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Response of two marine bacterial isolates to high CO_2 concentration

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ABSTRACT: Experimental results related to the effects of ocean acidification on planktonic marine microbes are still rather inconsistent and occasionally contradictory. Moreover, laboratory or field experiments that address the effects of changes in CO_2 concentrations on heterotrophic microbes are very scarce, despite the major role of these organisms in the marine carbon cycle. We tested the direct effect of an elevated CO_2 concentration (1000 ppmv) on the biomass and metabolic rates (leucine incorporation, CO_2 fixation and respiration) of 2 isolates belonging to 2 relevant marine bacterial families, Rhodobacteraceae (strain MED165) and Flavobacteriaceae (strain MED217). Our results demonstrate that, contrary to some expectations, high pCO_2 did not negatively affect bacterial growth but increased growth efficiency in the case of MED217. The elevated partial pressure of CO_2 (pCO_2) caused, in both cases, higher rates of CO_2 fixation in the dissolved fraction and, in the case of MED217, lower respiration rates. Both responses would tend to increase the pH of seawater acting as a negative feedback between elevated atmospheric CO_2 concentrations and ocean acidification.

KEY WORDS: Bacterial metabolism · Flavobacteriaceae · Ocean acidification · Rhodobacteraceae

INTRODUCTION

In the last 200 yr, the oceans have absorbed about 50% of the anthropogenic CO_2 (Sabine et al. 2004), which has resulted in a reduction of surface seawater pH of 0.1 units (Royal Society 2005). Caldeira & Wickett (2003) estimate that surface ocean seawater pH will decrease by 0.7 units over the next 200 yr.

Ocean acidification could have severe consequences for marine biota, including both calcifying and non-calcifying organisms (Raven et al. 2005, Fabry et al. 2008), but there is no solid evidence about how the different organisms will react to the coupled pCO_2-pH change (Hendriks et al. 2010). In the case of marine microbes, the experimental results are inconsistent and occasionally contradictory (Joint et al. 2011, Liu et al. 2010).

Compared to experiments on phytoplankton, far fewer laboratory or field experiments have assessed the effects of changes in CO_2 concentrations on heterotrophic microbes (Joint et al. 2011), despite these playing a major role in the marine carbon cycle, mineralizing organic carbon in the oceans to CO_2 (del Giorgio & Williams 2005). A few mesocosm experiments have tested the effect of high CO_2 concentrations on the abundance and/or production of natural bacterioplankton populations (Rochelle-Newall et al. 2004, Grossart et al. 2006, Allgaier et al. 2008). These studies have found either no or indirect (linked to phytoplankton dynamics) effect of elevated partial pressure of CO_2 (pCO_2) on bacterial production (BP). Only a few studies have demonstrated a direct effect of CO_2 on natural prokaryotic plankton from the deep ocean (Coffin et al. 2004, Yamada et al. 2010) or

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on marine bacterial isolates (Takeuchi et al. 1997, Labare et al. 2010). The latter studies found a decrease in the production and growth rates at pH < 7 — values far from the usual pH observed in ocean waters under present or future scenarios of elevated pCO_2.

Most microorganisms, particularly heterotrophic bacteria, are able to assimilate CO_2 as part of their metabolism through anaplerotic reactions (Roslev et al. 2004). Although light-independent or dark CO_2 assimilation has been usually assumed to be insignificant in oxygenated marine waters, a recent work by Alonso-Sáez et al. (2010) suggests that the global relevance of this process could have been underestimated. Those results show for the first time that high ambient CO_2 concentrations could stimulate CO_2 fixation rates by increasing the CO_2 flux into the cells.

A comprehensive understanding of the effect of elevated CO_2 concentration on carbon cycling in the ocean requires the analysis of both production and respiration rates to provide a total carbon budget. However, to the best of our knowledge none of the published studies have simultaneously addressed the effect of CO_2 on BP and respiration, which are essential variables for bacterial growth efficiency (BGE) calculations. Allgaier et al. (2008) did find changes in bacterial taxonomic composition in response to high CO_2 concentrations, which suggest that the effects of elevated pCO_2 are likely to vary among species. Therefore, the aim of the present study was to test the direct effect of elevated CO_2 concentrations (1000 ppmv) on the biomass and metabolic rates (leucine incorporation, CO_2 fixation and respiration) of 2 bacterial isolates.

In order to test the direct effect of pCO_2 on bacterioplankton, it is essential to isolate them from the rest of the microbial food web components. The best approach is working with isolates or strains that are abundant and/or relevant in the ocean and that are likely to respond in a different way to a similar stress. We selected different bacterial isolates representative of 2 important families in marine surface waters: Rhodobacteraceae (MED165) and Flavobacteriaceae (MED217).

Rhodobacteraceae includes the *Roseobacter* lineage, which is adapted to use monomers, such as sugars or amino acids, and typically constitute about 20% of the bacterial community in eutrophic coastal waters (Buchan et al. 2005, Alonso-Gutiérrez et al. 2009). On the other hand, members of the family Flavobacteriaceae are successful in the degradation of polymeric substances and are particularly abundant during decaying phytoplankton blooms (Pinhassi et al. 2004, Alderkamp et al. 2006, Teira et al. 2008), possibly accounting for up to more than 50% of total bacterial abundance.

MATERIALS AND METHODS

Bacterial cultures and experimental setup

The 2 strains (MED165 and MED217) were isolated in May 2001 from the surface waters (5 m) of Blanes Bay (Mediterranean Sea) and were kindly provided by J. M. Gasol (ICM-CSIC). MED165 belongs to Rhodobacteraceae and is 99.8% similar to *Roseobacter* sp. AY576690; whereas MED217 belongs to Flavobacteriaceae and is 99.6% similar to *Cytophaga* sp. AY745817. Both MED165 and MED217 were isolated from 1:20 seawater dilution cultures enriched with inorganic phosphorus. Hereafter we refer to these isolates as *Roseobacter* (MED165) and *Cytophaga* (MED217).

The strains were initially grown in Zobell liquid medium (4 g of peptone [Bacto™ Peptone; Difco 211677] and 0.8 g of yeast extract [Bacto Yeast Extract; Difco 212750] dissolved in 600 ml of 0.2 µm filtered seawater and 200 ml of sterile MQ water) for 1 wk. Thereafter, they were grown in 0.2 µm filtered seawater amended with 0.5 ml of Zobell medium per litre of seawater in 2 l glass culture flasks.

In order to determine the periodicity of dilution for establishing a semi-continuous culture, we first studied the growth characteristics of each strain in the diluted media used (Fig. 1). Both strains approached the stationary phase after 28 h; therefore, 80% dilutions were done every 24 h. The derived growth rate, calculated using a logistic model, was slightly higher for *Roseobacter* than for *Cytophaga*, with doubling times of 4.1 and 5.3 h, respectively.

Semi-continuous cultures (n = 4) for each strain were simultaneously maintained by daily 80% dilutions with fresh medium. Culture flasks were kept in an incubation chamber at 18°C under 16 h light:8 h dark cycles. Light was provided by cool white fluorescent tubes and irradiance, measured in air in a position close to the centre of the culture flasks, was 250 µmol photons m^{-2} s^{-1}. The cultures were allowed to acclimate to the experimental conditions by continuously bubbling with the target CO_2 levels for 5 d under the light and temperature conditions previously described. To ensure sterile conditions mixed gases were supplied after filtering through a 0.2 µm membrane filter. The pCO_2 of fresh medium used for daily dilutions was also adjusted to the experimental

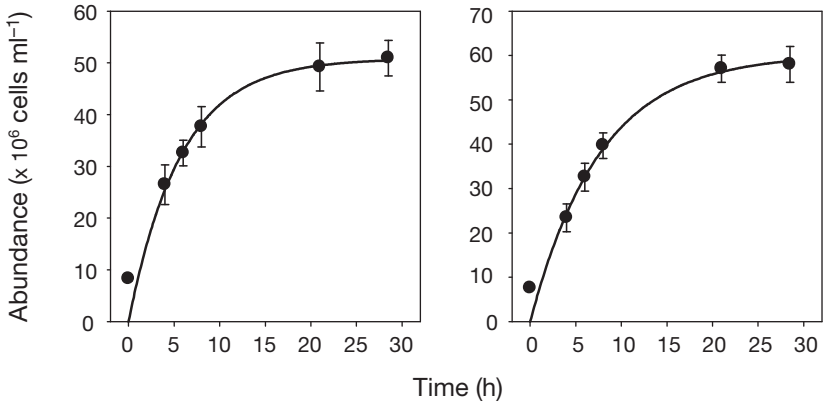

Fig. 1. Growth of (A) *Roseobacter* (MED165) and (B) *Cytophaga* (MED217) cultures in Zobell medium under light-dark cycles. Error bars represent ± SE of microscope cell counts (n = 20)

conditions. Cultures were aerated with air pumped from an open area outside the building, for the samples incubated under regular atmospheric CO_2 levels (Ambient CO_2: 380 ppmv) (n = 2), and with a mix of air and CO_2 from a 2000 to 3000 ppmv CO_2 gas tank (Air Liquide), for the samples incubated under elevated CO_2 levels (Elevated CO_2: 1000 ppmv final concentration) (n = 2). The mixture air:CO_2 reaching the cultures was controlled by a double tube flow meter, and the flow in each flask was regulated by individual flow meters (Aalborg). After the acclimation period, a single sample was taken at 09:00 h (approx. 12 h after dilution). Sampling was carried out by siphonation using the flow generated after sucking out the air with a syringe connected via silicon tube to a glass tube inserted in each flask. Samples were taken for the estimation of inorganic and organic carbon system variables and fluorescence of coloured organic matter, as well as for bacterial abundance and activity (leucine incorporation, CO_2 fixation and respiration rates).

Dissolved inorganic carbon and pCO_2

Water samples (n = 3) for the analysis of dissolved inorganic carbon (DIC) in each flask were collected in 5 ml glass serum vials and analysed with a Non-Dispersive Infrared Gas Analyzer (LiCOR 7000) within a few hours after collection. The system was standardised with Na_2CO_3 solutions. pH and temperature were measured with a Crison pH 25 pH meter and salinity with a Pioneer 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 (DOE 1994). The pCO_2 in the water samples was calculated from salinity, temperature, pH, and DIC measurements using the inorganic carbon equilibrium constants from Merhbach et al. (1973) as refit by Dickson & Millero (1987), the boric acid constant from Dickson (1990), the ionic product of water from Millero (1995), and the dissociation constant of bisulfate ion from Dickson (1990).

Fluorescence of particulate and dissolved organic matter (DOM)

Samples for dissolved organic carbon (DOC) and fluorescence of coloured dissolved organic matter (FDOM) quantification were collected in 250 ml acid-cleaned Winkler flasks and filtered through acid-rinsed 0.2 µm filters (Pall Supor membrane disc) in an acid-cleaned, all-glass filtration system under low N_2 flow pressure within 1 h of collection. Aliquots for the analysis of DOC were drawn into pre-combusted (450°C, 12 h) 10 ml glass ampoules, acidified with 25% H_3PO_4 to pH < 2, heat sealed, and preserved at 4°C until determination with a Shimadzu TOC-VCS analyzer under the principle of high temperature catalytic oxidation. The catalyser was 0.5% Pt on Al_2O_3 balls. The DOC concentration of each sample was obtained by subtracting the average (± SD) peak area of 3 to 5 injections (150 µl) from the average (± SD) peak area of the freshly produced Milli-Q water used as a blank and dividing by the slope of the standard curve with potassium hydrogen phthalate (Álvarez-Salgado & Miller 1998).

Aliquots for the quantification of FDOM were collected directly in a quartz cell of 1 cm path-length and measured in a Perkin Elmer LS 55 luminiscence spectrometer. The fluorescence of particulate organic matter (FPOM) was obtained by subtracting the FDOM from the fluorescence of the unfiltered sample. The Perkin Elmer LS 55 was equipped with a xenon discharge lamp, equivalent to 20 kW for 8 µs duration. Discrete excitation/emission (Ex/Em) pair measurements were performed at Coble's (1996)

peak-M, characteristic of marine humic-like substances (Ex/Em: 320 nm/410 nm) and peak-T, characteristic of protein-like substances (Ex/Em: 280 nm/350 nm). Four replicate measurements were performed for each Ex/Em pair. The system was calibrated with a mixed standard of quinine sulphate (QS) and tryptophan (Trp) in sulphuric acid 0.05 M (Nieto-Cid et al. 2005). The equivalent concentration of every peak was determined by subtracting the average peak height from the corresponding Milli-Q water blank height and dividing by the slope of the standard curve. Fluorescence units were expressed in ppb equivalents of QS (ppb QS) for peak-M and ppb equivalents of Trp (ppb Trp) for peak-T. The precision was ±0.1 ppb QS and ±0.6 ppb Trp, respectively.

Cell abundance and biovolume

One or 2 ml samples were fixed with 0.2 µm filtered formaldehyde (1 to 2% final concentration) and subsequently stored at 4°C in the dark for 12 to 18 h. Thereafter, each sample was filtered through a 0.2 µm polycarbonate filter (Millipore, GTTP; 25 mm filter diameter) supported by a cellulose nitrate filter (Millipore, HAWP; 0.45 µm), washed twice with Milli-Q water, dried, and stored in a microfuge vial at −20°C. For total bacterial counts, cells were stained with a DAPI (4′,6′-diamidino-2-phenylindole) mix (5.5 parts of Citifluor [Citifluor], 1 part of Vectashield [Vector Laboratories] and 0.5 parts of PBS with DAPI [1 µg ml^{-1} final concentration]). The slides were examined with an epifluorescence microscope equipped with a 100 W Hg-lamp and appropriate filter sets for DAPI. More than 200 DAPI-stained cells were counted per sample. Bacterial biovolumes were estimated from DAPI images as $(\pi/4)W^2(L - W/3)$, where L is length, and W is width. Although bacterial biovolume is commonly examined by using DAPI staining for DNA, it is important to note that DAPI images may underestimate cell sizes (Suzuki et al 1993).

Catalysed reported deposition-fluorescence *in situ* hybridisation (CARD-FISH)

In order to control potential cross-contamination of the cultures we used catalysed reported deposition-fluorescence *in situ* hybridisation (CARD-FISH) with oligonucleotide probes specific for the *Bacteroidetes* group (CF319a) (Manz et al. 1996) and the *Roseobacter* lineage (Ros537). Filters for CARD-FISH were embedded in low-gelling-point agarose and incubated with lysozyme (Teira et al. 2008). Filters were cut in sections and hybridized for 2 h at 35°C with horseradish peroxidase (HRP)-labelled oligonucleotide probes. Tyramide-Alexa488 was used for signal amplification (20 min) as previously described (Pernthaler et al. 2002). We used 55% formamide for both probes. Cells were finally counter-stained with a DAPI-mix. The slides were examined with an epifluorescence microscope equipped with a 100 W Hg-lamp and appropriate filter sets for DAPI and Alexa488. More than 200 DAPI-stained cells were counted per sample. For each microscope field, 2 different categories were enumerated: (1) total DAPI-stained cells and (2) cells stained with the specific probe. The counting error, expressed as the percentage of standard error between replicates (SE/mean × 100), was <2% for DAPI counts and <10% for FISH counts.

The CARD-FISH using probes of the lineage *Roseobacter* (Ros537) and for the class Bacteroidetes (CF319a), indicated that problems of cross-contamination were negligible in the case of *Roseobacter* cultures (ca. 100% of the DAPI-stained cells hybridized with the Ros537 probe both in the High and in the Low CO_2 treatments), whereas a minor cross-contamination with *Roseobacter* cells was detected in the *Cytophaga* cultures (ca. 90% of the DAPI-stained cells hybridized with the CF319a probe, and ca. 10% with the Ros537; both in the High and in the Low treatments) (data not shown).

Leucine incorporation

The [^{3}H] leucine incorporation method, modified as described by Smith & Azam (1992), was used to determine BP. Samples (4 replicates and 2 killed controls) were incubated for 40 min in the same incubation chamber as the cultures. The theoretical leucine to carbon conversion factor (CF) was used to calculate bacterial biomass production (BP) rates from Leu uptake rates (3.1 kg C mol Leu^{-1}).

CO_2 fixation

Eight 5 ml acid-cleaned glass vials were filled with culture, inoculated with 180 kBq (5 µCi) of $NaH^{14}CO_3$ and then incubated for 1 h in a temperature-controlled photosynthetron incubator (CHPT). Each 2 replicates were exposed to a range of 4 irradiance levels (0, 100, 250, and 1000 µmol photons m^{-2} s^{-1}) in

order to assess the effect of light on bacterial CO_2 fixation. After the incubation period, the samples were filtered at very low vacuum pressure (<50 mm Hg) through 0.2 µm polycarbonate filters using a system that allows the recovery of the filtrate. The filters and the filtrates were decontaminated by exposing them to fumes of concentrated HCl for 12 h and by adding 100 µl of 50% HCl and shaking for 12 h, respectively. Two extra replicates were inoculated and immediately decontaminated (without incubation) in order to obtain a time zero value. A multipurpose liquid scintillation cocktail was used for both filters and filtrates (Insta-Gel plus, Perkin Elmer). Quenching corrections were made using an external standard. As no significant effect of irradiance was found, either for *Roseobacter* or for *Cytophaga* (ANOVA test, $p > 0.5$, $n = 16$), we averaged the disintegrations per minute (DPMs) of the 8 bottles and subtracted the mean DPMs of the time zero value for CO_2 fixation calculations. The amount of ^{14}C fixed as particulate organic carbon (P-CO_2 fix) and the amount of ^{14}C subsequently released from the cells to the dissolved fraction (D-CO_2 fix) was obtained as the mean value in the filter and mean value in the filtrate, respectively.

Respiration

Oxygen consumption rates were determined by dark-bottle incubations. Six dark, 50 ml, gravimetrically calibrated, borosilicate bottles were carefully filled from each culture, using silicone tubing, overflowing >100 ml. An initial set of 3 dark bottles was fixed immediately for initial oxygen concentration, the remainder being kept in the dark for 1 to 2 h in the same incubation chamber as the cultures. Dissolved oxygen concentration was measured through automated precision Winkler titration performed with a Metrohm 721 DMS Titrino, using a potentiometric end point detector as described in Serret et al. (1999). Bacterial respiration (BR) was converted into C units by using a respiration quotient (RQ) of 0.8 (Williams & del Giorgio 2005).

Statistical analysis

Data were log or arcsin transformed to attain normality and homocedasticity (tested by Kolmogorov-Smirnov and Levene tests, respectively). The unpaired *t*-test was then used for comparisons of 2 datasets (Elevated vs. Ambient treatments). When homocedasticity failed, we used a *t*-test assuming unequal variances. An ANOVA test was used for comparison of more than 2 datasets. All statistical analyses were computed using SPSS statistics ver. 19.0 software. In order to correct for the small sample size ($n = 4$), we applied the correction proposed by Good (1982), substituting p by $p\sqrt{0.5}$.

RESULTS

CO_2 system variables

At the beginning of the acclimation period, the pCO_2 of the air bubbled into the cultures was adjusted to 1000 ppmv in the Elevated CO_2 treatment and to 380 ppmv in the Ambient CO_2 treatment. Due to bacterial activity at the sampling time, i.e. 12 h after 80% dilution with acclimated fresh medium, the pCO_2 of seawater in the Elevated CO_2 treatment was 1162 ± 84 ppmv in the *Roseobacter* and 1137 ± 51 ppmv in the *Cytophaga* cultures. By contrast, in the Ambient CO_2 treatment, the seawater pCO_2 was significantly (*t*-test, $p = 0.047$, $n = 4$) higher in the *Cytophaga* cultures (412 ± 27 ppmv) than in the *Roseobacter* cultures (242 ± 11 ppmv). The mean pH in the Elevated CO_2 treatment was 7.60 in both cultures, whereas the pH in the Ambient CO_2 treatment was significantly higher (*t*-test, $p = 0.026$, $n = 4$) in *Roseobacter* (8.17 ± 0.03) than in *Cytophaga* cultures (7.99 ± 0.02).

DOC and induced fluorescence of particulate and DOM

DOC concentration at the sampling time was higher in the *Roseobacter* (372 ± 5 µmol C l^{-1}) than in the *Cytophaga* (286 ±10 µmol C l^{-1}) cultures, and no significant differences were found between the Elevated and the Ambient CO_2 treatments. The protein-like fluorescence of particulate organic matter (FPOM-T) at the sampling time was higher in the *Roseobacter* than in the *Cytophaga* cultures (Fig. 2A). There were no significant differences between the mean FPOM-T in the Elevated and Ambient CO_2 treatments. The protein-like fluorescence of dissolved organic matter (FDOM-T) followed a very similar pattern than the FPOM-T (Fig. 2B). By contrast, the humic-like fluorescence of dissolved organic matter (FDOM-M) in the *Cytophaga* culture was significantly higher (*t*-test, $p = 0.03$, $n = 4$) in the Ambient than in the Elevated CO_2 treatment (Fig. 2C).

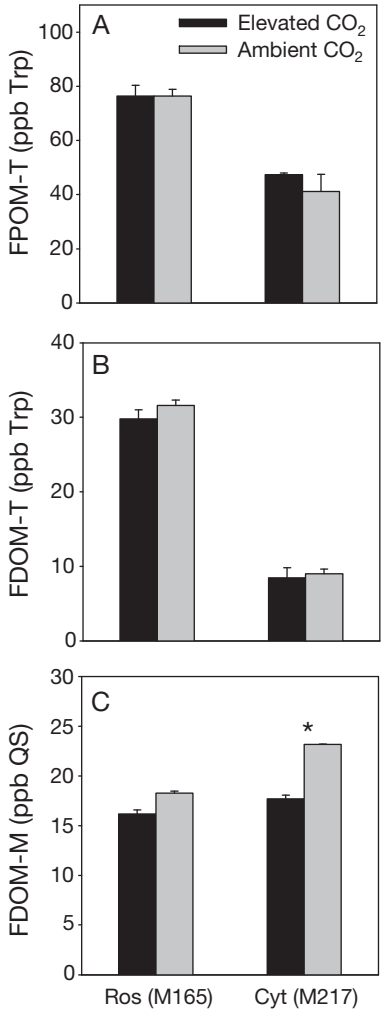

Fig. 2. (A) Protein-like fluorescence of particulate organic matter (FPOM-T), (B) protein-like fluorescence of dissolved organic matter (FDOM-T), and (C) humic-like fluorescence of the dissolved organic matter (FDOM-M) in the *Roseobacter* and *Cytophaga* cultures in the Elevated (black bars; n = 2) and Ambient (white bars; n = 2) CO_2 treatments. Significant differences between the Elevated and Ambient CO_2 treatments are marked with an asterisk (*p < 0.05)

Bacterial abundance and activity rates

After the acclimation period, the abundance of *Roseobacter* at the sampling time was ca. 50 × 10^6 cells ml^{-1}, whereas that of *Cytophaga* was ca. 80 × 10^6 cells ml^{-1} (Fig. 3A). There were no significant differences between the Elevated and the Ambient CO_2 treatments. The mean biovolume of *Roseobacter* cells (0.71 ± 0.05 μm^3) was higher than that of *Cytophaga* cells (0.35 ± 0.05 μm^3), and no significant differences were found between the Elevated and Ambient CO_2 treatments.

Despite lower cell abundance, rates of leucine incorporation BP were almost twice as high in the *Roseobacter* than in the *Cytophaga* culture (Fig. 3B). There were no significant differences between CO_2 treatments. Similarly, anaplerotic CO_2 fixation rates (both dissolved and particulate) were also higher in *Roseobacter* than in *Cytophaga* (Figs. 3C,D). There were no significant differences in the rates of CO_2 fixation measured in the particulate fraction (P-CO_2 fix; Fig. 3C) between treatments, whereas those measured in the dissolved fraction (D-CO_2 fix) were significantly higher in the Elevated than in the Ambient CO_2 treatment (Fig. 3D) in both isolates. Overall, P-CO_2 fixation represented 8 to 9% of the BP in both isolates.

On the other hand, BR was higher in the *Cytophaga* than in the *Roseobacter* cultures, particularly in the Ambient CO_2 treatment (Fig. 3E). BR was significantly higher in the Ambient than in the Elevated CO_2 treatment in the case of *Cytophaga* (*t*-test, p = 0.02, n = 4). The derived BGE, estimated as BP/(BP + BR), was higher for *Roseobacter* than for *Cytophaga* (Fig. 3F). *Cytophaga* showed a significantly (*t*-test, p = 0.005, n = 4) higher BGE in the Elevated CO_2 (0.57 ± 0.01) than in the Ambient CO_2 (0.49 ± 0.02) treatment, while *Roseobacter* growth efficiency did not respond to the elevated CO_2.

DISCUSSION

Both detrimental and stimulatory effects of seawater acidification associated with elevated pCO_2 on marine plankton have been widely demonstrated at the organism level; including both phytoplankton and zooplankton species (see review by Riebesell et al. 2008). However, to the best of our knowledge, the effect of high pCO_2 has been scantily tested on single marine heterotrophic bacteria species (Takeuchi et al. 1997, Labare et al. 2010). Moreover, none of these studies tested the effect of elevated CO_2 on respiration or CO_2 fixation rates. Both processes are relevant in terms of dissolved inorganic carbon system chemistry and carbon cycling, as CO_2 fixation reduces, and respiration increases, the concentration of CO_2 in seawater. The objective of our study was to evaluate the direct effect of elevated pCO_2 levels on catabolic and anabolic processes rates of 2 relevant marine strains. Our results revealed that *Roseobacter* and *Cytophaga* strains, presumably carrying out contrasting functions in the ecosystem (Teira et al. 2008, 2009), responded differently to high pCO_2.

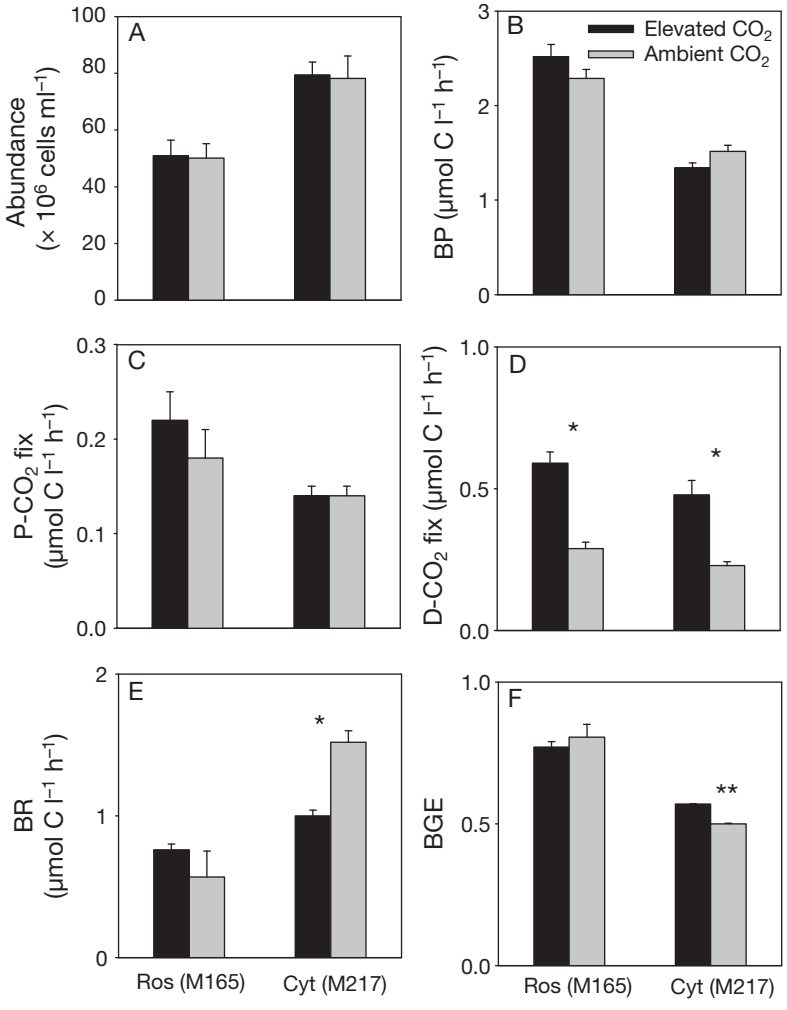

Fig. 3. (A) Bacterial abundance, (B) leucine incorporation rates (BP), (C) CO_2 fixation measured in the particulate fraction (P-CO_2 fix) rates, (D) CO_2 fixation measured in the dissolved fraction (D-CO_2 fix) rates, (E) bacterial respiration (BR), and (F) bacterial growth efficiency (BGE) in *Roseobacter* and *Cytophaga* cultures in the Elevated (black bars; n = 2) and Ambient (white bars; n = 2) CO_2 treatments. Significant differences between the Elevated and Ambient CO_2 treatments are marked with an asterisk (*$p < 0.05$; **$p < 0.01$)

Roseobacter and *Cytophaga* metabolism under present-day pCO_2 levels (380 ppmv)

The bacterial yield in terms of cell abundance was lower for *Roseobacter* than for *Cytophaga* (50 vs. 80 × 10^6 cells ml^{-1} at the sampling time; Fig. 3A). The lower bacterial yield of *Roseobacter* contrasts with its higher BP and P-CO_2 fixation rates. A possible explanation could be a larger cell size of *Roseobacter* compared to *Cytophaga*. Based on microscope estimates, we found that the mean biovolume of *Roseobacter* cells was twice that of *Cytophaga* cells. The higher FPOM-T in *Roseobacter* than in *Cytophaga* cultures also suggests higher bacterial biomass in the *Roseobacter* cultures.

Cell-specific BP rates in the Ambient CO_2 treatment (5.4 and 13.1 fg C $cell^{-1}$ d^{-1} for *Cytophaga* and *Roseobacter*, respectively) are within the range of cell-specific BP rates measured in coastal waters where members of Rhodobacteraceae and Flavobacteriaceae are particularly abundant (Reinthaler & Herndl 2005, Lamy et al. 2009, Lekunberri et al. 2010).

Heterotrophs can assimilate CO_2 in various carboxylation reactions as part of central and peripheral pathways (Dijkhuizen & Harder 1985). We found that CO_2 fixation was independent of light in both strains, which points out to a purely heterotrophic assimilation process. The daily cellular rates of P-CO_2 fixation in the Ambient CO_2 treatment by *Roseobacter* (1.2 fg C $cell^{-1}$ d^{-1}) and *Cytophaga* (0.43 fg C $cell^{-1}$ d^{-1}) were about 1 order of magnitude higher than those reported by Alonso-Sáez et al. (2010) in arctic waters. The P-CO_2 fixation represented 7 to 8% of the total carbon anabolism, which agrees well with most previous estimates that suggest that 1 to 8% of the organic carbon in some heterotrophic bacteria can be attributed to CO_2 assimilation in carboxylation reactions (Romanenko 1964, Doronia & Trotsenko 1985, Roslev et al. 2004).

The higher D-CO_2 fixation and the lower respiration rates measured in *Roseobacter* compared to *Cytophaga* may explain the differences in pH and pCO_2 observed in the Ambient CO_2 treatment (8.17 in *Roseobacter* vs. 7.99 in *Cytophaga*), as CO_2 fixation would tend to increase seawater pH and respiration would tend to decrease pH.

Roseobacter and *Cytophaga* metabolism under high pCO_2 future levels (1000 ppmv)

In the case of *Roseobacter*, the only significant response to elevated pCO_2 was an increment in the rate of D-CO_2 fixation, a pattern also observed in *Cytophaga*. Although the effect of elevated pCO_2 on

the heterotrophic CO_2 fixation has never been tested, an increase in photosynthetically produced-DOC release, as a consequence of high pCO_2, has been previously observed in phytoplankton (e.g. Engel et al. 2004, 2005, Riebesell et al. 2007). Although the mechanism of CO_2 fixation differs between phytoplankton and bacterioplankton, our results indicate that the fraction of recently fixed carbon released as DOM by heterotrophic bacteria could also increase under high seawater pCO_2. By contrast, neither P-CO_2 fixation nor leucine incorporation (BP) were significantly affected by elevated CO_2, which is also in accordance with there being no differences in cell abundance and biovolume. Several studies with natural microbial plankton populations, testing similar high CO_2 levels, either failed to find a significant effect on bulk leucine incorporation rates (Allgaier et al. 2008) or did find an increase related to a higher DOC excretion by phytoplankton (Grossart et al. 2006).

Interestingly, a significant decrease in bacterial respiration occurred in the Elevated CO_2 treatment in *Cytophaga* (Fig. 3E), which produced an increase in its growth efficiency under high pCO_2 conditions (Fig. 3F). As far as we know, the effect of high CO_2 on the growth efficiency of single marine bacterial species or natural bacterial communities has never been tested before (Liu et al. 2010). The higher respiration rates measured in the Ambient than in the Elevated CO_2 treatment strongly concurs with the significantly higher concentration of humic substances observed in the Elevated CO_2 treatment (Fig. 2C). Several studies have demonstrated the correlation between microbial respiration rates and rates of humic matter formation (Nieto-Cid et al. 2006, Lønborg et al. 2010). A decrease in respiration rates of soil bacteria with increasing CO_2 concentration (0 to 1000 ppmv) was reported by Koizumi et al. (1991), although they did not provide an explanation for this metabolic response.

A decrease in phytoplankton respiration under high pCO_2 conditions has been recently suggested by Hopkinson et al. (2010). These authors speculated that this respiration decline could be most likely related to a reduced energy cost on intracellular pH homeostasis. Most non-extremophilic bacteria grow over a broad range of external pH values, from 5.5. to 9.0, and maintain a cytoplasmatic pH within the narrow range of 7.4 to 7.8 (Booth 1985, Padan et al. 2005). Surface ocean bacteria grow in alkaline environments (pH ~8.2) and there is a large number of adaptive strategies for alkaline pH homeostasis (Padan et al. 2005), including increased metabolic acid production, increased ATP synthase that couples proton entry to ATP generation, changes in membrane properties, and increased expression and activity of monovalent cation/proton antiporters. Thus, energy savings when pH approaches the desired intracellular pH likely vary among different bacterial species, depending on the strategy for pH regulation. Unfortunately, we do not have information about the mechanisms implied in pH regulation in the studied strains. The lower external pH in the Elevated CO_2 treatment (pH = 7.60) could imply a reduced energy expense and thus, a higher growth efficiency and a lower total carbon demand, for the *Cytophaga* but not for the *Roseobacter* strain.

Our results contrast with recent observations of increases in bacterial polysaccharide degradation under pH lowered by 0.2 to 0.3 units, which has the potential to enhance respiratory CO_2 production under high pCO_2 future scenarios (Piontek et al. 2010). In the case of *Cytophaga* cultures, the implication of a reduced respiration under high CO_2 concentration is a negative feedback to rising CO_2. We have shown that laboratory experiments with cultured organisms may provide valuable information on physiological responses to the perturbation of CO_2 concentrations. We have demonstrated here that some metabolic rates of 2 important representatives of marine bacteria do change in response to a higher CO_2 concentration, and, contrary to some expectations, lowering pH did not negatively affect bacterial growth or even increased growth efficiency in the case of *Cytophaga*. In both cases, the bacterial activity under high pCO_2 (higher D-CO_2 fixation or higher D-CO_2 fixation plus lower respiration rates) would increase the buffering capacity of seawater.

The complex interactions among microbial plankton organisms and different environmental factors imply that experiments under *in situ* conditions and with natural plankton communities are also essential to understand how the pelagic ecosystems will react to an increase in surface ocean CO_2 concentration. Our results suggest the need for including simultaneous measurements of several key metabolic processes in CO_2 perturbation experiments with natural microbial populations to better understand the net effect of human-induced rising seawater CO_2. Moreover, we have shown that the response may vary among different bacteria taxa, and thus it is crucial to simultaneously analyze changes in bacterial function and taxonomic composition, as well as to conduct experiments over a wide range of different bacterial community structures.

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