently, it is reasonable to assume that the changes in thymidine incorporation rates in our experiments reflected changes in bacterial activity. Accordingly, the high initial bacterial community production in our experiments (2.5 µg C l^{-1} h^{-1}) is consistent with the high bacterial abundance. These relatively high values of both biomass and BP would indicate that the *in situ* bacterial community was less affected than phytoplankton by nutrient limitation. Some experiments of nitrate and phosphate addition performed with natural plankton samples from the Eastern Mediterranean suggest that bacteria are more affected by limitation of phosphate than nitrate (Thingstad et al. 2005, Zohary et al. 2005). In our initial sample, phosphate concentration was low but the nitrate:phosphate molar ratio was much lower than the Redfield ratio, indicating that phosphate could have a reduced role in limiting plankton growth. The values of BP and RR obtained in our study can be used for calculating the bacterial growth efficiency (BGE; i.e. the amount of new bacterial biomass produced per unit of assimilated organic C substrate). It has been demonstrated that BGE varies systematically with the trophic status of the system (del Giorgio & Cole 1998). If it is assumed that bacterial contribution to community respiration ranges from 50 to 100% (as reported for communities in the Western and Eastern Mediterranean Sea; Navarro et al. 2004, Lagaria et al. 2011), BGE in the initial sample would vary between 0.10 and 0.18, i.e. one order of magnitude higher than BGE reported by del Giorgio & Cole (1998) for oligotrophic systems (i.e. 0.01) and lower than that of the most eutrophic systems (0.5).

Effects of enclosure and treatments on phytoplankton metabolism

Bottle effect and other experimental artefacts could potentially affect the results of the microcosm experiments. In our experimental setup, the samples were pre-screened by 200 µm mesh before incubation. Abundance of total diatoms in the initial sample (non-filtered) was 218.8 cells ml^{-1}, compared to 205.5 cells ml^{-1} for the screened samples. Furthermore, abundances of *Rhizosolenia* and other large diatoms were lower than 5 cells ml^{-1} in both pre-filtered and filtered samples. Therefore, pre-screening did not substantially affect the abundance or composition of the phytoplankton community. However, filtration brings with it removal of mesozooplankton, which could explain the increase in the abundance of >6 µm cells (ultraphytoplankton) produced from Day 0 to 2. Reul et al. (2014) attributed this change to relaxation of the top-down control exercised by mesozooplankton on ultraphytoplankton. These unspecific effects were also notable in the metabolic rates of the plankton community, as PP increased by 3.5 times on average by Day 2 in comparison with Day 0, irrespective of the treatment (including the treatment that simulated *in situ* conditions, i.e. LC LN HL; Fig. 2a). The data presented in this article demonstrate that the PP increase was not only due to increasing phytoplankton biomass but also to changes in PP^B (Fig. 2b). Irradiance during PP incubations averaged 539 and 232 µmol photons m^{-2} s^{-1} in HL and LL treatments, respectively (Neale et al. 2014). These irradiance values were probably not limiting for photosynthesis. If this is the case, PP is representative of the maximal photosynthetic capacity (P_{max}) of the community that depends on the Calvin cycle activity. Consequently, our results indicate that the activity of the Calvin cycle increased in all treatments by Day 2, as expected due to the increase in the abundance of large relative to small cells (Raven & Kübler 2002, Litchman et al. 2009). The differences in PP^B from Days 0 to 2 (Fig. 2b) can be explained by higher photosynthesis rates per carbon unit (Geider et al. 1986) and lower metabolic costs of the diatoms in comparison with flagellates and other phytoplankton groups. Furthermore, the phytoplankton assemblage experienced high light at the sampling station; therefore, relief from high-irradiance stress could have contributed to the increase in PP observed by Day 2 (Neale et al. 2014, Sobrino et al. 2014).

In addition to the changes produced by the enclosure effect, notable changes in phytoplankton metabolism due to the treatments can be discerned. Note that repeated measures ANOVA were carried out by using the data obtained from Days 2 to 6 as repeated measurements. This approach controlled for the day-to-day variability associated with the above-described experimental artefacts. Furthermore, some of the physiological responses attributable to the treatments are similar to those described previously for experiments done with phytoplankton cultures and natural assemblages. For instance, phytoplankton growth under nutrient limitation normally reduces

the chl *a* per cell content (Stramski et al. 2002). Consistently, chl *a* per phytoplankton biomass was reduced at LN in our experiments. Furthermore, nutrient limitation affected both *R*DIC and RNO_3^- (at least by Day 6; Fig. 7). The reduction of RNO_3^- compared to *R*DIC was stronger at LN than HN. A consequence of this finding is that DIC over-consumption was produced at LN (i.e. higher DIC uptake than expected from nitrate or phosphate removal). DIC over-consumption is frequent in phytoplankton growing under limited nutrients (Sorensen & Siegel 2001, Geider & LaRoche 2002, Mei et al. 2005) and brings with it formation of organic matter with a C:N ratio exceeding the Redfield ratio (Schartau et al. 2007), as was obtained in our experiments (Neale et al. 2014). It is normally assumed that the DIC excess assimilated by the phytoplankton is channelled through the pool of photosynthetic DOC (e.g. Kähler & Koeve 2001) or alternatively used to produce secondary metabolites (Berman-Frank & Dubinsky 1999) and accumulation of lipids in diatoms growing in nutrient starvation (Hockin et al. 2012, Yang et al. 2013).

Changes in bacterial metabolism and plankton community respiration

Zooplankton grazing is an important source of dissolved organic matter in the natural environment (Nagata & Kirchman 1991). Absence of meso- and macrozooplankton in the experiments (caused by the pre-screening of the samples) would imply that there was less DOC available for bacterial metabolism in all treatments. However, BP increased at high nutrients and decreased at low nutrients (Fig. 3a). Consequently, the enclosure effect on bacterial metabolism appeared to be lower than on the phytoplankton community.

In the HN treatments, BP decreased steeply on Day 4 and recovered on Day 6 (Fig. 3a). A linear relationship between bacterial biomass and BP was obtained ($BP = 0.04BB + 0.26$; $r = 0.77$, $p < 0.01$, $n = 24$), therefore the changes in BP reflected changes in biomass. Consequently it can be assessed that nutrient addition produced higher bacterial growth rates and efficiency. Increases in heterotrophic production following the addition of nutrients have also been described in experiments performed with Mediterranean plankton assemblages (Lagaria et al. 2011).

Apparently, these changes in bacterial metabolism did not affect the respiration rates of the whole community, as the differences in RR among high and low nutrient treatments were not significant (Fig. 4). RR and plankton biomass were not correlated, indicating that respiration was not a simple function of biomass—likely because of community acclimation to the experimental conditions (Wu et al. 2010, Teira et al. 2012) as well as changes in the composition of the community over time and among treatments. Several studies have indicated that the contribution to community respiration of bacteria relative to phytoplankton decreases with increasing nutrient availability because of increases in phytoplankton respiration (Biddanda et al. 2001, Chan et al. 2004). Furthermore, Roberts & Howarth (2006) found that the bacterial contribution to respiration is lower when light availability for photosynthesis increases. According to the results of nutrient addition experiments performed by Roberts & Howarth (2006), the variability patterns of community respiration following nutrient enrichment are related to the higher metabolic needs of phytoplankton growing actively, while bacterial respiration remains more stable. In our experiments, the main factor contributing to variability of RR was light (Table 1), supporting the hypothesis that the changes in RR were related to changes in phytoplankton respiration (direct effects of irradiance level on bacterial respiration can be discarded).

Effects of high CO_2 on plankton metabolism and carbon balance

It has been proposed that high CO_2 could modulate the response of phytoplankton to changing light and nutrient conditions. The results of our experiments indicate that the effect of high CO_2 on the metabolic responses of the phytoplankton to nutrient additions was limited. Among the variables measured in the present study, only ^{13}C uptake rate by Day 6 was affected by CO_2 level irrespective of nutrient and light levels (see Reul et al. 2014 and Sobrino et al. 2014 for a more complete description of other high CO_2 effects). Most articles reporting effects of high CO_2 on primary production describe slight increases or absence of effects (see Riebesell & Tortell 2011 for a review). However in our experiments, the DIC uptake rates in phytoplankton acclimated to high CO_2 reduced in comparison with present CO_2. Decreases in *R*DIC under high CO_2 are generally a consequence of the down-regulation of carbon concentrating mechanisms (Beardall & Raven 2004). Additionally, higher photo-inhibition at high CO_2 has been described for natural samples under full solar radiation and PAR exposures (Sobrino et al. 2009, Gao et al. 2012). Nitrate uptake was not modified

at high CO_2 (as obtained by Bellerby et al. 2008). Consequently, the uptake of DIC relative to nitrate decreased under acidification conditions. A further consequence of this change is that the composition of the organic matter produced by phytoplankton was modified at high CO_2. It could be expected that these changes in organic matter quality would produce changes in bacterial production and respiration. Concordantly, BP was significantly affected by the interaction of C × L (Table 1), indicating that reduction of BP at LL was mitigated at HC. However, RR was affected significantly by the interaction of C × N (Table 1). Particularly, reduction of RR at LN was less pronounced at HC than at LC. Total DOC concentration in our experiments was higher at HC (see Neale et al. 2014), which could explain this interactive effect that was apparently independent of the interaction of C × L.

The effect of the physiological changes induced by CO_2 on POC and PON balance in the experiments was limited, as demonstrated in Fig. 9. At LC, the addition of nutrients induced increases in POC and PON (estimated from the *R*DIC and RNO_3^-) by 2 to 5 times compared with the increases produced at LN (Fig. 9a). BP and plankton community respiration integrated over time were also higher at HN compared with LN. At HC, addition of nutrients also promoted higher rates of bacterial and phytoplankton production integrated over time compared with LN (Fig. 9b). However, the differences between HN and LN were comparatively less than those obtained at LC, particularly for PON production by phytoplankton and at LL. Consequently, amounts of POC and PON accumulated in the treatments were similar under the 2 CO_2 conditions, indicating that the differences in the metabolic rates of the plankton did not reflect differences in the amount of produced POM. If the results of our experiments are extrapolated to the natural environment, it can be concluded that high CO_2 will have a limited effect on POM production in the Alboran Sea, at least during the phytoplankton blooms triggered by upwelling episodes. However, other aspects such as changes in biomass and phytoplanktonic DOC productions should also be taken

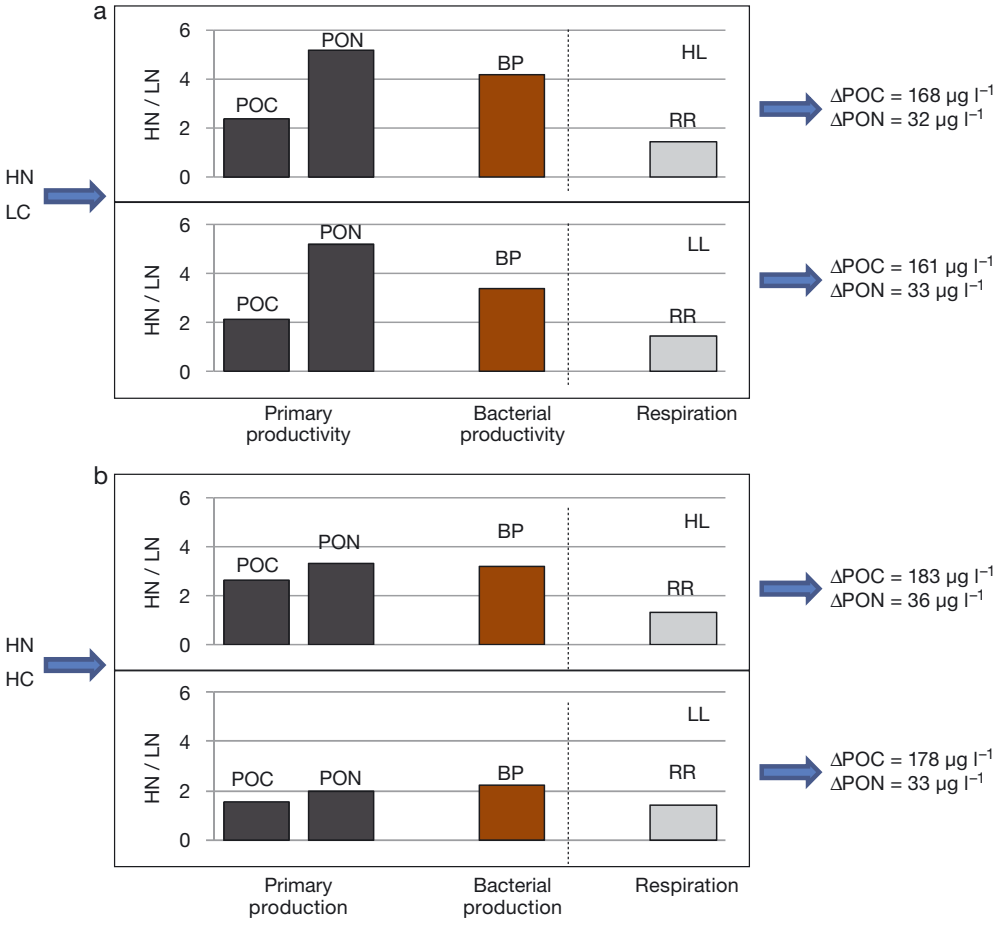

Fig. 9. Schematic diagram showing the increase in plankton metabolic rates produced by the nutrient addition compared with the changes produced in the low nutrient (LN) treatments. The bars represent cumulative increases of particulate organic carbon (POC) and nitrogen (PON) primary production, bacterial production (BP) and respiration rates (RR) in the high nutrient (HN) treatments relative to the increases obtained in the low nutrient treatments. Cumulative values of BP and RR were calculated by integrating (linear interpolation method) over the whole experiment time, using the values obtained on Days 0, 2, 4 and 6. POC and PON primary production was calculated from the uptake rates of dissolved inorganic carbon and nitrate obtained on Day 6. Net balances of POC and PON (ΔPOC and ΔPON) under the different experimental conditions are also shown

into account. If it is assumed that the biological pump activity in the northern Alboran Sea depends on the occurrence of these upwelling episodes, our results would indicate that the role of this area as an atmospheric CO_2 sink will not be substantially affected by ocean acidification. However, extrapolation of these results to the natural environment has to be done with caution as it is possible that changes in the planktonic composition to high CO_2 requires more prolonged experiments.

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