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Elevated pCO_2 Alters Marine Heterotrophic Bacterial Community Composition and Metabolic Potential in Response to a Pulse of Phytoplankton Organic Matter

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Originality-Significance Statement

This is the first study to directly link measurements of bacteria-mediated carbon cycling to changes in community composition and physiology in response to elevated pCO_2 during simulated phytoplankton bloom conditions. This work contributes to our understanding of the

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metabolic functions of heterotrophic bacteria that are influence by the ongoing global enrichment of inorganic carbon in the ocean.

Abstract

Factors that affect the respiration of organic carbon by marine bacteria can alter the extent to which the oceans act as a sink of atmospheric carbon dioxide. We designed seawater dilution experiments to assess the effect of pCO_2 enrichment on heterotrophic bacterial community composition and metabolic potential in response to a pulse of phytoplankton-derived organic carbon. Experiments included treatments of elevated (1000 ppm) and low (250 ppm) pCO_2, amended with 10 µmol L^{-1} dissolved organic carbon from *Emiliana huxleyi* lysates, and were conducted using surface-seawater collected from the South Pacific Subtropical Gyre. To assess differences in community composition and metabolic potential, shotgun metagenomic libraries were sequenced from low and elevated pCO_2 treatments collected at the start of the experiment and following exponential growth. Our results indicate bacterial communities changed markedly in response to the organic matter pulse over time and was significantly affected by pCO_2 enrichment. Elevated pCO_2 also had disproportionate effects on the abundance of sequences related to proton pumps, carbohydrate metabolism, modifications of the phospholipid bilayer, resistance to toxic compounds and conjugative transfer. These results contribute to a growing understanding of the effects of elevated pCO_2 on bacteria-mediated carbon cycling during phytoplankton bloom conditions in the marine environment.

Introduction

Heterotrophic bacteria play a critical role in the marine carbon cycle. They consume 50 % or more of the dissolved organic carbon (DOC) produced in the surface ocean by photosynthesis (Azam *et al.* 1983; Ducklow 1999). At low growth efficiencies bacterial respiration results in the conversion of the majority of consumed organic carbon to carbon dioxide (CO_2). Collectively, these processes decrease the amount of DOC and can affect the rate at which recently produced DOC accumulates in the surface ocean. Reduced accumulation of DOC can diminish the contribution of DOC to the vertical export of carbon via the biological pump (Passow and

Carlson 2012). Thus, alterations to the marine system that affect bacterial consumption and subsequent respiration of organic carbon can have profound impacts on the marine carbon cycle.

We recently reported that increases in pCO_2 (e.g. 1000 ppm) in short-term experiments enhanced bacterial removal of organic carbon and increased bacterial respiration from the start of the experiments to stationary growth phase (James *et al.* 2017). This result was observed for natural bacterial communities growing on a range of organic carbon compounds, from glucose to more complex phytoplankton products and naturally occurring DOC. Additionally, enhanced bacterial respiration and removal of organic carbon were observed for experiments conducted at various sites, and which represented oceanic regions that varied in the frequency and magnitude of their exposure to elevated pCO_2 (e.g. subtropical gyres versus coastal upwelling systems). Collectively, these experiments suggest that short-term increases in pCO_2 can lead to enhanced rates of bacteria-mediated removal of organic carbon and respiration of DOC to CO_2, thus decreasing the rate at which DOC accumulates in the surface ocean. Despite the biogeochemical implications of this result, a mechanistic understanding of the phylogenetic and physiological response of marine heterotrophic bacteria to elevated pCO_2 remains uncertain.

Though recent experiments using microcosms and mesocosms have provided significant insight to the effects of elevated pCO_2 on natural bacterial communities, the results are often conflicting. For example, multiple studies report significant shifts in the composition of free-living bacterial communities in response to elevated pCO_2 and low pH (Krause *et al.* 2012; Maas *et al*. 2013; Zhang *et al*. 2013; Roy *et al.* 2013; Siu *et al.* 2014; Bunse *et al.* 2016), while others observe negligible effects (Sperling *et al.* 2013; Newbold *et al.* 2012; Oliver *et al.* 2014; Allgaier *et al.* 2008). These studies were conducted at various locations throughout the world's oceans, such that variability in the response of these microbial communities to elevated pCO_2 and low pH may reflect differences in the extant communities. In addition, the majority of these studies incorporate organisms from multiple trophic levels and likely reflect both the direct effects of

elevated pCO_2 on marine heterotrophic bacteria and the indirect effects mediated through other food web processes.

Recent studies of the consequences of elevated pCO_2 on bacterial physiology and metabolism also revealed variable results. Some studies suggest that low pH conditions lead to increased bacterial degradation of carbohydrates through enhanced extracellular enzymatic rates of ß-glucosidase (Grossart *et al.* 2006; Piontek *et al.* 2010; Maas *et al.* 2013; Piontek *et al.* 2013; Endres *et al.* 2014), while Yamada and Suzumura (2010) observed no effect of pCO_2 on rates of extracellular ß- glucosidase. The response of bacterial abundance to elevated pCO_2 is also variable, with some studies observing an increase in bacteria (Endres *et al.* 2014; Arnosti *et al.* 2011), in contrast to others which observed no difference in cell number (Grossart *et al.* 2006; Allgaier *et al.* 2008, Yamada & Suzumura 2010). These studies suggest that the effects of elevated pCO_2 on the ecological function of bacteria are variable, and likely reflect differences in experimental design, manipulation of inorganic carbon parameters, and bacterial communities resulting from various experimental sites.

Variability in the response of marine bacteria to elevated pCO_2 highlights the need for studies that directly link measurements of bacteria-mediated carbon cycling to changes in community composition and physiology to gain an understanding of the ecological function of heterotrophic bacteria during exposure to elevated pCO_2 in various oceanic regions. Bunse *et al.* (2016) conducted an elegant mesocosm experiment that provides insight to the physiological response of Mediterranean bacterial communities to pCO_2 enrichment during phytoplankton blooms. In the presence of phytoplankton communities, Bunse *et al.* (2016) showed that elevated pCO_2 stimulated transcript abundance of respiratory proton pumps that aid in translocating protons across the cell membrane, suggesting that bacteria upregulate respiratory proton pumps to export protons that invade the cell as a result of high external pCO_2. Consistent with our results from seawater culture experiments (James *et al.* 2017), Bunse *et al.* (2016) suggest that upregulating

proton pumps may decrease bacterial growth efficiency. Though this study offers insight to the physiological and phylogenetic responses of bacteria to changes in pCO_2, predicting how elevated pCO_2 will affect carbon bacteria-mediated cycling requires measurements of organic carbon removal and bacterial growth dynamics.

Here we present a metagenomic shotgun sequence analysis of the metabolic pathways encoded by marine heterotrophic bacterial communities in the presence of freshly produced phytoplankton-derived organic matter (simulating post-bloom dynamics) and the effect of exposure to elevated pCO_2. Shifts in the overall community composition and functional potential induced by pCO_2 enrichment, concomitant with enhanced bacterial removal of phytoplankton-derived organic carbon and decreased bacterial growth efficiency, provide new insight to phylogenetic and physiological adaptations of marine bacterial communities to elevated pCO_2 conditions.

Experimental Procedures

A seawater culture experiment was conducted using subsurface seawater (25 m) collected in July 2014 near French Polynesia in the South Pacific Subtropical Gyre (17˚ 26'S, 149˚ 43'W). A mixture of one-third whole surface seawater and two-thirds 0.2 µm- gravity filtered seawater (0.2 µm mixed cellulose ester filter, GSWP, Millipore, Billerica, MA) was combined to provide naturally occurring heterotrophic bacterial communities and freshly produced phytoplankton-derived dissolved organic carbon (DOC). Filtered and unfiltered seawater was combined and equally divided between two polycarbonate containers. In order to obtain the desired pCO_2 levels, water was bubbled at approximately 100 mL min^{-1} with CO_2-mixed air (Scott Marrin Inc.) to achieve low (250 ppm) or elevated (1000 ppm) levels. We were unable to measure pCO_2 at sea but applied the same flow rate and bubbling time that resulted in effective pCO_2 adjustment previously (James *et al.* 2017). Using ship-board measurements of total alkalinity (~2340 µmol kg^{-1}) and assuming successful alteration of pCO_2 to 1000 ppm we were able to estimate pH in the

elevated pCO_2 incubations - this calculation indicates that at most, external pH values may have dropped from ~ 8.1 to ~7.7.

pCO_2-adjusted seawater was transferred into new polycarbonate carboys and a very small volume of *Emiliana huxleyi* lysate (1.2 mL to 11.5 L of experimental seawater) was added to a final concentration of approximately 10 µmol C L^{-1}. Duplicate incubations were placed in a dark, temperature-controlled incubator at 22 ˚C. Incubations were sampled for TOC, cell abundance, and community DNA over a ten-day period. These samples were collected twice daily, for five days, followed by less frequent sampling for the remainder of the experiment (Fig 1A). Measured values of TOC include both DOC and particulate organic carbon as bacterial biomass. As such, the change in TOC between two time points is a measure of the amount of carbon lost to bacterial respiration (James *et al.* 2017). Two-way analysis of variance (two-way ANOVA) was performed to assess the effects of pCO_2 and time point on bacterial growth dynamics including cell abundance, cell-specific TOC removal and bacterial growth efficiency.

Metagenomic samples were collected on days 0, 2 and 3 of the experiment. These time points coincided with the start of the experiment, and two time points following exponential growth phase (Fig 1A). Samples were collected by filtering 300 mL of seawater through a 0.2 µm polyethersulfone filter (Supor-200, Pall, Port Washington, New York) under low vacuum pressure (≤ 10 mm Hg). Filters were loaded into sterile cryovials and stored frozen (-80 ˚C at sea, -40 ˚C onshore). Samples were lysed in sucrose lysis buffer (40 mmol L^{-1} EDTA, 50 mmol L^{-1} Tris-HCl, 750 mmol L^{-1} sucrose, 400 mmol L^{-1} NaCl, pH adjusted to 8.0) with 1% sodium dodecyl sulfate and 0.2 mg mL^{-1} proteinase-K at 55˚C for two hours. Genomic DNA was extracted using the MOBIO DNEasy PowerSoil Kit (QIAGEN, Carlsbad, California) and normalized to 0.2 ng μL^{-1}. Metagenomic libraries were prepared using Nextera XT (Illumina, San Diego, USA) and sequenced on the MiSeq2 Platform at San Diego State University using the 600 cycle PE sequencing reagent kit (Illumina, San Diego, USA).

A total of 8,276,624 reads were obtained for samples collected on days 0, 2, and 3. The average length of pre-quality controlled forward sequence reads per sample ranged from 200 ± 81 to 259 ± 62 base pairs, while reverse reads ranged from 203 ± 83 to 261 ± 62 base pairs.

Bacterial Community Structure & Metabolic Potential

Metagenomic sequence reads were uploaded to the MG-Rast server (Meyer et al. 2008). Briefly, the MG-Rast pipeline trims low quality sequence regions using SolexaQA (Cox *et al.* 2010), removes artificial duplicate reads using a k-mer based dereplication step (Gomez-Alvarez *et al.* 2009), and removes sequences with near-exact matches to model organisms and humans using Bowtie (Langmead et al. 2009). Features are then identified using FragGeneScan to identify protein coding regions (Rha *et al.* 2010), and finally annotated and compared with the SEED protein database (Overbeek *et al*. 2005; Aziz *et al.* 2008). The average length of quality controlled forward sequence reads ranged from 194 ± 78 to 245 ± 70 base pairs, while reverse reads were relatively shorter and ranged from 171 ± 69 to 214 ± 72 base pairs. We chose to analyze only forward reads as these were consistently longer in length. Taxonomic and metabolic functional annotations were analyzed using the default requirements in MG-Rast (e-value: 5, identity: 60 %, length: 45 bp, and minimum abundance: 1). For taxonomic analysis, relative abundances of bacterial taxa were normalized to the total number of significant sequence similarities. For metabolic analysis, the relative abundances of metabolic functional genes were normalized using the total number of Identified Protein Features (i.e. predicted protein coding regions).

Relative abundance of sequences represents the average of duplicate samples at the start of the experiment (day 0) and at two time points following exponential growth phase (days 2 and 3), unless otherwise stated. Statistical analysis of the community taxonomic and metabolic response to the addition of phytoplankton lysate (i.e. comparing samples from the start of the experiment

[n=2] to those following exponential growth phase [n=4]) was evaluated using one-way permutational multivariate analysis of variance (PERMANOVA) with the 'adonis' function in the R package 'vegan' (Oksanen *et al*. 2013). Following exponential growth phase, two-way PERMANOVA was performed to assess the community taxonomic and metabolic response to the independent and combined effects of pCO_2 (low and elevated) and time point (days 2 and 3). Two-way ANOVA was performed to assess if there were significant differences between the mean relative abundances of individual bacterial groups with the independent and combined effects of pCO_2 (low and elevated) and time point (days 2 and 3) following exponential growth phase. PERMANOVA and two-way ANOVA statistical analyses were performed on arcsine square root transformed relative abundance and bacterial growth efficiency data; cell-specific TOC removal and cell abundance data was log10 transformed.

Evaluation of significantly differentially abundant metabolic functions was conducted using EdgeR (Robinson *et al.* 2010; Jonsson *et al.* 2016). EdgeR applies a negative binomial distribution to sequence count data and identifies differential abundance using an exact test based on the quantile-adjusted conditional maximum likelihood method. For this analysis, the time points following exponential growth phase (i.e. days 2 and 3) were combined in order compare the effects of elevated pCO_2 on bacterial community metabolism. Significant metabolic functions required the log2 fold change between elevated and low pCO_2 treatments to be ≥ 1.5, corresponding to a difference in relative abundance of 2.8, or greater. These sequences are publicly available through the MG-Rast server under the project name OA8_OA11_2016_AJames (http://metagenomics.anl.gov/linkin.cgi?project=mgp19414).

Results

Changes in cell abundance of the natural heterotrophic bacterial communities demonstrated typical dilution culture patterns of lag, exponential, and stationary growth (Fig 1A). Mean bacterial abundance yield on days 2 and 3 showed no difference between pCO_2 treatments (p

value = 0.34, two-way ANOVA) or time points (p value = 0.51, two-way ANOVA; Fig 1A). In contrast to cell abundance, the mean magnitude of cell–specific TOC removal increased from day 2 to 3 (p value < 0.01, two-way ANOVA), and was significantly enhanced with elevated pCO_2 (day 2: 127.7 ± 6.1 fg C $cell^{-1}$, day 3: 168.6 ± 19.9 fg C $cell^{-1}$), compared with low pCO_2 (day 2: 103.4 ± 9.0 µmol C $cell^{-1}$, day 3: 129.9 ± 6.1 fg C $cell^{-1}$; p value = 0.01, two-way ANOVA; Fig 1B). Cell-specific TOC removal was calculated as the change in TOC (ΔTOC) from the start of the experiment to days 2 and 3, divided by the concomitant change in cell abundance. Measured values of TOC include both DOC and the carbon associated with bacterial biomass. As such, ΔTOC represents the oxidation of organic carbon substrates and thus, bacterial respiration, indicating greater cell-specific bacterial respiration with elevated pCO_2 (James *et al.* 2017). Enhanced cell-specific respiration and similar bacterial abundance yield led to significantly depressed bacterial growth efficiencies on days 2 and 3 with elevated pCO_2 (day 2: 7.3 ± 0.3 %, day 3: 5.6 ± 0.6 %), compared with low pCO_2 (day 2: 8.9 ± 0.7 %, day 3: 7.2 ± 0.3 %; p value = 0.01, two-way ANOVA), and a significant decrease from day 2 to 3 (p value < 0.01, two-way ANOVA). The interaction between pCO_2 and time point was non-significant for all two-way ANOVA and PERMANOVA tests (below). As a result, the interaction term was removed from all multivariate models prior to calculating reported p values.

Bacterial Community Composition

Comparison of sequences to the SEED database indicates a significant shift in the bacterial community composition from the start of the experiment (T_0) to following exponential growth phase (p value = 0.03, one-way PERMANOVA; Fig 2A) in response to the pulse of phytoplankton lysate. Higher relative abundances of Prochlorococcaceae (33.5 ± 0.7 %) and SAR 11 (15.3 ± 0.9 %) at the beginning of the experiment were superseded following exponential growth phase, as Gammaproteobacteria families, Pseudoalteromonadaceae (day 2: 35.7 ± 6.1 %, day 3: 32.1 ± 7.7 %), Alteromonadaceae (day 2: 26.0 ± 5.4 %, day 3: 22.6 ± 4.5 %), Shewanellaceae (day 2: 6.9 ± 1.1 %, day 3: 6.2 ± 1.2 %), and Vibrionaceae (day 2: 5.2 ± 0.8

%, day 3: 4.1 ± 0.4 %), became more relatively abundant in the later timepoints (Fig 3, Table 1). Rhodobacteraceae remained among the most relatively abundant families over the course of the experiment, increasing from 6.6 ± 0.1 % at the beginning of the experiment to 16.0 ± 4.5 % on day 3 (Fig 3, Table 1).

Changes in pCO_2 (p value = 0.01, two-way PERMANOVA) and time point (p value < 0.01, two-way PERMANOVA) also led to significant shifts in the bacterial community composition following exponential growth phase. Of the five most abundant taxa, four exhibited a significant response to pCO_2 (Table 1): Psuedoalteromonadaceae and Shewanellaceae were significantly more abundant with elevated pCO_2, compared with low pCO_2 (p values = 0.01, two-way ANOVA; Fig 3, Table 1). In contrast, relative abundances of Alteromonadaceae and Vibrionacea were significantly reduced with elevated pCO_2, compared with low pCO_2 treatments (p values = 0.01, two-way ANOVA; Fig 3, Table 1). Rhodobacteraceae did not show a significant response to changes in pCO_2 (p value 0.3 Table 1); however, Rhodobacteraceae increased significantly from 5.6 ± 0.5 % on day 2, to 16.0 ± 4.5 % on day 3 (p value = 0.03, two-way ANOVA; Fig 3, Table 1). In contrast, Vibrionaceae decreased significantly from day 2 (5.2 ± 0.8 %) to day 3 (4.1 ± 0.4 % ; p value = 0.03; two-way ANOVA, Fig 3, Table 1). Thirty-two, less abundant taxa including Idiomarinaceae, Flavobacteriaceae, Aeromonadaceae, Alcanivoracaceae, also exhibited significant positive or negative responses to elevated pCO_2 (Table 1).

Bacterial Metabolic Potential

Comparison of sequences to the SEED database led to the identification of 71,784 to 197,399 protein coding regions across samples (referred to as Identified Protein Features). These Identified Protein Features were then annotated as putative metabolic functional genes based on sequence homology. Metabolic functional gene assignments comprised 4961 putative metabolic functions for all sequence libraries in this dataset. For the remainder of the paper, reference to *metabolic functions* refers to the number of sequence reads assigned to these putative metabolic

functions relativized by the number of Identified Protein Features. Microbial community metabolism was compared between samples by classifying metabolic functions (Subsystem Level: Function) into three hierarchical groups of decreasing granularity termed Subsystem Levels 1-3 (metabolic pathways annotated into a stepwise hierarchy; Overbeek *et al.* 2005).

The composition of metabolic functions (Subsystem Level: Function) at the start of the experiment was significantly different from that following exponential growth phase (p value = 0.02, one-way PERMANOVA; Fig 2C). In addition, the composition of metabolic functions encoded following exponential growth phase differed by pCO_2 level (p value = 0.01, two-way PERMANOVA) and time point (i.e. day 2 versus day 3; p value = 0.02, two-way PERMANOVA; Fig 2D). Hierarchical grouping of metabolic functions into the broadest designations (Subsystem Level 1; e.g. Carbohydrate metabolism) reveals significant shifts in 21 out of 28 metabolic functional categories from the start of the experiment (n=2) to following exponential growth phase (n=4; p values $\leq$ 0.02, one-way ANOVA; Fig 4, Table 2), indicating a pronounced change in community metabolic potential over time. No Subsystem Level 1 categories showed significant shifts in mean relative abundance from day 2 to day 3 (p values > 0.05, two-way ANOVA; Fig 4, Table 2). Comparing relative abundance of metabolic functions grouped by Subsystem Level 1 and obtained following exponential growth phase also shows significant differences as a result of pCO_2 level (p values $\leq$ 0.01, two-way ANOVA; Fig 4, Table 2), indicating an effect of elevated pCO_2 on community metabolic potential.

To further characterize the differences in metabolic function between elevated and low pCO_2 treatments, the statistical package EdgeR was used (Robinson *et al.* 2010). A total of 415 metabolic functions (8.3 % of identified metabolic functions) showed significant differential abundance between pCO_2 treatments. 153 metabolic functions exhibited a log2 fold change of 1.5, or greater (SI Table 1). The majority of these metabolic functions had greater abundance with elevated pCO_2 (104), compared to low (49) pCO_2 (Fig 5, SI Table 1).

Metabolic functions with significantly higher relative abundance in elevated pCO_2 incubations corresponded to different metabolic functional categories than those with significantly higher relative abundance in low pCO_2 incubations (Fig 5, SI Table 1). Elevated pCO_2 had a pronounced effect on metabolic functions related to carbohydrate metabolism (21 out of 104 metabolic functions), clustering-based subsystems (15), fatty acids, lipids, and isoprenoids (15), and membrane transport (9) (Fig 5, SI Table 1). Significantly enriched metabolic functions related to carbohydrate metabolism were dominated by monosaccharide (48 %) and amino sugar (29 %) utilization (SI Table 1). 87 % of significantly enriched metabolic functions categorized as fatty acids, lipids, and isoprenoids were related to fatty acid and phospholipid biosynthesis (SI Table 1), and 87 % of the significantly enriched metabolic functions belonging to the "clustering-based subsystems" category were related to conjugative transfer (SI Table 1). Additionally, 8 out of 9 (89 %) significantly enriched metabolic functions related to membrane transport were associated with conjugative transfer. Collectively, following exponential growth phase, 20 % of metabolic functions that were significantly more abundant with elevated pCO_2 were related to conjugative transfer (SI Table 1). Though elevated pCO_2 had less pronounced effects on metabolic functions related to virulence, disease, and defense, and respiration, genes associated with resistance to antibiotic and toxic compounds (80 % of metabolic functions categorized in the subsystem of "virulence, disease, and defense") and electron transport machinery (i.e. cytochromes) were significantly enriched with elevated pCO_2 (SI Table 1).

Discussion

Our results indicate that in the presence of labile organic matter elevated pCO_2 can directly influence the trajectory of dominant bacterial populations and overall community metabolic potential of natural surface heterotrophic bacterial communities in the South Pacific Subtropical Gyre. Elevated pCO_2 led to greater relative abundances of Pseudoalteromonadaceae and Shewanellaceae, two of the dominant taxa responding to the lysate amendment. In addition,

elevated pCO_2 had disproportionate effects on numerous metabolic functions, including those related to carbohydrate use, phospholipid and fatty acid biosynthesis, conjugative transfer, resistance to antibiotics and toxic compounds, and electron transport machinery (Fig 6, SI Table 1). These results suggest that enhanced removal of recently produced phytoplankton-derived organic carbon and enhanced bacterial respiration with elevated pCO_2 were driven by shifts in key heterotrophic bacterial groups and concomitant shifts in community metabolic potential.

We did not conduct direct measurements of oxygen concentrations over the course of the experiment. However, using direct measurements of bacterial respiration, and assuming a respiratory quotient of 1, we estimate the differences in oxygen concentration between low and elevated pCO_2 following exponential growth phase did not exceed 1 % of oxygen saturation (assuming 200 μM O_2 saturation). Based on recent publications (see review by Wright *et al.* 2012 and citations therein), we do not expect these differences in oxygen concentration to have impacted respiration rates or community structure.

Bacterial Community Composition

The bacterial community composition shifted in response to a pulse of phytoplankton lysate from more oligotrophic bacterial clades such as Prochlorococcaceae and Pelagibacteraceae at the start of the experiment to communities that were dominated by copiotrophic Gammaproteobacteria following exponential growth phase, with both low and elevated pCO_2 (Fig 3). This shift was in part influenced by the elimination of light, which substantially depressed the relative abundances of cyanobacterial families (Table 1). Despite the dominant role of cyanobacteria families in oligotrophic gyre ecosystems (e.g. Chisholm et al. 1992, Goericke and Welschmeyer 1993), our goal for this study was to assess the direct effects of pCO_2 on heterotrophic removal of organic carbon in the presence of phytoplankton lysate. Future studies of the combined effects of pCO_2 on autotrophic and heterotrophic bacterial groups are required to assess the effects of elevated pCO_2 on marine carbon cycling in the oligotrophic gyres. In addition to an absence of light,

dominance by gammaproteobacterial clades following exponential growth phase is not surprising, as groups such as Alteromonadaceae and Pseudoalteromonadaceae have been shown to rapidly grow in seawater enriched with phytoplankton organic matter (Nelson and Carlson 2012; Nelson and Wear 2014) and other labile components of dissolved organic material (Carlson *et al.* 2002, Sarmento & Gasol 2012; Wear *et al.* 2015). Dominance by gammaproteobacterial clades in a phytoplankton mesocosm experiment was also observed by Bunse *et al.* (2016), with 30 – 50 % of annotated transcript reads being dominated by Gammaproteobacteria in both low and elevated pCO_2 treatments.

Despite the overwhelming dominance by traditionally copiotrophic gammaproteobacterial clades (approximately 70 % of total sequences) in low and elevated pCO_2 incubations, a significant shift in the dominance of main clades of bacterial populations as a function of pCO_2 was still evident (Fig 3, Table 1). The relative abundances of the closely related Pseudeoalteromonadaceae and Alteromonadaceae were roughly equivalent in low pCO_2 treatments but Pseudoalteromonadaceae were significantly enriched (and Alteromonadaceae concomitantly depleted) in the elevated pCO_2 treatment, suggesting selection for Pseudoalteromonadaceae by pCO_2 enrichment (SI Fig 1). Bacterial community composition has been shown to influence the type and amount of organic carbon that is removed by heterotrophic bacteria (Cotrell & Kirchman 2000, Carlson *et al.* 2004; Nelson & Carlson 2012, Newbold *et al.* 2012; Nelson *et al*. 2013; Letscher *et al.* 2015; Wear *et al.* 2015) and it is possible that the decreased growth efficiency induced by pCO_2 enrichment was associated with selection for increased Pseudoalteromonadaceae and Shewanellaceae. Calculations of marine bacterial growth efficiencies, which provide an estimate of how much consumed organic carbon is respired by bacteria, range from 0.05 to as high as 0.6 (delGiorgio & Cole 1998), and have been shown to vary based on bacterial community composition (Reinthaler *et al.* 2005; Nelson *et al.* 2013). Thus, the shift in key bacterial populations in our experiment provides a potential mechanism to explain the observation of enhanced removal of phytoplankton-derived organic carbon and

bacterial respiration with elevated pCO_2. Why elevated pCO_2 selects for Pseudoalteromonadaceae and Shewanellaceae in the presence of coccolithophore culture lysate remains unclear and requires further investigation.

Bacterial Metabolic Potential

In addition to a shift in the relative abundance of dominant bacterial populations, enhanced removal of recently produced phytoplankton-derived organic carbon removal by bacteria and increased bacterial respiration may be driven by a direct effect of pH on bacterial physiology. Our analysis reveals clear effects of elevated pCO_2 on metabolic functions related to carbohydrate metabolism, fatty acid and phospholipid biosynthesis, conjugative transfer and antibiotic and toxic compound resistance. Additionally, we observed effects of pCO_2 on multiple metabolic functions related to regulation of intracellular pH and the membrane potential.

Carbohydrate Metabolism

pCO_2 enrichment had the most pronounced effect on metabolic functions related to carbohydrate metabolism (Fig 5, SI Table 1), where nearly one third of the differentially abundant carbohydrate metabolic functions were related to bacterial use of chitin, N-acetyl-glucosamine, and N-acetyl-galactosamine. Chitin is one of the most abundant polymers in the marine environment and potential sources include structural components of invertebrates, phytoplankton, and marine fecal pellets (see review by Souza *et al.* 2011), suggesting that the addition of whole surface seawater (i.e. containing particles) may have led to the availability of chitin in our experiment. N-acetyl-glucosamine is a monomeric unit of chitin and similar to N-acetyl-galactosamine– both are major components of peptidoglycan and capsular polysaccharides, as well as the outer membrane lipopolysaccharides of Gram negative bacteria (Brinkkotter *et al*. 2000; Riemann & Azam 2002; Leyn *et al.* 2012), suggesting that dying bacterial cells may have provided a source of carbon and nitrogen to surviving cells in elevated pCO_2 incubations. A recent mesocosm experiment conducted in the Ross Sea, Antarctica,

investigating the effects of elevated pCO_2 on mixed-surface communities, found that in some cases, activities of chitinase, the enzyme involved in chitin utilization, were enhanced in low pH mesocosms (Maas et al. 2013). This result is consistent with our finding that elevated pCO_2 increased the metabolic potential of bacterial communities for using chitin, and oligomers similar to chitin, and suggests that short-term exposure to elevated pCO_2 may lead to greater bacterial degradation of chitin and related amino sugars, such as N-acetyl-glucosamine and N-acetyl-galactosamine, present in fecal pellets and phytoplankton and bacterial cell walls.

Fatty Acid and Phospholipid Biosynthesis

Numerous studies illustrate bacterial modifications in the phospholipid bilayer of their cellular membranes as a critical mechanism for enabling cells to cope with low pH stress (Brown *et al.* 1997, and reviews by Slonczewski *et al*. 2009, Zhang and Rock 2008, and Krulwich *et al.* 2011). For our experiment, 13 out of the 15 metabolic functions related to fatty acids metabolism that were enriched with elevated pCO_2, were involved in phospholipid and fatty acid biosynthesis (Fig 6, SI Table 1). Further, the average relative abundance of each of these metabolic functions with elevated pCO_2 was more than 2.5 times the average relative abundance in low pCO_2 incubations, and we found no differentially abundant metabolic functions related to phospholipid and fatty acid biosynthesis in low pCO_2 incubations (SI Table 1). Metabolic functions that were more abundant with elevated pCO_2 included multiple genes required for the elongation of fatty acid chains in bacteria, including enoyl-acyl carrier protein (ACP) reductase (FabI), 3-hydroxydecanoyl-ACP dehydratase (FabA), and 3-oxoacyl-ACP synthase (FabB). FabI is required to complete the cycle of fatty acid elongation, while FabA and FabB work together to introduce double bonds into the growing acyl chain, resulting in the production of unsaturated fatty acids (Zhang & Rock 2008). Unsaturated fatty acids are more fluid than saturated fatty acids because the double carbon-carbon bonds insert kinks into the hydrocarbon chain that disable the lipids from packing tightly together. Multiple studies point to bacterial modification of the phospholipid bilayer to incorporate cyclopropane fatty acids, which mimic unsaturated

fatty acids and increase the impermeability of the phospholipid bilayer to protons, as a critical mechanism for enabling cells to cope with low pH stress (Brown *et al.* 1997, Chang & Cronan 1999, and reviews by Slonczewski *et al.* 2009, Zhang and Rock 2008, and Krulwich *et al.* 2011). Exposure to reduced pH in our experiment may have conferred advantage to bacterial populations that are able to alter the permeability of their membrane in response to changing pH. This result is consistent with previous observations in which bacteria alter the structure and composition of their cellular membranes as a mechanism for coping with low pH stress.

Conjugative Transfer

Metabolic functions related to conjugative transfer made-up approximately 20 % of the differentially abundant metabolic functions that were more abundant with elevated pCO_2 (Fig 6). Through direct transfer of single stranded DNA, conjugative transfer represents one of the dominant modes of genetic recombination in bacteria, and is a mechanism by which bacteria share fitness-enhancing traits (Chen *et al.* 2005). As such, conjugative transfer can enhance survival of bacteria in response to environmental stress (Burrus & Waldor 2004, Krause *et al.* 2000, Frost and Koraimann 2010 and citations therein). In addition to transferring beneficial DNA between cells, conjugative transfer was shown to enhance pathogenesis of a wide range of bacteria (Green & Mecsas 2016 and citations therein). Further, numerous food-safety studies report enhanced abundance of pathogenic bacteria in response to low pH stress, and suggest that exposure to low pH through anti-bacterial sanitization methods confers advantage to pathogenic bacteria that are capable of conjugative transfer and thus able to exchange stress-tolerant genes during exposure to exceptionally low pH environments (Chung *et al.* 2006 and citations therein). The substantial number of metabolic functions related to conjugative transfer enriched in response to elevated *p*CO2 in our study suggests that direct transfer of genetic material may play a critical role in the response of marine heterotrophic bacteria to elevated pCO_2. Further studies of the bacterial populations conducting conjugative transfer, as well as the specific genes being

transferred, are required to assess the effects of pCO_2 on the potential for enhanced transfer of stress-response genes and/or bacterial pathogenicity.

Antibiotic and Toxic Compound Resistance

Elevated pCO_2 also had disproportionate effects on metabolic functions related to antibiotic and toxic compound resistance (Fig 6, SI Table 1). Specifically, multiple metabolic functions regulating efflux of metal cations, and multidrug efflux pumps were significantly more abundant in elevated pCO_2 incubations (SI Table 1). A copper chaperone that aids in copper homeostasis exhibited relative abundance 47.5 times greater with elevated pCO_2, as compared with low pCO_2 (SI Table 1). Copper chaperones prevent exposure of the cytoplasm to copper ions during transit through the cell (Harrison *et al.* 1999), and were shown to deliver copper to a membrane fusion protein for export from bacterial cells (Franke *et al.* 2003). Hayes *et al.* (2006) observed a similar response by *E. coli* to low pH conditions – low pH led to upregulation of metal cation efflux proteins required to rid the cell of silver and copper. The researchers posit that the solubility of metals such as copper increased with decreasing pH. Other metals including zinc and iron were also shown to increase in solubility as a result of low pH (Millero *et al.* 2009), suggesting that bacteria may need to induce metal efflux pumps to regulate the concentration of these heavy metals in response to low pH. In addition to removing metal cations, greater abundance of multidrug efflux pumps suggest that bacteria require methods of removing other toxic compounds and antimicrobial agents from the cell (Anderson *et al.* 2015) during exposure to elevated pCO_2.

Regulation of Intracellular pH and Membrane Potential

Consistent with the enhanced bacterial respiration observed in this study, a recent phytoplankton mesocosm study showed that marine bacteria upregulate transcription of respiratory proton pumps in response to decreasing pH due to elevated pCO_2 (Bunse *et al.* 2016). Decreasing external pH may result in enhanced proton uptake and acidification of the cytoplasm, causing

cells to upregulate proton export to reestablish internal pH values (see review by Slonzcewski *et al.* 2009). We observed greater relative abundance of proton pumps that may enable cells to enhance transmembrane proton export in response to acidification from elevated pCO_2. For example, metabolic functions related to cytochrome c biogenesis were significantly enriched with elevated pCO_2 (relative abundance up to 4 times greater with elevated pCO_2; SI Table 1). Our findings are consistent with Bunse *et al.* (2016) where cytochrome c biogenesis was shown to be one of the most responsive proteins to elevated pCO_2 for marine Rhodobacteriaceae bacteria. Cytochrome c is one of the main components of the electron transport machinery within bacterial cells and aids in the active export of protons from the cytoplasm (Maurer *et al.* 2005, Slonczewski *et al.* 2009 and citations therein, Krulwich 2011, Bunse *et al.* 2016; Fig. 6).

Enhancing proton export out of the cytoplasm in response to low pH would increase the concentration of positively charged ions on the outer surface of the cell membrane. Marine bacteria maintain a charge gradient across the cell membrane that is positive outside the cell and negative inside (see review by Krulwich *et al.* 2011). This charge gradient, referred to as the electrical membrane potential, is a critical component of the proton motive force, which drives cellular metabolic functions. Enhanced proton export in response to low pH would augment the membrane potential and rapidly diminish the ability of cells to sustain proton export against the concentration gradient. To maintain respiratory export of an excess of cytoplasmic protons at low pH, cells must swap protons for positively charged cations such as potassium and sodium (see review by Krulwich *et al.* 2011). This swapping of positively charged ions (electroneutral proton pumping) preserves the membrane potential and enables continued export of protons. For our study, the only metabolic function enriched with elevated pCO_2 for potassium regulation was related to potassium uptake (i.e. relative abundance of the KdpE gene was 3.7 times greater), suggesting that cells possessed the metabolic potential to sustain proton export through exchange with potassium ions in response to low pH.

In addition to coupled proton/cation pumps, several studies suggest that bacteria alter their catabolism of carbohydrates in order to regulate intracellular proton concentrations and maintain pH homeostasis (Slonczewski & Foster 1996, Blankenhorn *et al.* 1999, Stancik *et al.* 2002, Foster 2004, Yohannes *et al.* 2004, Maurer *et al.* 2005, Hayes *et al.* 2006). A study of the metabolic response of *E.coli* to changes in pH under low oxygen conditions revealed enhanced regulation of catabolic enzymes and transporters such as those for arabinose, fuculose, and gluconate in response to low pH (Hayes *et al.* 2006). The catabolism of these sugars and sugar derivatives produced fewer acids internally as compared to catabolism of glucose and maltose, which produced a burst of fermentation acids upon catabolism (Stancik *et al*. 2002; Hayes *et al.* 2006). Elevated pCO_2 incubations for our study were also significantly enriched with metabolic functions related to arabinose and fucose metabolism (SI Table 1), indicating the potential of cells to alter their preference for various organic substrates, possibly choosing to use substrates that produce more or fewer acids as a mechanism for pH homeostasis.

Enhanced bacterial respiration for our study, as well as the observations by Bunse *et al.* (2016), suggest that respiratory proton export may be a common response to acidification. Bunse *et al.* (2016) posit that decreasing external pH may cause an influx of protons across cellular membranes. Studies of marine bacteria suggest that intracellular pH is maintained at approximately 7.6 (Nakamura *et al.* 2005; see review by Krulwich *et al.* 2005) and because external pH in our experiment was reduced from approximately 8.1 to 7.7 (see Experimental Procedures section), this decrease in external pH would not result in an influx of protons. Alternatively, intracellular proton concentrations could be augmented via enhanced import and/or intracellular production of protons. For example, the Resistance-Nodulated-Division (RND) efflux system was enriched with elevated pCO_2 (Fig 6) and requires the import of a proton to drive the proton motive export of toxic substrates (Lewinson *et al.* 2004). The import of protons would elevate cytoplasmic proton concentrations and possibly require cells to increase respiratory processes to pump protons out of the cell. Import and catabolism of more highly

protonated DOM is another process that may result in greater intracellular proton production under low pH conditions. Though this mechanism is highly speculative, studies of the effects of low pH on organic and inorganic speciation of trace metals indicate the potential for increased competition between protons and free ion metals such as copper and iron for complexation with dissolved organic matter at low pH (Gledhill *et al.* 2015; Stockdale *et al.* 2016). Greater protonation of marine DOM under low pH conditions might increase intracellular release of protons upon catabolism of organic matter, requiring cells to enhance proton export to counteract the effects of increased intracellular proton exposure. Studies of the effects of pH on protonation of naturally occurring marine DOM, as well as measurements of intracellular pH and the mechanisms energizing the proton potential in marine heterotrophic bacteria, would further our ability to predict the effects of low pH on bacteria-mediated carbon cycling and provide valuable insight to the underpinnings of marine bacterial physiology and bioenergetics in the sea.

Concluding Summary

This study demonstrates that during bloom conditions, short-term responses to a significant increase in pCO_2 by heterotrophic bacterial surface communities from the South Pacific Subtropical Gyre include a shift in the dominance of main clades of bacteria and enrichment of specific community metabolic functions. Elevated pCO_2 enriched metabolic functions related to carbohydrate metabolism and pH homeostasis mechanisms that have been previously described (including metabolic functions for proton pumps, modifications of the phospholipid bilayer, and resistance to toxic compounds; Fig 6; SI Table 1). In addition, we provide evidence for the potential enrichment of metabolic functions related to conjugative transfer in response to elevated pCO_2 (Fig 6; SI Table 1). The shift in the most dominant bacterial populations and disproportionate effects of elevated pCO_2 on carbohydrate metabolism provides a potential mechanism to explain why we observed enhanced removal of phytoplankton-derived organic carbon with elevated pCO_2 in this experiment. Moreover, greater abundance of metabolic functions related to phospholipid and membrane maintenance, and toxin and antibiotic

resistance, provide a potential mechanistic explanation for increased bacterial respiration with elevated pCO_2, as these mechanisms require energy that could otherwise be used for growth. Assemblages of heterotrophic bacterioplankton in the South Pacific Subtropical Gyre are likely to experience these changes in pCO_2 over long periods of time that span phytoplankton bloom and non-bloom conditions. However, our observations of enhanced metabolic potential for conjugative transfer indicate that short-term adaptation during phytoplankton bloom conditions via horizontal gene transfer in these communities may have long-term effects. Though studies of the effects of pCO_2 on heterotrophic bacterial communities during non-bloom conditions, as well as the long-term effects of elevated pCO_2 are required to elucidate the long-term implications of these short-term impacts on bacteria, our study suggests that short-term increases in pCO_2 may alter bacterial ecological function, enhancing rates of bacteria-mediated removal of phytoplankton-derived surface organic carbon and production of carbon dioxide in the surface ocean.

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Table Descriptions

Table 1: The average relative abundance (RA) (± standard deviation; SD) of Family level clades for bacterial communities sampled at the start of the experiment (T_0), and on days 2 and 3 following exponential growth phase; considering only groups that exhibited a significant (p value < 0.05) response to pCO_2 or time point, or RA > 5 % in T_0 samples or the samples collected following exponential growth phase. Statistical analysis was conducted using two-way ANOVA and represents differences in the arc-sine transformed RA of bacterial groups collected during stationary growth phase, as a function of pCO_2 treatment. Reported p values are False Discovery Rate (FDR) corrected. Asterisks indicate significant p values (< 0.05).

Table 2: Significance values reported for the following comparisons: relative abundance of metabolic functions at the start of the experiment compared to relative abundance of metabolic functions collected following exponential growth phase, as well as effects of pCO_2 (low and elevated) and time point (days 2 and 3) on the relative abundance of metabolic functions obtained following exponential growth phase. Metabolic functions were grouped by the broadest categories in the SEED database (Subsystem Level 1). Reported p values are False Discovery Rate (FDR) corrected.

Figure Descriptions

Fig 1. Mean bacterial abundance (BA; ± standard deviation) averaged across duplicate incubations and time as a function of pCO_2 (A). Panel B shows mean cell-specific TOC removal by day 2 and 3 as a function of pCO_2. Red denotes elevated (1000 ppm) pCO_2 while blue denotes

low (250 ppm) pCO_2. Gray arrows (A) indicate time points when metagenomic DNA samples were obtained. Data modified from James et al. 2017.

Fig 2. Dendrogram (A & C) and nonmetric multidimensional scaling ordination plots (B & D) of relative abundance of bacterial community composition (A & B), and bacterial metabolic functions (C & D) from DNA samples collected at the start of the experiment (T_0, green circles) and on day 2 (filled triangles), and day 3 (open triangles) of the experiment. Red denotes elevated (1000 ppm) pCO_2 while blue denotes low (250 ppm) pCO_2. The left panel includes samples from the start of the experiment and following exponential growth phase (n = 10), while the right panel includes only samples from post-exponential growth phase (n = 8). Results from the statistical analysis of the effects of pCO_2 and time point following stationary growth phase are reported in panels B & D (two-way PERMANOVA).

Fig 3. The average relative abundance of bacterial groups collected at the initiation of the experiment (T_0) and on days 2 and 3 for low (250 ppm) and elevated (1000 ppm) pCO_2 treatments. Sequence reads were taxonomically annotated by comparison with the SEED database. Coloration refers to bacterial clades – green hues correspond to Cyanobacteria (C), red and orange to Alphaproteobacteria (A), blues to Gammaproteobacterial (G) clades, and yellow represents to Flavobacteria (F).

Fig 4. The average relative abundance (± standard deviation) of metabolic functions grouped by the broadest functional categories in the SEED database. For samples collected at the start of the experiment, black bars represent the average of duplicate samples. For samples collected following exponential growth phase, averages represent duplicate samples obtained on days 2 and 3 of the experiment (n = 4 per pCO_2 treatment), for low (blue; 250 ppm) and elevated (red; 1000 ppm) pCO_2 treatments. Counts were normalized to Identified Protein Features. Statistical analysis of the community metabolic response to changes in pCO_2 was evaluated using analysis

of variance (ANOVA) – black asterisks denote significant difference in relative abundances between the start of the experiment and following exponential growth phase. Red asterisks denote a significant difference in the relative abundance of functions obtained following exponential growth phase in response to elevated pCO_2.

Fig 5. The number (count) of significantly differentially abundant (DA) metabolic functions encoded following exponential growth phase under low (blue) and elevated (red) pCO_2. Metabolic functions are grouped by the broadest functional categories in the SEED database (Subsystem Level 1). Statistical analyses were conducted using EdgeR and only the functions that exhibited a 1.5-fold change in relative abundance are reported.

Fig 6. Model of the potential physiological response of natural heterotrophic bacterial communities to elevated pCO_2. Colors denote whether the cellular process results in the acidification (dark orange; i.e. importing protons) or alkalization (purple; i.e. expelling protons) of intracellular pH. Light orange indicates functions that result in less intracellular pH change. See text for citations. Grey arrows refer to more than one process and are not colored by their effects on intracellular pH. Bolded values represent the proportion of total differentially abundant (DA) functions that were significantly more abundant with elevated pCO_2 (104 total) within each identified SEED category. See SI Table 1 for a full list of the DA metabolic functions identified using EdgeR. Large arrows indicate the relative consumption of dissolved organic carbon (DOC) and production of CO_2 (i.e. respiration) by bacteria growing at low (blue) and elevated (red) pCO_2 levels. RND refers to Resistance-Nodulated-Division efflux pumps.

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