The effects of elevated temperature and ocean acidity on bacterioplankton community structure and metabolism

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THE EFFECTS OF ELEVATED TEMPERATURE AND OCEAN ACIDITY ON BACTERIOPLANKTON COMMUNITY STRUCTURE AND METABOLISM

By

Nam Siu

Accepted in Partial Completion
Of the Requirements for the Degree
Master of Science

Kathleen Kitto, Dean of Graduate School

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MASTER’S THESIS

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Nam Siu
February 13th, 2013
THE EFFECTS OF ELEVATED TEMPERATURE AND OCEAN ACIDITY ON
BACTERIOPLANKTON COMMUNITY STRUCTURE AND METABOLISM

A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

By
Nam Siu
February 2013
ABSTRACT

By the end of the 21st century, mean sea surface temperatures are expected to increase 4°C, while atmospheric CO₂ concentrations are predicted to triple causing seawater to become more acidic. These compounding effects will undoubtedly have major consequences for the organisms and processes in the oceans. Bacterioplankton play a vital role in the marine carbon cycle and the oceans’ ability to sequester CO₂. We utilized pCO₂ perturbation experiments to investigate the effects of elevated temperature and acidity on bacterioplankton community structure and metabolism. Terminal-restriction fragment length polymorphism (T-RFLP) revealed that bacterioplankton incubated in lower pH conditions exhibited a reduction of species richness, evenness, and overall diversity, relative to those incubated in ambient pH conditions. Non-metric multidimensional scaling (MDS) of T-RFLP data resulted in clustering by pH suggesting that pH influenced the structure of these communities. Shifts in the dominant members of bacterioplankton communities incubated under different pH were observed in both T-RFLP and clone library analyses. Both ambient and low pH communities were dominated by sequences of γ-proteobacteria and α-proteobacteria, although abundance of α-proteobacteria increased in communities incubated at lower pH. Although the representatives from these two classes were distinctly different between the treatments, a few taxa were found to be persistent in all treatments. Changes in the structure of bacterioplankton communities coincided with significant changes to their overall metabolism. Bacterial production rates decreased, while bacterial respiration increased under lower pH conditions. This study highlights the ability of bacterioplankton communities to respond to ocean acidification both structurally and metabolically, which may have significant implications for their ecological function in the marine carbon cycle and the ocean’s response to global climate change.
ACKNOWLEDGEMENTS

I would like to express my gratitude to Drs. Brooke Love and Brady Olson, and the Ocean Acidification Lab at Shannon Point Marine Center (SPMC) for their help and expertise in CO$_2$ equilibration and experimentation, as well as the staff at SPMC and members of the Biology stockroom at Western Washington University (WWU) especially Peter Thut and Gene McKeen for providing laboratory space and logistical assistance. I would also like to extend my special thanks to Sean McAllister for his assistance with data analysis and thoughtful input aiding in the completion of this project. I am thankful to my summer research mentees for their help with data collection during their summer internships. Moreover, I am grateful for the members of my research lab and fellow graduate students who have provided help and support. Lastly, I would like to thank my thesis committee (Dr. Dietmar Schwarz) and advisors (Drs. Craig Moyer and Jude Apple) for their wisdom and advice, without which this project could not be possible. This project was funded in part by WWU’s Office of Research and Sponsored Programs, Biology Alumni Student Research Fellowship, and Biology Graduate Committee and Chair Funds. I would also like to thank the WWU Biology June and Charles Ross Graduate Scholarship for financial support during the course of this project.
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Introduction

Since the beginning of the industrial revolution, anthropogenic activities have rapidly increased the concentration of CO$_2$ in the atmosphere, contributing to global climate change (Pachauri and Reisinger, 2007). One major consequence of this change in global climate is the rise of temperatures in a variety of habitats including the oceans. The oceans have absorbed approximately one third of all CO$_2$ released into the atmosphere, offsetting the rapid increase of CO$_2$ in the atmosphere and ameliorating global climate change (Doney et al., 2009b). Despite oceanic sequestration of atmospheric CO$_2$, global temperatures are expected to increase up to 4°C and CO$_2$ concentrations are predicted to increase two to three fold over the next century (Pachauri and Reisinger, 2007; Doney et al., 2009a). Furthermore, the oceanic absorption of atmospheric CO$_2$ is causing seawater to become increasingly acidified. This rate and magnitude of ocean acidification has not been experienced in the past 300 million years (Caldeira and Wickett, 2003). Consequently, marine ecosystems are especially vulnerable to these changes to the environment as organisms are under the compounding stress of ocean acidification and warming.

The biomass of the oceans is largely microbial, with millions of bacterial cells in every milliliter of seawater (Hobbie et al., 1977). In some marine ecosystems, these microbes account for the majority of the overall respiration in the system (Pomerory, 1974; Robinson and Williams, 2005). Due to their great abundance and overall contribution to biological processes in the oceans, heterotrophic bacterioplankton are fundamental components of marine biogeochemical cycles (Kirchman, 1994; Bidle and
Azam, 2001). These bacterioplankton are also responsible for consuming the majority of organic carbon fixed by photosynthesis (del Giorgio and Duarte, 2002). In turn, organic carbon from these microbes are consumed by higher trophic levels through the ‘microbial loop’ (Azam et al., 1983). Heterotrophic bacterioplankton produce sinking organic aggregates, deposits and flocculent matter in the form of detritus or marine snow and are important in marine sediment formation. This microbially mediated process known as the ‘biological pump’ (Longhurst and Glen Harrison, 1989) transports organic carbon vertically through the water column for sequestration in the deep sea and is one of the largest oceanic carbon sinks (Siegenthaler and Sarmiento, 1993). By facilitating carbon sequestration, these bacterioplankton perform a vital role in the response of the oceans to global climate change (Joint et al., 2009). Moreover, the efficiency of carbon sequestration in the ocean is regulated by the carbon consumption and transport rates of bacterioplankton which is ultimately determined by the phylogenetic structure of these communities and their overall metabolism (Fuhrman, 2009).

Despite the important role of heterotrophic bacterioplankton with respect to the flux and sequestration of carbon, few studies have examined how these microorganisms will respond to increased ocean acidity and temperature over the next century, let alone the combined effects of both these factors (Liu et al., 2010; Joint et al., 2011). One of the first investigations utilized mesocosms to study the effects of increased partial pressure CO₂ (pCO₂) on entire planktonic communities. This study found that during the breakdown of a phytoplankton bloom caused by pCO₂ enrichment, bacterioplankton abundance increased significantly (Grossart et al., 2006). Alterations to the phylogenetic structure of bacterioplankton communities as a response to elevated pCO₂ have been detected by
denaturing gradient gel electrophoresis (DGGE) (Allgaier et al., 2008; Lidbury et al., 2012). A similar study using terminal restriction fragment length polymorphism (T-RFLP) to track the response of bacterioplankton communities to increased $p$CO$_2$ also suggested that ocean acidification can potentially affect the structure of the bacterioplankton communities (Zhang et al., 2012), however their findings might be limited by the use of only two restriction enzymes (Engebretson and Moyer, 2003). More recently, Ray et al. (2012) used pyrosequencing technology to identify bacterioplankton community composition and found that the communities in their study were dominated by representatives of $\gamma$-proteobacteria and $\alpha$-proteobacteria. This study, showed how these communities response to dissolved organic carbon (DOC) input could be affected by ocean acidification (Ray et al., 2012).

Shifts in the structure of heterotrophic bacterioplankton communities, in addition to the anticipated changes in temperature and ocean pH over the next century will undoubtedly have consequences for their overall metabolism and ecological function (Fuhrman, 2009; Liu et al., 2010; Joint et al., 2011). Even less is known about the link between changes in bacterioplankton community structure to the responses in the overall metabolism and in turn the ecological function of these communities (Riebesell et al., 2007; Lidbury et al., 2012; Teira et al., 2012). Such comprehensive and encompassing studies are necessary because most of the available models on ocean acidification exclude biological processes. Additionally, due to their contribution to carbon flux in the oceans, these bacterioplankton are immensely important in determining the ocean’s response to increased $p$CO$_2$ (Borges et al., 2005). One study has suggested that vertical carbon flux will increase due to elevated $p$CO$_2$ (Delille et al., 2005), while another predicted that the
vertical flux of marine aggregates will slow or even stop (Mari, 2008). Increased rates of extracellular enzymatic activity, protein production and polysaccharide degradation by bacteria under enriched pCO$_2$ conditions have also been documented (Grossart et al., 2006; Piontek et al., 2010; Piontek et al., 2012). Moreover, although it is difficult to imagine a future where increases in ocean temperature and acidity do not occur simultaneously, none of the previous studies have examined the combined effects of ocean acidification and warming on the structure and function of bacterioplankton communities.

As marine microbial assemblages have historically experienced variable pH in the environment, a practical null hypothesis is that there will be no change in the ecological function of microbial communities due to decreased pH (Joint et al., 2011). Alternately, a review of more recent investigations suggest that ocean acidification will affect microbial communities and their overall function will shift (Liu et al., 2010). It is clear that the changes in the marine environment predicted for the near-future are not going to catastrophically reduced bacterioplankton communities or their functions. However due to previously observed community shifts, it is expected that such changes in temperature, pH and their combined effects will drive shifts in the phylogenetic structure of bacterioplankton communities and the physiology of the communities as a whole, affecting metabolism and ecological function (del Giorgio and Bouvier, 2002; Apple et al., 2006b; Fuhrman, 2009).

The Salish Sea is a marine and estuarine ecosystem extending from the northern most point of the Strait of Georgia, to the western most extent of the Strait of Juan de Fuca and the southern reaches of Puget Sound. The Salish Sea is especially vulnerable to excessive
acidification as it experiences intrusions of acidified deep water from upwelling and higher rates of respiration due to eutrophication (Feely et al., 2010). Coastal marine and estuarine ecosystems, such as the Salish Sea are of both socio-economical and ecological importance, with their ‘value’ directly threatened by ocean acidification (Harley et al., 2006). So far, there has been no study evaluating the effects of climate change and ocean acidification on heterotrophic bacterioplankton and their function in maintaining the health of this valuable yet vulnerable ecosystem.

The purpose of this study is to investigate the compositional and metabolic response of heterotrophic bacterioplankton communities taken from the Salish Sea to both elevated temperature and acidity in perturbation experiments. The effects of these factors on bacterioplankton community structure and metabolism were determined by exposing bacterioplankton to anticipated near-future temperatures and $pCO_2$ concentrations in laboratory incubations. The objectives of this study were to 1) investigate the phylogenetic response of these bacterioplankton communities to increased temperature and acidity using molecular techniques targeting the bacterial SSU rDNA and 2) determine the metabolic response of these bacterioplankton communities by measuring rates of bacterial production and respiration.
Methods

Study Site and Sample Collection

Water samples were collected from the central Salish Sea (San Juan Channel, San Juan Islands, WA) utilizing a conductivity, temperature and depth (CTD) rosette from a research vessel (*R.V. Centennial*, Friday Harbor Laboratories, University of Washington) and the seawater intake system at Shannon Point Marine Center (SPMC) (48°30'32.78”N, 122°41'8.06”W). Three representative environmental bacterioplankton communities were collected from two locations (north and south) along the San Juan Channel and SPMC’s seawater intake system (Figure 1). Bacterioplankton assemblages were collected on 0.22 µm Sterivex filters (Millipore, Billerica, MA) and individually stored at 4°C in RNAlater nucleic acid preservative (Life Technologies, Grand Island, NY). Inoculum used in perturbation experiments was made by filtering SPMC intake seawater through a 3 µm capsule filter (Pall Life Sciences, Port Washington, NY).

Experimental Design

A gas equilibration and measurement system utilizing a LI-820 CO$_2$ Analyzer (LI-COR Biosciences, Lincoln, NE) was used to bubble autoclaved 0.22 µm filtered seawater to experimental $p$CO$_2$ concentrations of 380 ppm and 1050 ppm for one week (Riebesell et al., 2010). The pH was verified through direct probe measurements and Gran alkalinity titrations coupled with the use of a web-based alkalinity calculator (http://or.water.usgs.gov/alk/) and the program co2sys.xls version 14 (Lewis et al., 1998). Equilibrated seawater was inoculated and incubated in collapsible 4 liter cubitainers and
300 ml biological oxygen demand (BOD) bottles in incubators set on 24 hr dark cycles at experimental temperatures of 10°C and 16°C. The four experimental treatments resulted in a 4x4 factorial design allowing for the investigation of the direct and combined effects of \( pCO_2 \) concentration and temperature on bacterioplankton community structure and metabolism. One treatment of 380 ppm and 10°C represented present day conditions and one treatment of 1050 ppm and 16°C represented conditions predicted for the next century. For each treatment three replicates were performed in cubitainer incubations and four replicates were performed in BOD incubations. One liter fractions were sampled from the cubitainers on to 0.22 µm Sterivex filters for molecular analysis at 7 and 14 days, while BOD bottles were fixed using Winkler titration reagents at the beginning of incubations, 24 and 48 hrs for measurement of dissolved oxygen. Three replicate incubations for each of the two \( pCO_2 \) treatments (380ppm and 1050ppm), two temperature treatments (10°C and 16°C) and two sampling time points (T7 and T14) were pooled resulting in the analysis of eight experimental meta-communities designated “380ppm10°CCT7, 380ppm10°CCT14, 380ppm16°CCT7, 380ppm16°CCT14, 1050ppm10°CCT7, 1050ppm10°CCT14, 1050ppm16°CCT7, 1050ppm16°CCT14”. The pH in the 380 ppm treatments ranged between 7.895 and 7.855, while in the 1050 ppm treatments the pH ranged between 7.604 and 7.552.

A second perturbation experiment was conducted to quantify bacterial production of the different bacterioplankton communities that responded to the increase in environmental \( pCO_2 \) concentrations. Due to relatively minor changes in bacterioplankton community structure with regards to temperature, this second experiment focused only on
the effects of increased $p$CO$_2$ concentrations (380 ppm vs. 1050 ppm) for an incubation period of 7 days.

**Molecular Analyses**

**DNA extraction and amplification:** Genomic DNA (gDNA) of the bacterioplankton communities collected on 0.22 µm Sterivex filters were extracted using a FastDNA® SPIN Kit for Soil (Qbiogene, Carlsbad, CA) following the manufacturer’s specifications; with the modifications where the filters were aseptically and individually extracted from their housing and diced to fit into gDNA extraction tubes supplied by the kit, and DNA was eluted into filter sterilized 10mM Tris buffer at pH 8 (Tris buffer). A FastPrep instrument was used at an indexed speed of 5.5 for 30 seconds to mechanically lyse cells. The purity and concentration of gDNAs were quantified with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, South Logan, UT) and samples were diluted to a concentration of ~ 10ng/µl in Tris. Extracted gDNAs were PCR amplified using FAM labeled forward primer 68F (6=carboxyfluorescein) (5’ TNA NAC ATG CAA GTC GRR CG 3’) and 1492R (5’ RGNY TAC CTT GTT ACG ACT T 3’) for the bacterial SSU rRNA gene, where R is a purine analog, Y is a pyrimidine analog, and N is an equal mixture both analogs (Glen Research, Sterling, Virginia). PCR reactions were performed in duplicates using 10-20 ng of gDNA template, 5 U of Taq DNA polymerase, 1X PCR Buffer (100 mM Tris-HCl at pH 9.0, 500 mM, 1% Triton X-100), 2.5 mM MgCl$_2$, 200 µM of each dNTP, 10 µg BSA, 1 µM each of forward and reverse primers, and molecular grade water to a total volume of 50 µl. A hot start step (2 min at 95°C) was performed before the addition of BSA and Taq. The following conditions were used for the
amplification process: 30 cycles of denaturation (94°C for 1 min), annealing (57°C for 90 sec) and elongation (72°C for 3 min) with an additional final elongation (72°C for 7 min). The size of PCR amplicons were verified using 1% agarose gel electrophoresis against a 1-kb ladder (Invitrogen, Carlsbad, CA). Negative PCR controls were confirmed throughout experiments. Replicate PCR products were pooled and purified using 30K centrifugal filters (VWR International, Radnor, PA).

**Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis:** Cleaned and pooled PCR products from each sample were separated and individually digested with the following eight tetrameric restriction-endonuclease treatments (New England Biolabs, Ipswich, MA): *Alu I* (AG\^CU), *BstUI* (CG\^CG), *HaeIII* (GG\^CC), *HhaI* (GCG\^C), *HinfI* (G\^ANTC), *MboI* (\^GATC), *MspI* (C\^CGG) and *RsaI* (GT\^AC). T-RFLP reaction mixtures used 10 U of restriction enzyme, 1X NEBuffer 2 (New England Biolabs), 15 μl PCR product and molecular grade water for a 30 μl total volume. All treatments were incubated for 13 hrs at 37°C, except for BstUI which was at 60°C for the same incubation period. The T-RFLP products were cleaned using Sephadex G-75 columns by centrifuging at 750 x g at 4°C for 5 min into aseptic 96-well plates. Cleaned T-RFLP products were then dried in their 96-well plates at 60°C using a Vacufuge instrument and resuspended in 15 μl of formamide with 0.5 μl 1.1Z™-labeled GeneScan-500 internal size standard (Applied Biosystems, Foster City, CA). Samples were denatured at 95°C for 5 min, before separation by capillary electrophoresis via an ABI 3130 xl genetic analyzer with a 50 cm capillary array using POP 6 (Applied Biosystems). T-RFLP electropherograms were analyzed against the internal size standard using the program GeneMapper v3.7 (Applied Biosystems). Further T-RFLP analysis only
included terminal restriction fragments (T-RFs) between 50-500 bp in size in order to exclude primer-dimers (<50 bp) and inaccurate measurements of T-RFs (>500 bp) (Engebretson and Moyer, 2003). Samples containing peaks greater than 6000 relative fluorescence units (RFUs) were diluted in formamide and rerun, whereas those with peaks less than 1000 RFUs were amplified, digested and run again. The program BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium) was used for cluster analysis of the composite T-RFLP fingerprints by UPGMA and Pearson product-moment correlation (Häne et al., 1993). Cophenetic correlation coefficients were calculated to assess the robustness of the cluster analysis groupings. A similarity matrix was generated from the T-RFLP analysis and entered into a Bray-Curtis resemblance matrix in the program PRIMER version 6 (PRIMER-E Ltd, Lutton, UK) for 2D multidimensional scaling (MDS) and two-way analysis of similarities (ANOSIM) on the effects of pCO$_2$ concentrations and temperatures (Clarke and Gorley, 2006). A univariate analysis of variance (ANOVA) was performed using the program SPSS version 17 (IBM, Armonk, NY) on the number of T-RFs detected by T-RFLP analysis in each of three sample types, where T-RF counts from each restriction enzyme were used as separate replicates.

**Clone library construction and analysis:** Two representative clone libraries of 380 ppm and 1050 ppm communities were constructed from four replicate PCRs for SSU rRNA genes of the samples “380ppm10ºCT14” and “1050ppm16ºCT14”, using 25 to 50 ng of gDNA template, 5 U of AmpliTaq Gold (Applied Biosystems, Carlsbad, CA), 1X AmpliTaq Gold PCR buffer, 2.5 mM MgCl$_2$, 200 µM each dNTP, 10 µg BSA, 1 µg T4 gene 32 protein (T4g32p; Ambion, Austin, TX), 1 µM each of forward primer 68F (5’TNA NAC ATG CAA GTC GRR CG 3’) and 1492R (5’ RGNY TAC CTT GTT GTT ACG...
ACT T 3′) and molecular grade water to a total volume of 50 µl. The following conditions were used for the amplification process: a hot start (94°C for 5 min), 30 cycles of denaturation (94°C for 1 min), annealing (56°C for 90 sec) and elongation (72°C for 3 min). An additional final elongation (72°C for 7 min) was also used. The size of PCR amplicons were verified using 1% agarose gel electrophoresis against a 1-kb ladder. Negative PCR controls were confirmed throughout. The replicate PCR amplicon mixtures were pooled, concentrated, and purified with Ultracel 50K Amicon Ultra centrifugal filters (Millipore). The purified PCR amplicons were then ligated with a CloneJET blunt-end PCR cloning kit (Thermo Scientific) following the manufacturer’s instructions. Ligation reactions were transformed into One Shot Top10 cells (Invitrogen) and grid-plated on Luria-Bertani plates with 100 µg/ml ampicillin (LB-Amp). All putative clones were streaked to isolation and the inserts were assayed for size using Colony-PCR with the vector-specific pJET forward and reverse primers and by running the products against a 1-kb size standard by 1% agarose gel electrophoresis. Clones that screened positive for the correct insert size (~1500 bp) were then cultured overnight in a 96-well format using 1.5 ml of Terrific Broth with ampicillin in 96-well deep well microplates covered with a seal cover. Then, 100 µl of the cultures were transferred into 50 µl of 50% glycerol in 96-well UV microplates covered with aluminum seal and frozen at -80°C. Sequencing reads from the 5’ of the SSU rDNA were used to determine operational taxonomic unit (OTU) structure of each clone library, and one clone from each major OTU was selected for full-length sequencing using internal sequencing primers (Lane, 1991). Full length (>1,400 bp) sequences were assembled contiguously with a minimum of 2X coverage using the program BioNumerics. Full-length and partial-
length sequences were compiled into a single dataset for each clone library for OTU binning and clone identification. The full-length sequences were trimmed to include data between the forward (68F) and reverse (1492R) primers. Priming sites were excluded during sequence trimming in the dataset. The dataset was aligned with the Arb-SILVA database using the SINA Webaligner function (Pruesse et al., 2007). Alignments were edited and then masked so that clone identification could be restricted to unambiguously aligned nucleotide positions using the program BioEdit (Hall, 1999). A minimum similarity of 97% was used to bin the clones into their prospective OTUs (Schloss, 2005). OTUs were numbered in order from lowest to highest according to the abundance of clones, thus OTU 1 was the most dominant OTU containing the highest abundance of clones. Shannon diversity index, estimated OTU richness (ACE and Chao), and rarefaction curves were calculated using the program DOTUR with a 97% similarity cutoff (Schloss, 2005). Clone library coverage was calculated with the following equation: 

\[ C = 1 - \left(\frac{n}{N}\right) \]

where \( n \) is the number of unique OTUs and \( N \) is the total number of clones analyzed (Mullins et al., 1995). Clone sequences were identified to the lowest taxonomic group possible using both the Ribosomal Database Project (RDP) version 10.14 seqmatch algorithm (Cole et al., 2009) and NCBI’s Basic Local Alignment Search Tool (BLAST). Sequences were screened for chimeras with the program Mallard version 1.02 (Ashelford et al., 2006) using the *E. coli* SSU rDNA as a reference sequence, one chimera was detected and excluded from this study. Sequences from each clone library were digested *in silico* in order to assign phylogenetic affiliations to T-RFs from the pertubation experiment. The *in silico* digests were performed using the same eight enzymes as the T-RFLP analysis, with a variance of 2 bp allowed when comparing T-RF
sizes. A SIMPER analysis was performed in the program PRIMER version 6 to identify the taxa responsible for differences in the structure of bacterioplankton communities between the two clone libraries. SSU rDNA sequences obtained in this study were submitted to GenBank under the accession numbers KC139297 through KC139306.

**Metabolic Analyses**

**Bacterial respiration:** The decline of O\(_2\) concentrations over 24 and 48 hr periods was measured using Winkler titrations following Carignan et al. (1998) with a Metrohm Titrino automated titrator with a potentiometric titrode (Metrohm AG, Herisau, Switzerland). Initial O\(_2\) concentrations were measured from five replicate BOD bottles fixed at the beginning of experimental incubations the change in O\(_2\) concentrations in these controls were subtracted from the rate measurements of samples from those treatments. Final O\(_2\) concentrations were measure from four replicate BOD bottles. The oxygen measurements taken at 24 and 48 hrs from the two \(p\)CO\(_2\) treatments and two temperature treatments resulted in four averaged bacterial respiration rates from four replicates during each incubation period for each treatment designated: 380ppm10°C, 380ppm16°C, 1050ppm10°C, 1050ppm16°C. A multivariate ANOVA (MANOVA) was performed in the program SPSS version 17 (IBM) to examine the effects of incubation period, \(p\)CO\(_2\) concentration and temperature on bacterial respiration in the different treatments.

**Bacterial production:** The \(^3\)H-leucine incorporation and the centrifugation method as reported in Apple *et al.* (2004), was used to measure bacterial production of bacterioplankton communities in the second perturbation experiment. Briefly,
bacterioplankton were incubated with $^3$H-leucine for 1 hr, and then terminated with the addition of 100% trichloroacetic acid (TCA). Precipitation of incorporated leucine was achieved by addition of cold 5% TCA and centrifugation. Standardization of production rates were performed by subtracting mean change in leucine concentrations among controls for each treatment. Three replicates were performed for two $p$CO$_2$ treatments (380 ppm and 1050 ppm) and averaged to determine mean bacterial leucine uptake rate. Rates of leucine incorporation were converted to carbon using a conversion factor of 3.1 kg C mol per leucine (Kirchman et al., 1985). A univariate ANOVA was performed in the program SPSS version 17 (IBM) to test for differences in bacterial production rates between $p$CO$_2$ treatments.
Results

Terminal Restriction Fragment Length Polymorphism

Three distinct clusters of bacterioplankton communities were identified with UPGMA/Pearson product-moment correlation cluster analysis of composite bacterial T-RFLP community fingerprints. The first cluster was exclusively composed of environmental samples, while the remaining two clusters consisted of experimental samples clustering by pCO$_2$ treatments. These clusters are designated as the environmental cluster, the low pCO$_2$ cluster and the high pCO$_2$ cluster. Communities in the environmental cluster were most similar to those of the low pCO$_2$ cluster and most dissimilar to those of the high pCO$_2$ cluster (Figure 2). The three most revealing restriction enzymes in terms of T-RFs and community shifts were BstUI, HaeIII and HhaI (Figure S1). Enumerations of mean T-RFs ± SE among clusters found that the environmental cluster exhibited the highest richness with 17 ± 2 T-RFs; the low pCO$_2$ cluster contained 9 ± 1 T-RFs, while the high pCO$_2$ cluster was lowest in richness with 7 ± 1 T-RFs (Table 1). The number of T-RFs among the three clusters were found to be significantly different (P = 0.02). Multidimensional scaling of similarity values between samples in bacterial T-RFLP community fingerprints also revealed the same three distinct clusters in a 2D MDS plot with a 2D stress of 0.01 (Figure 3). The mean similarity (± SE) between samples was greatest within the environmental cluster at 82.37% ± 2.22%, followed by the low pCO$_2$ cluster and the high pCO$_2$ cluster at 80.23% ± 2.68% and 70.80% ± 3.20% respectively. This suggested that communities in the environmental cluster experienced the least amount of variability in community structure. Mean pairwise
similarity values (± SE) between were greatest between the environmental cluster and the low pCO$_2$ cluster at 28.68% ± 1.73%, however the environmental cluster was only 20.66% ± 0.63% similar to the high pCO$_2$ cluster. Lastly, the low pCO$_2$ cluster was 23.12% ± 1.98% similar to the high pCO$_2$ cluster. A two-way ANOSIM on the effects of pCO$_2$ and temperature between all samples found that pCO$_2$ concentration was most influential on the structure of bacterioplankton communities, their similarities and how they clustered (R = 1, P = 0.11), while temperature had little to no effect (R = 0, P = 0.67). A subsequent one-way ANOSIM isolating the effects of pCO$_2$ found that pCO$_2$ concentration significantly influenced the structure of bacterioplankton communities (R = 1, P = 0.004).

**Clone library analysis**

Clone library analysis of SSU rDNA from two samples representing the low and high pCO$_2$ clusters resulted in the partial and full-length sequencing of 75 clones from the 380 ppm treatments and 74 clones from the 1050 ppm treatments. Analysis of the two constructed clone libraries revealed distinctly different phylogenetic structure between the low and high pCO$_2$ clusters. Communities in the 380 ppm treatments were more rich and diverse relative to that of those in the 1050 ppm treatments (Table 2). In total, 32 and 23 OTUs were detected in the 380 ppm and 1050 ppm clone libraries respectively. The terms ‘OTU’ and ‘phylotype’ are synonymous in which both are defined as a group of organisms based on taxonomic and phylogenetic relationship. ACE and Chao indexes were used to estimate OTU richness, while the Shannon index was used to estimate diversity in both clone libraries; these values are reported in Table 2 followed by their
95% low and high confidence intervals in brackets. In the 1050 ppm clone library, a higher OTU coverage of 68.92% was found relative to the 57.33% OTU coverage in the 380 ppm clone library. Rarefaction analysis of the two clone libraries suggested greater diversity in terms of richness in the 380 ppm clone library representing the low pCO₂ cluster (Figure 4). Rarefaction curves also indicated sufficient sampling of both clone libraries as evident by the absence of overlap in 95% confidence intervals at the maximum number of clones sequenced. Both clone libraries were dominated by sequences of γ-proteobacteria and α-proteobacteria (Figure 5). The 380 ppm clone library contained 82.7% γ-proteobacteria and 9.3% α-proteobacteria sequences, yielding a higher γ vs. α ratio (Table 2) than that of the 1050 ppm clone library with 75.7% γ-proteobacteria and 18.9% α-proteobacteria sequences. α-proteobacteria were only detected in the 380 ppm clone library, while β-proteobacteria were detected solely in the 1050 ppm clone library. Greater richness among the γ-proteobacteria was observed in the 380 ppm clone library. Eleven γ-proteobacteria taxa were identified with the most abundant phylotype from the genus Colwellia. A total of six γ-proteobacteria were detected in the 1050 ppm clone library in which phylotypes from the genus Pseudomonas were most abundant. The opposite pattern is observed among the α-proteobacteria. Three taxa were identified in the 1050 ppm clone library with phylotypes from the family Rhodobacteraceae being most abundant. However Rhodobacteraceae was the only taxa of α-proteobacteria detected in the 380 ppm clone library. Several shared taxa were detected throughout both clone libraries. These were represented by unclassified γ-proteobacteria phylotypes and γ-proteobacteria phylotypes belonging to Alcanivorax, Glaciecola and Oceanospirillales; α-proteobacteria phylotypes of Rhodobacteraceae;
and phylotypes of the Flavobacteria *Cellulophaga*. In contrast, \(\gamma\)-proteobacteria phylotypes belonging to *Colwellia*, *Spongiispira*, *Spongiibacter*, *Oleispira*, *Amphritea*, *Psychro sperma* and *Marinobacter*, \(\epsilon\)-proteobacteria phylotypes related to *Arcobacter* as well as *Planctomyces* phylotypes were detected only in the 380 ppm clone library. Whereas \(\gamma\)-proteobacteria phylotypes belonging to *Pseudomonas* and *Porticoccus*, \(\alpha\)-proteobacteria phylotypes of *Sneathiella* and *Roseovarius*, and \(\beta\)-proteobacteria phylotypes related to *Ralstonia*, and *Hydrogenophaga* were detected exclusively in the 1050 ppm clone library. SIMPER analysis revealed *Pseudomonas*, *Colwellia* and *Porticoccus* to be the three taxa contributing to a total of 53.62% of the differences in the structure of bacterioplankton communities, their relative contributions were 22.46%, 20.29% and 10.87% respectively.

**Metabolic Analyses**

**Bacterial respiration:** Only results from 48 hr incubations will be presented, as the respiration rates in the first 24 hrs were an order of magnitude lower and did not significantly differ between treatments (Figure 6). After 48 hrs, significant differences were observed in bacterial respiration due to elevated \(p\text{CO}_2\) \((P < 0.001)\) and temperatures \((P = 0.018)\) (Figure 6B). The greatest difference was found between \(p\text{CO}_2\) treatments, where a 243% \(\pm\) 29% increase in respiration rates was observed from 380 ppm to 1050 ppm \(p\text{CO}_2\) treatments. A slight decrease in respiration rates of 38% \(\pm\) 5% was also observed from 10°C to 16°C temperature treatments. Respiration rates for the two \(p\text{CO}_2\) and two temperature treatments are reported in µgC/l/hr followed by \(\pm\) SE. These rates were 8.2 \(\pm\) 2.4 in the 380ppm10°C treatments, 5.5 \(\pm\) 2.2 in the 380ppm16°C treatments,
30.7 ± 1.2 in the 1050ppm10°C treatments, and 17.4 ± 3.9 in the 1050ppm16°C treatments.

**Bacterial production:** A significant reduction of 72% ± 13% in bacterial production due to increased pCO₂ concentration was observed (P = 0.002) (Figure 7). Production rates for the two pCO₂ treatments are reported in µgC/l/hr followed by ± SE. These rates were 3.2 ± 0.14 in the 380 ppm treatments and 0.97 ± 0.50 in the 1050 ppm treatments.


**Discussion**

Heterotrophic bacterioplankton represent a large portion of the biomass in the oceans (Kirchman and Mitchell, 2008). As such, this group of microorganisms are responsible for consuming most of the primary production from phytoplankton, and facilitating many important biological processes in the oceans, including the ‘biological pump’, one of the largest oceanic carbon sinks (Longhurst and Glen Harrison, 1989; del Giorgio and Duarte, 2002). In addition to being a fundamental component of the marine carbon cycle, bacterioplankton play a vital role in the ocean’s ability to sequester carbon and respond to global climate change (Joint et al., 2011). Despite of the importance of bacterioplankton, relatively little is known about how these microorganisms themselves will respond to the warmer and more acidified oceans of the future (Liu et al., 2010; Joint et al., 2011). The objective of this investigation was to be the first study on the effects of elevated temperature and ocean acidity on the composition and metabolism of heterotrophic bacterioplankton communities taken from the Salish Sea. Bacterioplankton from the Salish Sea were incubated at present-day (380 ppm) and predicted (1050 ppm) pCO$_2$ concentrations (Pachauri and Reisinger, 2007) in perturbation experiments. The manipulation of pCO$_2$ concentrations and pH in these perturbation experiments was achieved by equilibration of media through CO$_2$ gas bubbling. This technique is considered to be more representative of natural changes over the next century compared to the used of other methods such as acid base additions (Riebesell et al., 2010). As such, 380 ppm and 1050 ppm were the concentrations of pCO$_2$ during the equilibration of media and they represented a proxy for the change in pH/acidity. Accordingly, the
low/380 ppm $pCO_2$ treatments equated to a mean pH of 7.88, while 7.58 was the mean pH in the high/1050 ppm $pCO_2$ treatments. The difference between the two $pCO_2$ treatments of 380 ppm and 1050 ppm corresponded to a difference of 0.3 pH units or a threefold increase in acidity, replicating present-day conditions and those predicted for the next century (Caldeira and Wickett, 2003; Pachauri and Reisinger, 2007). Therefore, treatments designated as low/380 ppm $pCO_2$ will be refer to as less acidic or ambient pH conditions, while those of high/1050 ppm $pCO_2$ will be refer to as more acidic or low pH conditions.

T-RFLP community fingerprinting analysis revealed distinctly different structure among bacterioplankton communities incubated in ambient and low pH conditions. Bacterioplankton communities from ambient pH incubations exhibited a greater level of richness and structure relative to bacterioplankton communities incubated at low pH (Table 1). The first evidence of these changes in community structure arose from the comparison of T-RFLP community fingerprints among bacterioplankton communities in the ambient and low pH treatments, where significant population shifts were observed. UPGMA/Pearson product-moment correlation cluster analysis (Figure 2) and multidimensional scaling (Figure 3) of T-RFLP data revealed discrete clustering of bacterioplankton communities by $pCO_2$ concentrations with respect to treatments, suggesting that these changes in community structure were caused by the different pH conditions. In contrast to these findings, a recent study also utilizing T-RFLP and SSU rDNA clone library analyses found no such responses in bacterioplankton community structure due to increased $pCO_2$ (Zhang et al., 2012). However, only two restriction enzymes ($MspI$ and $RsaI$) were used in that T-RFLP analysis. This could have potentially
limited the ability to resolve differences in community structure (Engebretson and Moyer, 2003). Nevertheless other studies have also found little evidence of changes in bacterial community structure as a result of pCO$_2$ enrichments (Newbold et al., 2012; Sperling et al., 2012). On the contrary, in support of the findings in this study, several previous investigations have documented varying levels of response in the structure of bacterioplankton communities to elevated pCO$_2$ or reduced pH (Tortell et al., 2002; Grossart et al., 2006; Allgaier et al., 2008; Arnosti et al., 2011). Additionally marine biofilms (Witt et al., 2011; Lidbury et al., 2012) and picoeukaryotic plankton communities (Meakin and Wyman, 2011) have also been shown to alter community structure in response to increased ocean acidity.

Although the focus of this study was on the comparison of structure and metabolism between bacterioplankton communities under pH and temperatures associated with ambient and predicted conditions, environmental samples were taken and analyzed to serve as a reference. The most noteworthy difference between environmental and experimental communities was the decrease or loss of autotrophs as pCO$_2$ perturbations were all incubated in the dark (Table 1). It was also possible that losses of heterotrophic bacterioplankton from the environment occurred due to non-ideal conditions or inability to culture certain strains (Morris et al., 2012). This was evident in the T-RFLP analysis by the observed reduction of populations in experimental samples relative to the inoculum environmental reference (Figure S1). Despite this reduction in bacterioplankton populations, all the populations in ambient pH samples were found to be dominant populations in environmental references. This suggests that in the ambient pH samples, the same bacterioplankton from the environment were present and exhibited
relatively similar patterns of dominance and structure. More interesting was the considerable shifts in populations observed in the low pH samples relative to ambient pH and environmental samples (Figure 3). Many of the dominant bacterioplankton populations in the ambient pH and environmental samples were not detectable in the low pH samples, while populations that were not detectable previously emerged in the more acidic conditions (Figure S2). Lastly, SSU rDNA clone libraries were constructed on a single representative experimental sample from each low and ambient pH cluster observed in the T-RFLP analysis (Figure 2). However, the presence of homologous peaks between environmental and experimental samples, and matches of these T-RFLP peaks to in silico digests of SSU rDNA sequences allowed for the putatively identification of populations in samples which clone library analysis was not performed (Figures S1 and S2).

Clone library analysis of SSU rDNA corroborated the T-RFLP community fingerprinting analysis and helped to identify the phylogenetic affiliations of bacterioplankton in the two different community types associated with ambient and low pH conditions. Although fewer bacterioplankton populations were found across all samples in the T-RFLP analysis relative to numbers found in the clone libraries. Multiple phylotypes are known to occur in the same T-RFLP peak. This was observed in T-RFLP peaks when compared to in silico digests of SSU rDNA sequences (Figure S1). However the trend in both T-RFLP and clone library analyses were very similar in that bacterioplankton communities in acidified treatments exhibited lower levels of richness (Figure 4). Greater abundance of populations, higher levels of evenness and richness were all observed in the ambient pH clone library in comparison to the low pH clone
library. However, greater coverage was found in the low pH clone library and because the number of clones in the two libraries was very similar, this was due to the lower richness in the low pH clone library as coverage is determined by dividing the number of unique clones by the number of clones sequenced (Table 2). The phylogenetic affiliations of populations were resolved to the lowest taxonomic group possible, some populations were identified to genus level, some only to family level, while yet others remained unclassified. Although a few taxa were found to be ubiquitous in both clone libraries, the majority of taxa were found exclusively at either ambient or low pH treatments (Figure 5). Bacterioplankton that arose in the acidified conditions were likely present under ambient pH conditions in low abundance below detection thresholds until shifts in environmental conditions favored the growth and dominance of these bacterioplankton (Sogin et al., 2006). As indicated by the SIMPER analysis, it was the change in these exclusive taxa that significantly influenced the structure of bacterioplankton communities in this study. Lower numbers of taxa relative to populations in each clone library were observed as a result of assigning and binning phylotypes into the same taxa due to inability to resolve to the lower taxonomic levels. For instance in the ambient pH clone library, three populations were all found to be phylotypes of *Colwellia*, causing these three populations to be represented as only one taxa in the results. Regardless of this disparity in abundance of OTUs and taxa, the trend again is clear, lower levels richness and structure were exhibited in bacterioplankton communities incubated under low pH or acidified conditions.

Phylotypes of *γ*-proteobacteria and *α*-proteobacteria dominated the composition of bacterioplankton communities in both low and ambient pH incubations in this
experiment. However the relative proportion of $\gamma$-proteobacteria decreased while that of the $\alpha$-proteobacteria increased with increasing acidity (Figure 5). This was indicated by the reduced $\gamma$ vs. $\alpha$ ratio from ambient pH to low pH incubations (Table 2). Other investigations on the response of bacterioplankton communities to increased ocean acidity have also found $\gamma$-proteobacteria and $\alpha$-proteobacteria to dominate communities taken from the North Atlantic and Arctic Oceans (Ray et al., 2012; Zhang et al., 2012). In addition, natural bacterioplankton communities throughout the vertical water column and within the acidified zone of the North Pacific have been shown to be contain $\gamma$-proteobacteria and $\alpha$-proteobacteria occurring in a variable range of pH levels (DeLong et al., 2006). The $\gamma$ vs. $\alpha$ ratio between the clone libraries in this study was calculated as a means to signify the shift in dominance between bacterioplankton communities, as it was observed that $\alpha$-proteobacteria increased overall richness and abundance in the low pH or more acidic incubations (Table 2 and Figure 5). A possible explanation for this occurrence is that $\alpha$-proteobacteria are genetically and metabolically better adapted for the stress and acidic conditions expected for the future. Members of the $\alpha$-proteobacteria are known to exhibit opportunistic or generalist traits (Moreno, 1998; Teeling et al., 2012). For instance, members from Rhodobacteraceace, a family of $\alpha$-proteobacteria found in this study (Figure 5), in addition to performing nitrogen fixation, have the ability to obtain energy both heterotrophically or autotrophically and in a wide variety of conditions (Imhoff and H.G., 1984). Moreover, Roseobacter a genus found within the Rhodobacteraceace, has been characterized as versatile group of marine bacteria with unusually large numbers of genes encoding for various metabolic pathways (Moran et al., 2007; Brinkhoff et al., 2008; Newton et al., 2010). Perhaps by reducing pH
in experimental incubations, *α-proteobacteria* are not only being selected for, but can thrive in absences of competitors that are not well suited for acidified conditions. This was indicated by the loss of bacterioplankton abundance observed in reduced DNA yields (data not shown) and richness in low pH incubations. Increased rates of protein synthesis and polysaccharide degradation by extracellular enzymes as a consequence of acidification have been documented (Grossart et al., 2006; Piontek et al., 2010; Piontek et al., 2012). Moreover, considerable suppression of prokaryotic activities has been observed at low pH conditions (Yamada et al., 2010). It is possible that the changes in the composition and structure of bacterioplankton communities observed in this study were driven by the succession of bacterioplankton possessing enzymes and metabolisms that are better suited for low pH conditions. Perhaps these better adapted bacterioplankton are the opportunistic *α-proteobacteria*. A correlation between community structure and metabolism was observed in our study. Changes in the structure of bacterioplankton communities as a result of increasing acidity correlated with major changes in the overall metabolism of these communities. The production of bacterioplankton communities in low pH incubations was significantly depressed (Figure 6). This could have been caused by the observed reduction of richness in low pH incubations, and possibly the energetic and metabolic costs to bacterioplankton under acidic or stressful conditions (del Giorgio et al., 2011). The respiration of bacterioplankton communities significantly increased in response to low pH conditions, and was reduced to a lesser extent due to elevated temperatures (Figure 7). The effects of reduced pH on bacterial respiration were greater than those of increased temperature. It is well known that bacterioplankton under elevated temperatures respond by increasing respiration (Apple et al., 2006b; Vazquez-
Dominguez et al., 2007; Kirchman et al., 2009). However in this study, the overall respiration of bacterioplankton communities decreased due to elevated temperatures. Because there is a positive correlation between respiration and production of bacterioplankton communities (Karl et al., 2003; Apple et al., 2006b; Piontek et al., 2010), the reduced respiration rates may have been caused by suppressed metabolic activities as indicated by decreases in bacterial production in reduced pH incubations. Another contributing factor may be the loss of total bacterioplankton respiring, as reduced richness and biomass suggested by lower DNA yields (data not shown) were observed in the acidified conditions. Although the overall respiration of bacterioplankton communities decreased, if there were less bacterioplankton in these communities as suggested, the per-cell respiration might actually not have decreased, but rather remained constant or increased. Moreover, there may have been complex unknown interactions between pH and temperature that caused the observed suppression of respiration under elevated temperature.

In this study, the effects of elevated temperature on the structure of bacterioplankton communities were minor relative to those of reduced pH, although respiration was lower due to elevated temperature. The acidified conditions in incubations were a stronger driving force behind changes in the structure and metabolism of bacterioplankton communities. In contrast to the findings of our study, past investigations have documented shifts in the composition of freshwater and marine bacterioplankton communities induced by increased temperatures (Simon et al., 1999; Pearce, 2005; Hall et al., 2009; Adams et al., 2010; Dziallas and Grossart, 2011; Lindh et al., 2012). However a majority of these findings have been made in exceptionally cold
environments where bacteria are adapted to the cold and are more likely to respond to minor increases in temperatures. Additionally, elevated temperatures have been shown to increase bacterial production and respiration (Apple et al., 2006b). Although the independent effects of elevated temperatures have been previously studied, relatively little is known about the combined effects and interactions of pH and temperature on bacterioplankton community structure and metabolism. This represents a critical area for future investigation, if we are to fully understand the role of bacterioplankton in the warmer and more acidic oceans of the future.

Advances in technologies will allow future investigators to examine heterotrophic bacterioplankton communities at higher resolutions. For instance the use of membrane-inlet mass spectrometry can allow for very precise respiration measurements over short time scales (Kana et al., 1994; Apple et al., 2006b). Next generation sequencing technology can be used to produce large metagenomic and metatranscriptomic datasets providing deeper insights into the structure of bacterioplankton communities as well as the types and rates of their metabolisms. Previous investigations have overlooked the effects of dissolved (DOM) and particulate (POM) organic matter quantity and quality, which are important regulators of heterotrophic bacterioplankton community structure and metabolism. Findings from several studies suggest that ocean acidification has the potential to alter the C:N stoichiometry and therefore the quality of organic matter produced by phytoplankton (Bellerby et al., 2007; Hutchins et al., 2009; Losh et al., 2012). Such changes to the content and quality of organic matter can result in shifts in structure and metabolism of heterotrophic bacterioplankton communities (Apple et al., 2006a; Apple and del Giorgio,
Additionally, varying quantities and concentrations of DOM has also been shown to affect bacterioplankton community structure and metabolism (Eiler et al., 2003; Ray et al., 2012). Even increasing $pCO_2$ concentrations alone has been shown to increase the consumption of organic matter by heterotrophic bacterioplankton (Riebesell et al., 2007). It is predicted that these changes to the food source of bacterioplankton in addition to the effects of increased temperature and acidity will result in a greater loss of carbon in the upper ocean (Delille et al., 2005; Riebesell et al., 2007; Mari, 2008). This could have potentially significant consequences for carbon sequestration in the oceans and the oceans’ ability to respond to global climate change and acidification. Nonetheless, definitive knowledge regarding how bacterioplankton will structurally and metabolically respond to these combined and interactive effects remains limited (Finkel et al., 2010). This represents an area where future research is necessary to fully integrate biological processes into models of how the oceans will respond to future conditions. Although biological processes are fundamental in determining how the oceans will respond, most of the current models overlook these important components (Borges et al., 2005). Complex feedback cycles exist between the chemistry and biology of the oceans (Riebesell et al., 2009; Teira et al., 2012). In order to make reliable predictions for the future, ensuing models on the effects of climate change and acidification on the oceans should not overlook the biological processes and the potential feedbacks that exist between the various oceanographic processes that are simultaneously occurring. The Salish Sea is a unique marine and estuarine ecosystem with tremendous socio-economic and ecological value. Not only are such ecosystems under the direct threat of ocean acidification from the absorption of
atmospheric CO$_2$ (Harley et al., 2006), these ecosystems experience enhanced acidification from natural upwellings and anthropogenic sources of eutrophication increasing overall respiration in the system (Feely et al., 2010; Cai et al., 2011; Mucci et al., 2011). Future investigations on the effects of climate change and ocean acidification on ecosystem structure and function should be directed at these important ecosystems and should also establish a context of socio-economic and ecological importance to human populations.

This is the first study to focus on the effects of elevated temperature and acidity on the structure of heterotrophic bacterioplankton communities in the Salish Sea. Only a handful of similar studies have been carried out in other marine ecosystems (Liu et al., 2010; Joint et al., 2011). Furthermore, there are even fewer investigations linking shifts in community structure to changes in the metabolism and ecological function of these important microorganism as a result of warmer and more acidified conditions (Riebesell et al., 2007; Lidbury et al., 2012; Teira et al., 2012). In this study, temperature had little detectable effects on the structure of heterotrophic bacterioplankton communities taken from the Salish Sea. However, acidified conditions caused shifts in the structure of these bacterioplankton communities. In addition to the observable changes in dominance and composition, lowered pH also reduced richness and overall structure of manipulated communities. These changes in the diversity of bacterioplankton communities correlated with significant changes in their overall metabolic activity. Bacterial production rates decreased, while bacterial respiration increased under lower pH conditions. These findings suggest that the ecological function of heterotrophic bacterioplankton communities in the ocean will potentially be affected by the acidified conditions that are
predicted for the next century. Changes in the structure and metabolism of bacterioplankton communities have been shown to affect their ecological function (del Giorgio and Bouvier, 2002; Fuhrman, 2009). Based on the observations made in this study, in a more acidified ocean the efficiency of biological processes mediated by heterotrophic bacterioplankton may be reduced as less organics are cycled through bacterial production and more carbon is lost to respiration. Such alterations to the ecological function of heterotrophic bacterioplankton communities could have far reaching consequences for marine trophic interactions as well as the cycling and sequestration of carbon in the oceans. Results from the handful of previous investigations on the response of bacterioplankton communities to the effects of global climate change and ocean acidification are varying and sometimes contradictory, suggesting that this area of research is still at its infancy. As such, further research in this area is necessary and should focus on drawing strong connections between the structure and function of these ecologically important organisms. Nonetheless, the results from this study highlight the ability of heterotrophic bacterioplankton to respond to ocean acidification both structurally and metabolically, which will have significant implications for their ecological function in the marine carbon cycle and the ocean’s response to global climate change.


Figure 1. Sample locations include: environmental samples collected from the bottom waters of North (1) and South (2) San Juan Channel, San Juan Islands WA. Inoculum (3) for $p$CO$_2$ perturbation experiments were taken from the seawater intake system at Shannon Point Marine Center, Fidalgo Island, WA.
Figure 2. UPGMA/Pearson product moment correlation cluster analysis of bacterial T-RFLP community fingerprint analysis of three environmental samples and eight meta-communities from pCO$_2$ perturbation experiments. Scale bar is in percentage similarity calculated by the Pearson product moment correlation r-value X 100. Values at nodes represent the cophenetic correlation coefficients. Clone library analyses were performed on samples in boxes.
Figure 3. Bray-Curtis similarity multidimensional scaling plot generated from bacterial T-RFLP community fingerprint analysis with 20, 25 and 50 percentage similarity contours. Values adjacent to clusters represent mean similarity between samples within clusters.
Figure 4. Rarefaction analyses of sequences from constructed clone libraries. DOTUR was used to designate OTUs based on a 97% sequence similarity cut-off. Dotted lines represent 95% low and high confidence intervals.
Figure 5. Relative taxonomic composition identified by clone library analyses of 380 ppm and 1050 ppm treatments. Taxonomic affiliations were determined by RDP version 10.14 seqmatch algorithm. Representatives of γ-proteobacteria are identified by shades of blue, while those of α-proteobacteria are identified by shades of red.
Figure 6. Bacterial respiration rates (O₂mg/l/hr) of various treatments measured by the decline of O₂ in 24 hr (A) and 48 hr (B) incubations. Error bars represent standard error between replicates in each treatment. Note the difference in scale of bacterial respiration rates between graphs A and B.
Figure 7. Bacterial production rates (μgC/l/hr) of 380 ppm and 1050 ppm treatments estimated by the incorporation of [3H]leucine with a carbon conversion factor of 3.1 kg C mol per leucine. Error bars represent standard error between replicates in each treatment.
Table 1. T-RF counts from eight restriction enzymes in bacterial T-RFLP community fingerprint analysis. The number of T-RFs is a richness index representing the putative number of OTUs within a bacterial community. Dotted lines separate three clusters identified by UPGMA/Pearson product moment correlation cluster analysis of bacterial T-RFLP community fingerprints. Reported values are mean ± SE.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$AluI$</th>
<th>$BstUI$</th>
<th>$HaeII$</th>
<th>$HhaI$</th>
<th>$HinI$</th>
<th>$MboI$</th>
<th>$MspI$</th>
<th>$RsaI$</th>
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<th>Group mean</th>
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<td>23</td>
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<td>20</td>
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Table 2. Summary of clone library analyses results. Number of OTUs, estimated OTU richness (ACE and Chao), and Shannon diversity index were calculated at 97% sequence similarity using DOTUR, values in brackets represents 95% confidence intervals. γ:α ratios are estimated by the GenBank matches for sequences in each clone library. Clone library coverage was calculated with the following equation: $C=1-(n/N) \times 100$, where $n$ is the number of unique OTUs and $N$ is the total number of clones analyzed.

<table>
<thead>
<tr>
<th>Clone library</th>
<th>Clones sequenced</th>
<th>OTUs</th>
<th>γ:α ratio</th>
<th>Estimated OTU richness</th>
<th>Shannon diversity index</th>
<th>Clone library coverage</th>
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</thead>
<tbody>
<tr>
<td>380 ppm</td>
<td>75</td>
<td>32</td>
<td>8.89</td>
<td>46.8 (37.0; 75.4)</td>
<td>62.0 (40.7; 135.2)</td>
<td>3.79 (3.60; 3.97)</td>
</tr>
<tr>
<td>1050 ppm</td>
<td>74</td>
<td>23</td>
<td>4.00</td>
<td>59.2 (32.8; 145.0)</td>
<td>47.0 (27.8; 120.2)</td>
<td>2.98 (2.72; 3.25)</td>
</tr>
</tbody>
</table>
B) Inoculum – *Hae III*

- Colwellia [γ]
- Alcanivorax [γ]
- unclassified [γ]
- Pseudomonas [γ]
- Phaeobacter [α]
- Spongiispira [γ]
- Rhodobacteraceae [α]
- Porticoccus [γ]
- Oleispira [γ]
- Glaciecola [γ]

380ppm – pH 8.0 – *Hae III*

- OTU 1 Spongiispira [γ]
- OTU 6 Alcanivorax [γ]
- OTU 5 Colwellia [γ]
- OTU 7 unclassified [γ]
- OTU 2 Rhodobacteraceae [α]
- OTU 11 Oleispira [γ]
- OTU 4 Glaciecola [γ]
- OTUs 3/8/9 Colwellia [γ]

1050ppm – pH 7.7 – *Hae III*

- OTUs 2/5 Phaeobacter [α]
- unclassified [γ]
- OTU 3 Alcanivorax [γ]
- OTU 4 Porticoccus [γ]
- OTUs 1/6 Pseudomonas [γ]/ unclassified [γ]
Figure S1. Terminal-restriction fragment length polymorphism (T-RFLP) electropherograms showing \textit{BstUI} (A), \textit{HaeIII} (B) and \textit{HhaI} (C) digests for the samples (from top to bottom) Inoculum, 380 ppm, and 1050 ppm. Clone libraries were only constructed for experimental samples (380 ppm and 1050 ppm). Arrows indicate peaks corresponding to OTUs detected in each clone libraries with associated Ribosomal Database Project (RDP) matches.
Figure S2. Bacterial T-RFLP data from three environmental samples, as well as eight CO₂ and temperature experiment samples by the restriction enzyme HaeIII. Colored arrows indicate T-RFs from both environmental communities and communities associated with lower pCO₂ concentrations (Green), communities associated with higher pCO₂ concentrations (Red), and those of unculturable bacterial from environmental communities (Blue).