# Response of two marine bacterial isolates to high $CO_2$ concentration

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Running title: Bacterial metabolism under high $pCO_2$

ABSTRACT: Experimental results relative to the effects of ocean acidification on planktonic marine microbes are still rather inconsistent and occasionally contradictory. Moreover, laboratory or field experiments addressing the effects of changes in $CO_2$ concentrations on heterotrophic microbes are very scarce, despite their major role 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 two isolates belonging to two 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 $pCO_2$ 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 past 200 years the oceans have absorbed about 50% of the anthropogenic $CO_2$ (Sabine et al. 2004), which has resulted in a decrease of surface seawater pH by 0.1 units (Royal Society 2005). Caldeira & Wickett (2003) estimate that surface ocean seawater pH will reduce by 0.7 units over the next 200 years.

Ocean acidification could have severe consequences for marine biota, including both calcifying and non-calcifying organisms (Raven et al. 2005, Fabry et al. 2008), yet, there is not 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. 2010, Liu et al. 2010).

Compared to phytoplankton, much less laboratory or field experiments have assessed the effects of changes in $CO_2$ concentrations on heterotrophic microbes (Joint et al. 2010), despite playing a major role in the marine carbon cycle, mineralizing organic carbon in the oceans to $CO_2$ (del Giorgio & Williams 2005). A few mesocosms 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 $pCO_2$ on bacterial production. 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 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 nevertheless 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 bacterial production and respiration, which are essential variables for bacterial growth efficiency calculations. On the other hand, 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 our work 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 two 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. Moreover, the best approach is working with isolates or strains which 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 two important families in marine surface waters: Rhodobacteraceae (MED165) and Flavobacteriaceae (MED217).

The Rhodhobacteraceae 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-Gutierrez 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), eventually accounting for up to more than 50% of total bacterial abundance.

## MATERIAL AND METHODS

**Bacterial cultures and experimental setup.** The two 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 Dr JM 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 phosphorous. Thereafter, we will 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 one week. 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 ºC under 16h light: 8h 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 during 5 days 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 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 2000-3000 ppmv $CO_2$ gas tank (Air Liquide S.A.), 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 made 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 sampling took place at 9:00 a.m. (approx. 12 hours after dilution). It was carried out by gravity using the flow generated after sucking the air of a silicon tube with a syringe

connected to the tube and 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 LiCOR Non-Dispersive Infrared Gas Analizer 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 in 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 partial pressure of $CO_2$ in the water samples ($pCO_2$) 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 one hour of collection. Aliquots for the analysis of DOC were drawn into pre-combusted (450 ºC, 12 h) 10 mL glass ampoules, acidified with 25% $H_3PO_4$ to pH < 2, heat sealed and

preserved at 4 ºC 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 3-5 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 P.G. 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 (1-2% final concentration) and subsequently stored at 4 ºC in the

dark for 12-18 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 ºC. For total bacterial counts cells were stained with a DAPI (4', 6'-diamidino-2-phenylindole)-mix (5.5 parts of Citifluor [Citifluor, Ltd.], 1 part of Vectashield [Vector Laboratories, Inc.] 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. Despite 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 ºC with horseradish peroxidase (HRP)-labelled oligonucleotide probes. Tyramide-Alexa488 was used for signal amplification (20 min) as previously described (Perthaler 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: (i) total DAPI-stained cells and (ii) 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 bacterial production. Samples (four replicates and two 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 Inc.). Each two 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 a significant effect of irradiance was not found neither for *Roseobacter* nor for *Cytophaga* (ANOVA test, $p > 0.5$, $n=16$), we averaged the disintegrations per minute (DPMs) of the eight 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 1-2 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 two datasets (Elevated versus Ambient treatments). When homocedasticity failed we used a T-test assuming unequal variances. An ANOVA test was used for comparison of more than two datasets. All statistical analyses were computed using SPSS statistics 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 bubbling 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 hours 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 dissolved organic matter

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).

**Bacterial abundance and activity rates**

After the acclimation period, the abundance of *Roseobacter* at the sampling time was ca. 50 x $10^6$ cells $mL^{-1}$, whereas that of *Cytophaga* was ca. 80 x $10^6$ 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 $\mu m^3$) was higher than that of *Cytophaga* cells (0.35 ± 0.05 $\mu m^3$), and no significant differences were found between the Elevated and Ambient $CO_2$ treatments.

Despite lower cell abundance, rates of leucine incorporation (bacterial production, BP) were almost twice 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 8-9% of the BP in both isolates.

On the other hand, bacterial respiration (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 bacterial growth efficiency (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 $CO_2$ ($0.57 \pm 0.01$) than in the Ambient $CO_2$ ($0.49 \pm 0.02$) treatment, while *Roseobacter* growth efficiency did not respond to the elevated $CO_2$.

## DISCUSSION

Both detrimental and stimulatory effects of seawater acidification associated to 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 two 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 $pCO_2$.

**_Roseobacter_ and _Cytophaga_ metabolism under present-day $pCO_2$ levels (380 ppmv)**

The bacterial yield in terms of cell abundance is lower for *Roseobacter* than for *Cytophaga* (50 vs. 80 x $10^6$ 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* rather 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 one order of magnitude higher than those reported by Alonso-Sáez et al. (2010) in arctic waters. The P-$CO_2$ fixation represented 7-8 % of the total carbon

anabolism, which agrees very well with most previous estimates which suggest that 1-8% 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* versus 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 had 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 dissolved organic matter by heterotrophic bacteria could also increase under high seawater $pCO_2$. By contrast, both P-$CO_2$ fixation and leucine incorporation (BP) were not significantly affected by elevated $CO_2$ which is also congruent with 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 dissolved organic carbon 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 (0-1000 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 *Cytophaga* but not for *Roseobacter* strain.

Our results contrast with recent observations of increases in bacterial polysaccharide degradation under pH lowered by 0.2-0.3 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 two 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 implies 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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LITERATURE CITED

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**Figure legends.**

Figure 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).

Figure 2. (A) Protein-like fluorescence of the particulate organic matter (FPOM-T), (B) protein-like fluorescence of the 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 (*, $p < 0.05$; **, $p < 0.01$).

Figure 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 (*, $p < 0.05$; **, $p < 0.01$).

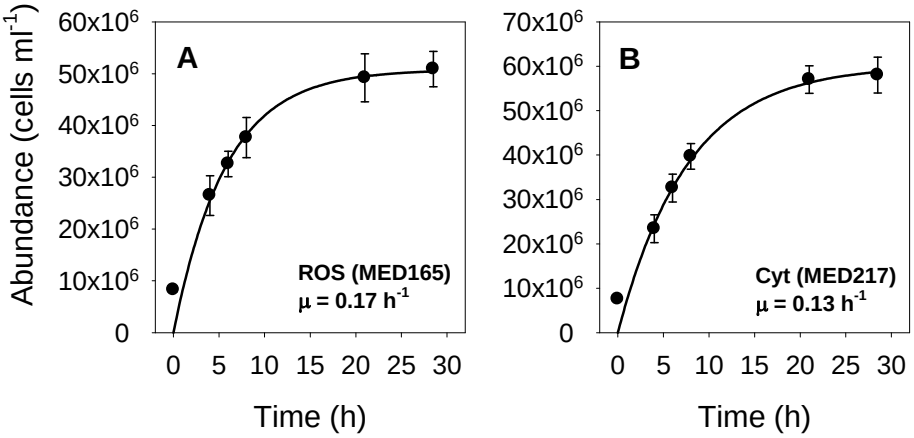

Abundance (cells ml-1)
60x10^6
50x10^6
40x10^6
30x10^6
20x10^6
10x10^6
0
A
ROS (MED165)
μ = 0.17 h-1
0 5 10 15 20 25 30
Time (h)
70x10^6
60x10^6
50x10^6
40x10^6
30x10^6
20x10^6
10x10^6
0
B
Cyt (MED217)
μ = 0.13 h-1
0 5 10 15 20 25 30
Time (h)

Figure 1

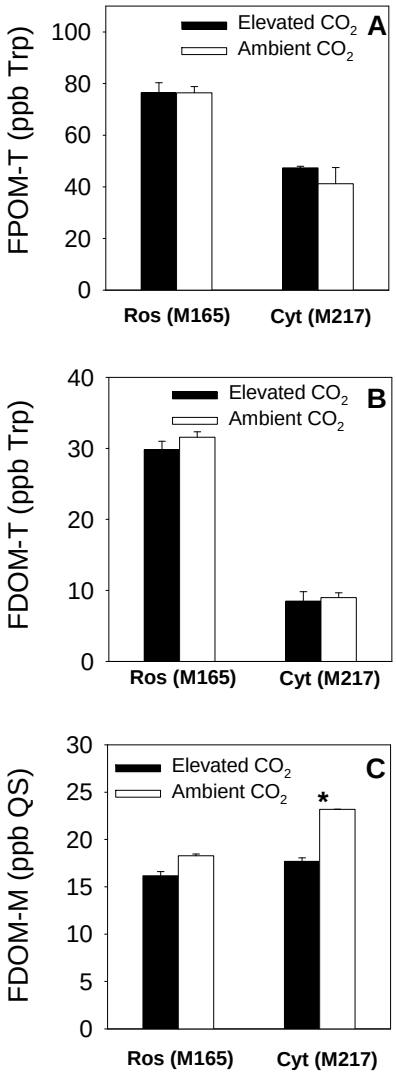
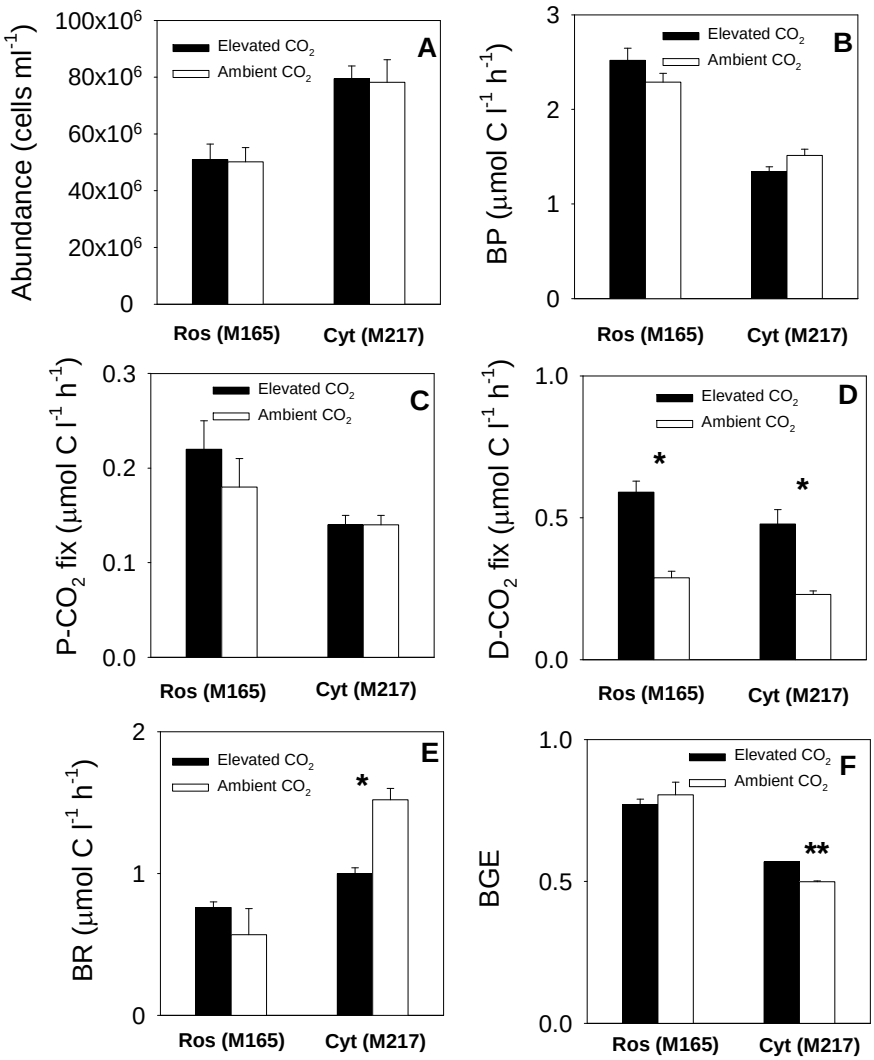

FPOM-T (ppb Trp)
Elevated $CO_2$
Ambient $CO_2$
A
100
80
60
40
20
0
Ros (M165)
Cyt (M217)
FDOM-T (ppb Trp)
B
40
30
20
10
0
FDOM-M (ppb QS)
C
30
25
20
15
10
5
*

Figure 2




1

2

3 Figure 3

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