SPECIALIZED RELATIONSHIPS BETWEEN ACTIVE BACTERIA AND THEIR ENVIRONMENT

by

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ABSTRACT OF THE DISSERTATION

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The question as to which environmental factors select for or influence the resident and active community within the global ocean is still unclear. While general trends have been established in overall community response to natural forces and the impact of particular environmental parameters on biodiversity, little has been done to examine how certain species react to environmental stimuli and how the percentage of metabolically active species correlates to the ambient conditions. This research aimed to address these understudied areas and provide insight into how particular alterations in the physical, chemical, and biological environment relate to species composition and activity. More specifically, this work utilized molecular techniques, such as 16S and 18S ribosomal RNA analysis, to track how changes within the active and resident bacterial populations correlate to changes in environmental drivers. By narrowing the focus to two widely spaced locations, a New Zealand Fjord and a Caribbean river plume, and then evaluating these relationships in the laboratory, it was our hope to find patterns in species-specific bacterial activity associated with salinity, DOM, and phytoplankton dynamics in model systems. Results of this work indicate that no single environmental parameter drives the

diversity of a system and that only a small percentage (< 30%) of the active bacterial population were correlated with the physical/chemical/biological parameters measured in our field studies. Furthermore, this data indicates that the appropriate parameters are not currently being measured to determine the regulatory force on the abundance and activity of the microbial population.

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Chapter 1: Introduction

1.1 The Relationship Between the Community Composition of Bacteria and the Environment

Understanding the mechanisms controlling the diversity and distribution of bacterial species remains one of the overarching themes in aquatic microbiology. While it has been shown in both laboratory and field experiments that bacterial species can react differently over temporal and spatial gradients (Riemann & Middleboe, 2002), the physical/chemical/biological drivers which influence microbial diversity and activity are still unclear. Changes in diurnal ectoenzymatic activity (Karner et al., 1992; Rath et al., 1993; Karner & Rassoulzadegan, 1995), bacterial production (Riemann & Sondergaard, 1984; Ducklow et al., 1993), amino acid assimilation (Jorgensen et al., 1983), and carbon flow (Nielson & Richardson, 1989) have all been linked to alterations in the abundance and activity of the bacterial community. As a result of this, it can be hypothesized that alterations within specific environmental parameters will also elicit a variety of responses from the bacterial community**.**

While these and similar works demonstrated the relationship between the bacterial populations and their surroundings, they typically utilized methods that characterized the entire microbial community (i.e. ^{14}C or leucine uptake), obscuring any variability among the different members within the system. This methodological homogenization prevents a mechanistic understanding of the flow of biomass and energy through a system, and precludes a comprehensive understanding of how changes within the microbial community impact an ecosystem. As a result, these bulk population analyses may also limit our ability to predict how natural and anthropogenic perturbations will impact an

ecosystem at the microbial level. Thus, it is necessary for researchers to narrow their focus to the species level when evaluating the response of the bacterial community to its environment.

Previous studies utilizing this more individualistic approach have suggested that environmental (i.e. Troussellier et al., 2002; Lami et al, 2009) and/or temporal elements (i.e. Fuhrman et al., 2006; Nelson et al, 2008) may contribute to the variability within microbial assemblages. However, discrepancies exist within the results of these investigations. The following sections outline the relationship between bacterial community composition and salinity, dissolved organic matter, nutrients, and phytoplankton and provide additional insight into these conflicting reports within the literature.

1.1.1 Salinity

The majority of the previous work investigating the effects of salinity on the bacterial population has been performed in the laboratory or by transplanting bacteria from one location to another. However, despite the controlled environment and similar methods, these studies have yielded differing results. For example, three reports of cultivated bacteria concluded that freshwater strains cannot survive in more saline environments (Valdes & Albright, 1981; Prieur et al., 1987; Hyun et al., 1999). However, another study found that freshwater bacteria grew just as well at 5 psu above ambient salinity and that they continued to grow at more than10 psu above ambient salinity, but at a reduced rate (Painchaud et al., 1995). Likewise, field experiments have also reported conflicting results with respect to salinity. One study on the phylogenetic and physiological structure along the Chesapeake Bay found that distinct populations

existed along the salinity gradient and that a potential relationship existed between cell physiology (i.e. bacterial production and respiration) and salinity (del Giorgio & Bouvier, 2002). Similar results have been published for the beta Proteobacteria in the Delaware estuary (Kirchman et al., 2004) and for the Columbia River system (Crump et al., 2004). In contrast, comparisons of surface water samples at various salinities from the Amazon/Orinoco River Plume indicate that alterations in community structure are not overwhelmingly related to salinity (Hewson et al., 2006). Perhaps one explanation for these differing results is that these studies generally focused on presence/absence and utilized group measurements to measure production and respiration. In examining how species react to alterations in salinity, it is necessary to differentiate those species that simply survive in the area versus those that are metabolically active in this environment (i.e. nutrient uptake, respiration, etc.).

1.1.2. Dissolved organic matter

Like salinity, the nature or quality of dissolved organic matter (DOM) is significantly linked to the heterotrophic bacterial community. The DOM both influences and is shaped by the active microbial population. Not all of the DOM present is available for utilization by bacteria; in fact some of the compounds present within the DOM pool are refractory. In addition to the organic content, size also excludes potential carbon sources. The majority of marine bacteria are obligate osmotrophs capable of transporting only very low molecular weight compounds (< 300 Daltons) into the cell (Hansell & Carlson, 2002). Therefore, it is assumed that bacteria are largely dependent upon the quality and quantity of DOM and should respond to the DOM accordingly. Meanwhile, it has also been demonstrated that shifts in the bacterial population impact the DOM

composition (Kirchman, 2000). In fact, one work indicated that bacteria are specialized in their ectoenzyme production and therefore, a number of species may be necessary to digest a suite of compounds (Martinez et al., 1996). Thus, bacterial diversity and activity is intimately linked to DOM quality and composition.

There have been a number of laboratory studies in support of the concept that DOM impacts bacterial dynamics. For instance, it has been shown that very different communities thrive on green algal detritus versus cyanobacterial detritus (van Hannen et al., 1999) and that specific bacterial groups respond differently when exposed to high-molecular weight than to low- molecular weight fractions of dissolved organic carbon (Covert & Moran, 2001). Still two more studies indicate that the DOM source and to a lesser extent inoculum source influence community structure (Kirchman et al., 2004; Judd et al., 2006). In one of the experiments, Judd et al. (2006) found that in 4 of 5 cases when DOM from an outside location was added to the inoculums, the community composition became more similar to the community from which the DOM was derived than the control of the population from which the inoculum originated.

However, despite the overwhelming evidence from the aforementioned laboratory studies and the conceptual understanding that the DOM affects the bacterial community and production, two field investigations seem to suggest otherwise. While researching a region of the Choptank River in Maryland known to contain an abrupt transition zone from β- to α-proteobacteria (Bouvier & del Giorgio, 2002), researchers found no overwhelming patterns within this transition zone connecting alterations in DOC and nutrient quantity to the decline in bacterial production and bacterial growth efficiency nor did they see an increase in bacterial respiration to carbon consumption (del Giorgio &

Bouvier, 2002). Likewise, in yet another study, after performing PCA analysis on their T-RFLP data and a number of environmental factors, including concentrations of bioavailable DOC and the fraction of humic, fulvic, and hydrophilic compounds within the DOC, Stepanauskas et al. concluded that it was random variation and not DOC controlling fluctuations within the bacterial population of a temperate delta system (Stepanauskas et al., 2003).

1.1.3 *Inorganic Nutrients*

It has been widely accepted that bacteria are responsible for a large portion of nutrient remineralization, releasing inorganic nutrients such as nitrogen, phosphorus, and silica back into aquatic systems. However, it has also been demonstrated that these organisms take up nutrients for growth. Numerous studies (i.e. Fuhrman et al., 2006; Kan et al., 2006; Lami et al., 2009) indicate that these elements are related to community composition; however they differ in their conclusions as to which particular inorganic nutrients are related to the structure of bacterial populations. Therefore, further research is necessary on the association between inorganic nutrients and bacteria, especially at the species level.

1.1.4 Phytoplankton Dynamics

Traditionally, the link between bacteria and phytoplankton was considered to be based solely upon the ability of bacteria to perform remineralization. The bacteria would break down dead organisms and feces via exoenzymatic activity and release the nutrients essential for photosynthesis. However, with the discovery of the microbial loop in 1974 the connection between bacteria and phytoplankton gained additional complexity (Pomeroy, 1974). According to this paradigm, organic matter produced by phytoplankton

is lost as dissolved organic material (DOM). In addition to amino acids, this material consists mostly of early products of photosynthesis such as glucose or glycolic acid. All of these are capable of being absorbed and incorporated by microorganisms (Pomeroy, 1974). In fact, later studies showed that 30 – 50% of the bacterial carbon requirements could come from the direct excretion of photosynthetically fixed carbon (Cole et al., 1988; Baines & Pace, 1991; Ducklow et al., 1999). Thus, it becomes apparent that the activity of the phytoplankton must be related to that of the bacteria.

To date, general trends have been established in the response of the entire bacterial community to phytoplankton (i.e. the linkage between bacterial and primary production in the open ocean [Cole et al., 1988]), yet only a few studies have been done to identify the regulating factors on individual bacterial species. The studies that have been performed, however, indicate a close association between species of each group. For example, it has been demonstrated that certain bacteria can promote cell death in phytoplankton (Mayali & Azam, 2004), can promote or reduce phytoplankton growth (Ferrier & Rooney-Varga, 2002; Yoshinaga et al., 1995), and can impact the production of algal toxins (Gallacher et al., 1997). Meanwhile, phytoplankton have been shown to impact the bacterial composition as they perform bacterivory during P stress (Nygaard & Tobiesen, 1993) and evidence has been gathered indicating that the quality of organic matter produced by different types of phytoplankton cause shifts in the bacterial species utilizing this organic matter (van Hannen et al., 1999). However, these works concentrate almost solely on specific harmful algal blooms so the relationship between a variety of phytoplankton and bacterial species still remains somewhat unclear.

Still, there have been a few studies outside of harmful algal blooms that demonstrate the concept that specific phytoplankton can impact the bacterial community. One study of an upwelling driven bloom occurring at LEO – 15 off the coast of New Jersey sought to determine if the decrease in phytoplankton diversity during the bloom would result in an alteration in bacterial diversity and promote specific bacteria. The data from this work indicated the emergence of new groups during the bloom. While 20 – 25% of the clones obtained during the dinoflagellate/diatom bloom were found to be related to the epsilon subdivision of proteobacteria, the specific SSU genes identified in the study were not seen in the non – bloom samples (Kerkhof et al., 1999). Meanwhile, Bidle et al. were also able to uncover a link between diatoms (*Skeletonema costatum, Chaetoceros* spp., and *Thalassiosira* spp.), particulate organic carbon, and a number of bacterial groups during a diatom bloom in Monterey Bay, California. In the course of their study to investigate the capacity of bacteria to solubilize the organic matrix that protects the siliceous frustules of diatoms, the authors also were able to determine which bacterial phylotypes colonized the diatoms. Using DGGE and rRNA, they discovered that particular attached bacterial groups, such as Cytophaga/Flavobacteria/Bacteriodes, α-proteobacteria, and γ-proteobacteria, were linked to the diatoms and controlled $bSiO_2$ dissolution (Bidle et al., 2003). While these and other studies support the concept that the close association between species of phytoplankton and bacteria impact the success of the species from each group, these works focus on a limited number of organisms (i.e. the particle associated bacteria or simply those appearing in clonal libraries), are centered on upwelling-driven or toxic blooms, and/or focus on colonization and pathogenic type responses. By incorporating the bacteria that appear within both the RNA and DNA

signatures and sampling under non-bloom conditions, this work aims to discover relationships between phytoplankton and a larger subset of bacteria.

1.2 Resident Versus Active Bacteria

Perhaps one reason that previous work has been unable to come to a consensus is due to the techniques employed. The molecular methods employed in the majority of studies infrequently address the question of presence versus activity. Molecular techniques, such as 16S rRNA gene fingerprinting and DGGE, have allowed for the rapid identification of organisms at higher resolutions, however, they do not designate which species are performing a metabolic function in a particular ecosystem. It has been demonstrated in numerous studies that individual activities differ from that of the community (i.e. del Giorgio & Bouvier, 2002; Kirchman et al., 2004) and that particular groups only thrive under certain conditions (i.e. van Hannen et al., 1999; Kirchman et al., 2004). Presence does not necessarily indicate activity. Even though an organism appears within a sample, it may not be actively involved in the environment. For example, marine bacterial cells may become dormant for periods of time during inhospitable conditions (Morita, 1980). Therefore, in order to investigate the influence of environment on the bacterial population, it is necessary to analyze both individual and community activity.

Previous studies have indicated that significant differences exist between the 16S rRNA gene and the16S rRNA phylotypes found within a sample (i.e. Troussellier et al., 2002; Moeseneder et al., 2005; Gentile et al., 2006; Lami et al., 2009) . Thus, in monitoring both the resident (rRNA gene) and active (rRNA) factions, researchers hope to obtain a clearer understanding of the microbial community and its potential. While

these works indicate that the environment plays an essential role that in structuring the microbial community, the methodologies employed may have biased these results. For instance, previous studies analyzed only on a single depth (Fuhrman et al., 2006; Gentile et al., 2006) ignoring the influence of vertical scale dynamics, such as mixing or stratification. Meanwhile other studies have overlooked the importance of the entire community, including the "rare biosphere" (Sogin et al., 2006), by focusing on select groups (Lami et al., 2009) or limiting their analysis to select OTUs which were readily resolved (Troussellier et al., 2002). Thus, no definitive explanation as to the structuring mechanism(s) regulating this diversity has been determined.

1.3 Objectives of Dissertation

The overall goals of this thesis were to evaluate the following hypotheses:

H_1: *The microbial community composition, the percentage of active bacteria, and specific individual active bacterial species, will change in parallel within a strong environmental gradient*

Ho: Any shifts in microbial parameters (composition, activity, etc.) within strong environmental gradients will be within the level of variability observed between replicate samples. [This finding would indicate we are not measuring the environmental parameters important to the microbial population (i.e. the impact of predatory behavior) or that neutral theory plays a larger role in determining diversity.]

To accurately evaluate these hypotheses, these specific tasks were completed using samples collected from two systems (the Orinoco River plume and Doubtful Sound, a New Zealand fjord).

1. Identify specific players within the microbial community that are changing in activity and/or cell abundance from field samples collected along the Orinoco River plume. Then, correlate these changes to selected environmental parameters (i.e. salinity, nutrients, and phytoplankton dynamics).
2. Evaluate whether any emerging patterns found along the Orinoco River plume (a low latitude system) also exist in Doubtful Sound (a high latitude system with an enhanced physical and chemical gradients). Moreover, to identify if alterations in additional environmental variables (i.e. dissolved organic matter and seasonality) increase the proportion of individual bacteria that change in activity or abundance.
3. Verify if the observed environmental parameters are associated with changing microbial activity and numbers by replicating similar patterns under laboratory conditions using water obtained from Doubtful Sound.

1.3.1 Dissertation Overview

The first objective, to assess whether the environment shapes bacterial diversity, is addressed in Chapter 2. This work focuses on the Orinoco River plume and the surrounding Caribbean basin, a region designated by horizontal and vertical salinity gradients. Here the influence of salinity, depth, distance from the mouth of the Orinoco, fluorescence, PAR, inorganic nutrients, and phytoplankton dynamics on the active percentage and on individual species from the active and resident bacterial populations is tested. These results are then compared to the relationship between the measured parameters and the overall community composition. The data indicates that while the composition of the overall resident and active populations were related to a number of

environmental factors, less than 30% of the individual species demonstrated a connection to the physical, chemical, and biological variables. Furthermore, no significant relationship was found between the percentage of active species or species richness and the measured parameters.

Two hypotheses can be made for the lack of significant connections between the observed environmental parameters and the members of the active and resident bacterial populations. The first hypothesis is that the range over which the samples were taken may not have been sufficient enough to elicit change. The salinity only changed from 18 to 36 and despite previous reports the phytoplankton community did not decrease in size moving away from the mouth of the Orinoco. The second hypothesis for the negligible number of associations between the bacteria and the environment is that the appropriate variables were not monitored. The study outlined in Chapter 3 was established to test these potential explanations.

In Chapter 3, an area with more pronounced environmental gradients was monitored and the effect of additional motivating environmental factors on the bacteria, such as organic carbon and seasonality, were evaluated. Doubtful Sound, one of 14 fjords on New Zealand's southwestern coast, exhibits extreme salinity, nutrient, and organic matter variations on both horizontal and vertical scales. After studying the influence of salinity, depth, distance from the tailrace, CDOM components, inorganic nutrients, and phytoplankton dynamics on the individual species from both the active and resident bacterial populations, it was determined that a more significant gradient did not elicit a greater response by the bacterial community. Despite the extreme gradients and the additional variables, the results were similar to those in Chapter 2. Approximately

30% of the members detected in either the resident or active bacterial communities demonstrated a significant correlation to the measured parameters. Likewise, although some environmental variables were found to be related to the overall resident and active community structure, no correlation was found between species richness and distance from the head of the fjord, depth, or salinity. However, unlike the percentages of active bacteria in the Orinoco River Plume, the portion of the active bacteria residing at a location within Doubtful Sound was found to increase moving away from the freshwater source.

While many of the results from Chapter 3 were similar to those in the study on the Orinoco River Plume, new influential environmental features emerged. The majority of active OTUs from 2007 that demonstrated a statistically significant relationship with the measured physical/chemical/ biological variables were related to inorganic nitrogen and terrestrial fulvic-type and humic-like components of the CDOM. This finding implies that nitrogen and terrestrial input of humics are very important to a subset of the active microbial community in Doubtful Sound during the winter. Moreover, seasonality may play a role in determining community composition. Individuals may appear throughout the fjord regardless of the season, however the active population at each station differs drastically depending upon the time of year. This indicates that species composition at a location may be reliant upon temporal forcing, a concept that has been seen at other locations as well (i.e. Fuhrman et al., 2006; Nelson 2008). These results indicate that while the enhanced gradient at Doubtful Sound may not have increased the number of individual correlations between environmental variables and bacterial species, monitoring

additional factors can improve our understanding of the relationship between bacteria and their surroundings.

Chapter 4 of this thesis attempts to address one of the potential reasons for the limited number of statistically significant correlations detected in Chapters 2 and 3. Along the Orinoco River Plume and in Doubtful Sound, a number of variables were changing simultaneously over a short spatial range. As a result, the relationships between members of the bacterial community and the environment may have been obscured. Chapter 4 outlines a transplant experiment, which was undertaken to reveal any highly specific relationships and verify those associations that were previously identified. Bacteria from low and high salinity waters within Doubtful Sound were subjected to a variety of salinities to uncover any connections between salinity and bacteria at the species level. It was determined that while the overall community structure, percent active, and species richness remained somewhat similar despite the salinity, certain members of the active and resident bacterial populations did respond to the experiment fluctuations.

Along with salinity, nutrient concentrations were also manipulated in the transplant experiments detailed in Chapter 4. When nutrient concentration was the only variable that was altered, both with and without larger organisms present, the population structure generally remained intact and only a select few species were affected. Instead, the results of this work indicate that species – species interactions between bacteria from different locations may be more influential in shaping the microbial community than some environmental factors.

1.4 Implications

There are two essential questions that must be addressed in order to achieve an understanding of how biomass and energy are produced and utilized in marine ecosystems. The first is what is the composition of the community? Who is there? The second, and perhaps more difficult question, is what are the ecological roles of each member within the community? Are the relationships between species and the ecological forces that drive them specialized? While a great deal of work has been done on the first question using direct counts, etc., the association between activity and environment still requires further investigation. This study will be one of the first to explore the relationship between elements within ecosystem and bacterial activity at the species level.

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Chapter 2. Monitoring Active Microorganisms in the Orinoco River Plume by TRFLP Profiling of Intact Ribosomes

2.1 Abstract

A study of the Orinoco River Plume was initiated to elucidate how physical, chemical, and biological gradients influence planktonic bacterial communities. The Orinoco plume forms an isolated surface layer with a sharp salinity gradient and high chl *a* signals extending thousands of kilometers into the Caribbean Basin. Samples were collected on September 18-27, 2006 from 13 stations at 4 depths along the plume axis and screened using ribosomal RNA genes by terminal restriction fragment length polymorphism (TRFLP) analysis. Both intact ribosomes and DNA (16S and 18S rRNA genes) were analyzed to separate the active microbial community from the populations that were present but not growing. Results indicate that a number of measured environmental parameters can be linked to TRFLP signal from the overall populations and a small proportion of individual phylotypes/species, however, no single factor was found that regulates most of the members of the bacterial community. This result supports the concept that it is essential to monitor specific members of both the active community and the resident population to discern the mechanisms driving overall microbial community change in the ocean.

2.2 Introduction

In "The Paradox of the Plankton," G.E. Hutchinson (Hutchinson, 1961) confronted a major contradiction between ecological theory and the natural world. According to the competitive exclusion principle, two species that are competing for the same resources cannot stably coexist. However, tens or hundreds of phytoplankton

species can be found within a small volume of water, despite the limited room for niche specialization and intense competition for the same basic resources (Hutchinson, 1961; Scheffer, 2003). One of the major reasons for this non-compliance, Hutchinson surmised, is that aquatic environments may not be a habitat where competing species are at equilibrium. In aquatic systems, environmental conditions can change very rapidly. These dynamic environments ensure that no one species has the advantage long enough to promote the extinction of others. Therefore, a single group cannot competitively exclude others, in a changing environment (Hutchinson, 1961; Richerson, 1970)

While the main focus of Hutchinson's paper was phytoplankton, it has also been argued that plankton serves as a model or other ecosystems, including the bacterial realm (McCauley & Murdoch, 1987; Scheffer, 2003). For example, changes in diurnal ectoenzymatic activity (Karner et al., 1992; Rath et al., 1993; Karner & Rassoulzadegan, 1995), bacterial production (Riemann & Sondergaard, 1984; Ducklow et al., 1993), amino acid assimilation (Jorgensen et al., 1983), and carbon flow (Nielson & Richardson, 1989) have all been linked to alterations in the abundance and activity of the bacterial community and attributed to changes in the physical/chemical/ biological conditions in the water column.

Although these studies demonstrated the relationship between overall bacterial populations and their surroundings, the methods cited above assay the entire microbial community (e.g. community ^{14}C or leucine uptake). This approach can obscure any variability that may exist among the different members within the system. Such methodological homogenization of the entire community prevents a mechanistic understanding of the role of individual participants in the flow of biomass and energy

through a system, and precludes a comprehensive understanding of how specific changes within particular members of the microbial community impact an ecosystem. As a result, bulk population analyses limit our ability to predict how natural and anthropogenic perturbations will impact an ecosystem at the single species level.

The purpose of this study was to evaluate if changes within the active and resident marine bacterial population, represented by specific operational taxonomic units (OTUs), correlate with physical/chemical parameters, such as alterations in the salinity, nutrient concentration, or the structure of the eukaryotic community. This research concentrates on the Orinoco River Plume in the Caribbean Sea, an area known to exhibit fluctuations in salinity, nutrients, and phytoplankton species (Lewis and Saunders, 1989; Bidigare et al., 1993; Corredor et al., 2003). The Orinoco River plume, like other tropical river plumes, can extend great distances into the open ocean due to weak Coriolis forcing at low latitudes [Joint Australian-US Group, 1994]. The Orinoco is the fifth largest river in the world and delivers nearly 70 x 10^3 m^3/s of water to the Caribbean Basin, carrying with it an average of 5 mg/L of dissolved organic carbon and 0.22 mg/L of dissolved organic nitrogen (Lewis and Saunders, 1989). When the freshwater from the Orinoco River reaches the ocean, it stimulates primary productivity and produces high chlorophyll concentrations out into the Caribbean (Bidigare et al., 1993, Corredor et al., 2003). The buoyant plume forms an isolated surface layer that gradually mixes with the ocean water below establishing sharp vertical salinity gradients. Horizontally, a decaying gradient of chlorophyll content, colored dissolved organic matter, and diffuse attenuation matching increasing salinity in a plume extending northeast as far as Puerto Rico (Del Castillo et al., 1998; Morell and Corredor, 2001). These features make the Orinoco River Plume an

ideal location to investigate specific associations between particular bacteria and their environment.

The goal of this study was to uncover how specific causal mechanisms may be influencing specific bacterial members of the community by testing for correlations between environmental drivers and presence/absence or activity. For our study, the relationships linking the active and resident bacterial populations (as measured by genetic fingerprinting of intact ribosomes and their genes) to salinity, nutrients, fluorescence, photosynthetically available radiance (PAR), and eukaryotic community structure were determined using multivariate analyses. The results indicate that less than half of the members of the resident community are metabolically active and growing and that individual OTUs vary in their response to specific environmental factors. Ultimately, the research described here will help to elucidate the mechanisms driving microbial diversity in the marine realm.

2.3 Methods

2.3.1 Sample Processing

Thirteen CTD casts were performed at sample sites selected inside and outside of the Orinoco River Plume based upon near real-time satellite imagery (coastwatch.noaa.gov; see Figure 2.1). Water was collected at 4 depths (two depths within the plume, one depth below, and one at the deep chlorophyll maximum) from each sampling site (Figure 2.2), except in the shallow regions at the mouth of the Orinoco River. For each depth, microbial biomass from 4 liters was concentrated onto 0.2 µm SUPOR filters, frozen and shipped at liquid nitrogen temperatures, then placed at -80°C

prior to laboratory extraction. Duplicates from a select set of stations and depths were also collected.

2.3.2 Chemical Analysis

Water samples for chemical analyses (50 mL) were collected and stored at 4^{o} C in Teflon-Coated polyethylene bottles until analysis. Analytical procedures for silicate, nitrate plus nitrite, and phosphate are those described by Strickland and Parsons [1972].

2.3.3 SSU Gene Fragment Amplification and TRFLP

Total nucleic acids were extracted from biomass samples using a modified phenol chloroform DNA extraction (Kerkhof et al., 1993) for microbial community characterization. Genomic DNA and RNA were used to amplify the 16S and 18S rRNA genes via PCR (polymerase chain reaction) and RT-PCR (reverse transcriptase PCR), respectively. For the 16S rRNA genes from DNA, 50 µl PCR reactions were set up with 10ng template and 20 pmol of the universal primer 27 Forward (5' AGA GTT TGA TCC TGG CTC AG 3') and the bacterial specific primer 1100 Reverse (5' GGG TTG CGC TCG TTG 3') per reaction. The amplification parameters were as follows: 94ºC for 5 min followed by 25 cycles of 94ºC for 30 sec, 57ºC for 30 sec, and 72ºC for 1 min and finally and extension period of 72ºC for 10 min. The 18S rRNA genes from DNA were similar with 20 pmol of the 18S forward primer cited in Moon-van der Staay et al. 2000 (5' ACC TGG TTG ATC CTG CCA G 3') and 20 pmol of Euk516r (5' ACC AGA CTT GCC CTC C 3') (Amann et al., 1990) under the following conditions: 94ºC for 5 min followed by 35 cycles of 94ºC for 45 sec, 56ºC for 45 sec, and 72ºC for 2 min, with a final extension period of 72ºC for 10 min.

To obtain purified RNA for the RT-PCR reactions, total nucleic acid samples were diluted 1/100 and treated with the Turbo DNA-free™ kit (Applied Biosystems, Foster City, CA) to remove any contaminating DNA. The RNA was further diluted to 10^{-5} times the initial level to provide comparable target molecule concentration as the DNA amplifications. Two microliters of the diluted extract were used with the Titan One Tube RT-PCR kit (Roche, Basal, Switzerland) for amplification of 16S and 18S rRNA gene fragments from the most abundant, intact ribosomes. The amplification parameters were the same with the exception of the 30 min incubation at 50ºC for reverse transcription. Concurrent PCR controls were run without RT to identify any DNA contamination. No amplification was observed in the no-RT controls.

For TRFLP profiling, all forward primers were labeled with 6-carboxylfluorescein (6-FAM; Applied Biosystems, Foster City, CA). These fluorescently labeled amplicons were run on a 1% agarose gel for quantification via image analysis and 10 ng were digested with the endonucleases *MnlI* for the 16S amplicons and *HaeIII* for the 18S amplicons (New England Biolab, Beverly, MA) at 37ºC for 6 hours. The 20 µL digestion reactions were then precipitated using 2.3µL of 0.75 M sodium acetate, 5 µg of glycogen, and 37 µl of 95% ethanol. The reactions were then dried briefly and resuspended in 19.7 µL of deionized formamide and 0.3µL of ROX 500 size standard (Applied Biosystems, Foster City, CA) for 15 minutes before analysis. TRFLP profiling was carried out using Genescan software and an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA). TRFLP peak detection was set at 50 arbitrary fluorescent units. All detected peaks, or OTUs (operational taxonomic units), were downloaded into spreadsheets and those peaks representing <1% of the total peak area were discarded. The remaining peaks were

parsed and normalized to a uniform total profile area to adjust for small loading differences between samples.

In order to identify the major bacterial species, a clone library was established utilizing a TA cloning kit (Invitrogen, Carlsbad, CA) and 80 clones were screened using TRFLP. Those clones containing a specific OTU were identified using an ABI 310 genetic analyzer and 27 Forward and 519 Reverse primers (Lane, 1991). The resulting sequences were compared with entries in the GenBank database by using Blast (Altschul et al., 1990) and with the Ribosomal Database Project (RDP) classifier (Wang et al., 2007).

2.3.4 Statistical Analyses

The presence or absence of an OTU was used to compare samples with a Sorenson similarity index included within the COMbinatorial Polythetic Agglomerative Hierarchical clustering package, Sim = [2(∑peaks in common between samples x & y)] / (∑peaks in x + ∑peaks in y) (http://alpha.es.umb.edu/faculty/edg/files/edgwedp.htm#COMPAH). Similarity matrices were compared to matrices of distance from the mouth of the Orinoco, salinity, depth, fluorescence, and irradiance using a Mantel test (Mantel 1967) and the XLStat package by Addinsoft. The results are based upon 1000 random distributions and a significance level (α) of 0.05.

Principal component analysis was performed to assess covariance between individual OTUs and salinity, depth, nutrients, fluorescence, irradiance, and eukaryotic OTUs. PCA was chosen due to the overwhelming number of observations and variables compared to the number of samples This data reduction technique was conducted

utilizing the environmental parameters and the adjusted peak areas from those OTUs that appeared in three or more samples. After determining those environmental parameters that were significantly correlated to the first five principalcomponents, representing 85+% of the total variance, OTUs were identified that exhibited a p value less than 0.05 and an r value > 0.8 in relation to the same principal components.

2.4 Results

2.4.1 Water Column Properties

Nearly all stations along the transect line displayed similar water column properties, with a lower salinity lens in the upper 5 m, a steep halocline (5m< x <50m), a shallow chlorophyll maximum, and a deep chlorophyll maximum (Figure 2.2). Biomass samples were collected in these features as indicated by the arrows in Figure 2.2. Nutrient profiles indicated nitrate + nitrite concentrations were generally very low in surface waters with subsurface maxima more apparent for the inshore stations (Figure 2.3). Higher silicate concentrations existed at the surface, with varying surface values showing a steady decline from 20 µM at station 13 to 0.8 µMat station 3 (Figure 2.3). Phosphate concentrations demonstrated a decrease at the surface moving away from the mouth of the River, beginning at 60 nM at station 13 and reaching levels that were below detection by station 3 (data not shown). Finally, the light field demonstrates a distinct difference in PAR attenuation for inshore vs. offshore stations (Figure 2.4; Table 2.1).

2.4.2 Profiling of the Bacterial Community

PCR and RT-PCR on genomic DNA and intact ribosomes from 12 of the 13 stations, using 16S rRNA gene primers, were performed and TRFLP (terminal restriction fragment lengths polymorphism) profiles were generated to assess the metabolically

active/growing component and the resident portion of the population (Figure 2.5). After excluding those peaks with areas less than 1% of the total profile, 91 and 81 different OTUs were observed in the active and resident profiles, respectively. Slightly less than a third these OTUs were detected in only a single sample. The singleton OTUs comprised less than 10% of the total peak area in any profile in which they were detected. Still fewer OTUs were found to be present in >80% of all fingerprints. These abundant OTUs included: 83, 101, 166 and 175 bp peaks from the intact ribosome profiles and 83, 101, 106, 139, 166, 205, and 247 in the 16S rRNA gene profiles. The largest peaks (comprising more than 20% of the total profile area) were strikingly similar in many of the intact ribosome and the 16S rRNA gene profiles (i.e. the 83, 101, and 150 bp peaks).

Using the Orinoco River Plume profile data, it was possible to create similarity indices between the active and resident profiles using the COMPAH96 software program. Analysis of 24 different intact ribosome and 16S rRNA gene sample pairs indicated 35% +/- 10% similarity between these groups, suggesting a third of the resident population appears to be active at any one site. This percentage of active species displayed little variation across the river plume and no correlation was observed with distance from the mouth of the Orinoco, salinity, depth, or nutrient level. Species richness, as measured by number of OTUs in a sample, also did not display a relationship to depth, distance from plume, nor salinity as all r^2 values were less than 0.4 for both populations (Figure 2.6 and Table 2.1).

Unlike the proportion of active individuals and the species richness, community similarity appeared to be linked to some of the measured parameters. Results of a test comparing the various datasets (Mantel 1967), indicated a small number of statistically

significant relationships between community composition and geographic distance, salinity, depth, fluorescence, and irradiance. The overall resident community was related to depth, salinity, and fluorescence (Table 2.2). Meanwhile, similar analysis of the overall active community shows a statistically significant relationship to depth, salinity, salinity at the surface, fluorescence, and PAR (Table 2.2).

In order to uncover any potential associations between specific OTUs and the environmental parameters, PCA was performed using the peak areas for each OTU in the resident and the active populations detected in three or more samples. The PC scores of these OTUs were examined for highly significant correlations ($r > 0.8$) with depth, salinity, silicate concentrations, phosphate concentrations, nitrate + nitrite concentrations, PAR, fluorescence, and the OTUs present in over half of all profiles of the active and resident eukaryotic community. In all, only 21% of the OTUs in the bacterial resident population were correlated with respect to the principal components (Figure 2.7A). Specifically, OTUs 247, 263, and 281 demonstrated positive responses to increases in phosphate and/or nitrite + nitrate concentrations, while OTUs 83, 101, 103, and 178 appeared to have negatively responded to an increase in silicate. When surface samples are considered (to represent the influence of the freshwater plume), additional OTUs (96, 103, 126, 150, 167, 176, 227, and 277) exhibited negative relationships with salinity while OTU 205 was positively correlated. Tests to determine if the OTUs in the 16S resident community were related to the most frequent OTUs in the eukaryotic population (i.e.18S intact ribosome and rRNA genes) revealed that OTUs 83 and 101 were negatively related to a member of the active eukaryotic community and that OTUs 140,

205, and 248 bp were significantly linked to members of the resident eukaryotic population.

In contrast to the resident population, approximately 13% of the active bacterial community correlated with the various environmental parameters (Figure 2.7B). When all stations are included, regardless of depth, OTU 101 was found to correlate positively with salinity. Meanwhile, four more peaks (OTUs 96, 103, 167, and 176) showed statistically significant increases in peak area as samples were collected away from the mouth of the Orinoco River. When the analysis was restricted to surface samples, OTUs 179 and 247 decreased in peak area and OTU 83 increased in peak area with increasing salinity. Likewise, PAR at the surface was found to be positively related to the activity of OTUs 96, 103, and 167. Activity was also shown to be related to surface nutrients for two peaks. OTU 247 demonstrated a positive relationship with increasing concentrations of phosphorus and silicate, while OTU 178 exhibited a negative relationship these nutrients. As for the relationship with eukaryotic species, active OTUs 101, 133, 150, and 284 were related to an active eukaryotic OTUs/species. All of these OTUs, except OTU 101 were negatively correlated to this eukaryote.

In order to identify individual OTUs, clonal libraries were established and screened. Examples of specific OTUs, the environmental driver with which they correlate, and their identification can be found in Table 2.3 and Figure 2.8. Unfortunately, a few select major OTUs dominated the clonal libraries and many of the correlating OTUs from both the resident and active communities were not found in the clonal libraries. This has also been seen in studies done by Phelps et al., 1998 and Vetriani et al., 2003.

2.4.3 The Eukaryotic Community

A similar analysis was run on a subset of samples in order to profile the resident and active eukaryotic community (Figure 2.9). A total of 63 OTUs in the 18S resident community and 82 OTUs in the active population were detected. An average of 14+/- 3 OTUs were found in each resident profile and approximately 17+/-5 OTUs in each of the active profiles. As with the bacterial profiles, approximately one-third of the eukaryotic OTUs found in both the resident and active eukaryotic factions appear only once. However, unlike the bacterial community, these singletons represent a much larger fraction of the total fingerprint area, up to 34% in the resident and up to 6% in the active communities. The major OTUs, those detected in more than half of the samples, were not the same between the resident and active factions. In the resident fraction only OTU 337 was found to recur, while in the active fraction, OTUs 258, 267, 270, 276, 331, and 337 were frequently detected. The OTUs with large peak areas (more than 20% to the total area) for any profile also differed between the resident (OTUs 267, 335, and 336) and the active (OTUs 267, 268, and 273) fingerprints.

Comparison of 15 different intact ribosome and 18S rRNA gene sample pairs indicate that 34% (+/- 7 %) of the eukaryotes present in any particular sample were active. Further analysis revealed no relationship between the percentage of active eukaryotic species within a population and any of the measured geographical/physical/chemical parameters. Unfortunately, a re-occurring contamination of the RT-PCR kit with eukaryotic DNA precluded a robust characterization of active eukaryotes and no individual correlation coefficients were calculated.

2.5 Discussion

Molecular ecologists seek to assess how environmental parameters influence bacterial species composition. Specific processes that have been linked to shifts in overall community or increases in individual species include: salinity gradients (i.e. Valdes & Albright, 1981; Prieur et al., 1987; Painchaud, 1995; del Giorgio & Bouvier, 2002; Kirchman et al., 2004; Crump et al., 2004), phytoplankton blooms (i.e. Kerkhof et al., 1999; Gonzales et al., 2000; Bidle et al., 2003; Pinhassi et al., 2004), seasonality (i.e. Nelson et al., 2008), and DOM composition (i.e. van Hannen et al., 1999). However, most of these studies do not target the active bacterial population in their measurements. Furthermore, it has been difficult to establish causal mechanism(s) structuring the populations. Our results demonstrate that while the overall fraction of active bacteria throughout the Orinoco River Plume transect remains fairly constant, only a small percentage of individual members within the community (<25% of the overall population) are changing in growth parameters (as measured by ribosome content) or abundance (as measured by the 16S rRNA gene) with respect to the measured environmental gradients (i.e. salinity, silicate concentration, etc.).

There are a number of explanations for why such a small percentage of OTUs are related to the measured parameters. For instance, it is possible that we are not measuring the correct parameters, especially in respect to nutrients. While the bacterial population has been shown to take up inorganic nutrients (i.e. Horrigan et al., 1988; Caron et al., 1994), this study suggests that that non-traditionally measured oceanographic parameters, such as dissolved organic matter composition, are a stronger factor in shaping the community. It is also possible that the physical and chemical range over which the measurements were made was insufficient to reveal how these parameters relate to

community structure. While the sampling stations were fairly close together, the samples were not collected from a single tagged water mass to follow changes in bacteria over time and space.

Our findings are similar to a prior study of the Amazon and Orinoco River plumes that was conducted 650 km east of our transect in 2003 (Hewson et al., 2006). Both studies found a few phylotypes/species that related in abundance to plume conditions. In addition, neither work found any significant relationship between species richness and salinity within the resident community. Lastly, both works concur that parameters influencing changes in community composition exist on scales less than 50 km (Hewson et al., 2006), even within the plume. However, the analysis presented here indicates that the scales are probably much smaller than 50 km, as surface samples collected 8 km apart were only 31.2% and 40.0% similar for the resident and active communities, respectively.

Despite these similarities, a few contradictions exist between the two investigations. For example, we did not find the highest species richness at the surface. Moreover, in the prior study, similarities between samples were found to exhibit a non-linear decrease with distance (Hewson et al., 2006) while, in this study, no significant relationship was found between community similarities and distance for either the resident or active community. While both works noted that no statistically significant association exists between the community composition (similarity) of the resident population and salinity at the surface (Hewson et al., 2006), we find that the resident and active bacterial communities were significantly related to salinity when all depths (0 – 100m) were included and that the active population at the surface alone did show a correlation. A number of possible explanations exist for the discrepancies between the

studies, including different sampling locations (this study focused on the Orinoco Plume only and was farther west) and possibly different genetic foci (this study examined the 16S rRNA gene, while Hewson et al. looked into the intergenic transcribed spacer region).

From this work in the Orinoco River Plume, a number of environmental parameters can be linked to individual phylotypes/species, but no single factor appears to regulate the entire community. Furthermore, the active and resident community investigations exhibit different patterns, further demonstrating the need to consider both intact ribosome and DNA templates when analyzing a community (i.e. Moesender et al., 2005). From our analysis three major microbial archetypes become apparent. The first group is composed of singeltons, or those species that appear only once throughout the entire sampling site. These individuals are a part of the "rare biosphere" (Sogin et al., 2006). With the exception of three OTUs (267, 335, and 338) found in the active population of station 7 at 48m, the singletons make up less than 5% of the overall peak area. A second group, representing the majority of OTUs/species in both the resident or active community, appear sporadically throughout the transect and are quite "patchy". Only 23% of the patchy resident community and 16% of the patchy active population demonstrate a significant correlation with the selected environmental drivers. This finding indicates the more likely mechanisms for the distribution of these patchy species are advection from an area of higher growth rate or abundance, association with macrobes or large particles, or selective removal via grazing. The final faction of the community consists of abundant OTUs, which are widely distributed (i.e. > 80% of the samples) or make up 20% or more of the total peak area in a sample. Most of these

abundant OTUs in both the resident and active populations correlate to at least one of the measured environmental parameters. These results support the concept that those bacteria, which are especially abundant, are also readily growing and dividing. Overall, the approach of discerning both the resident and the active microbial community will help to elucidate the environmental drivers of bacteria in the ocean and lead to a better understanding of the mechanisms most important to maintaining the overwhelming bacterial diversity within the marine system.

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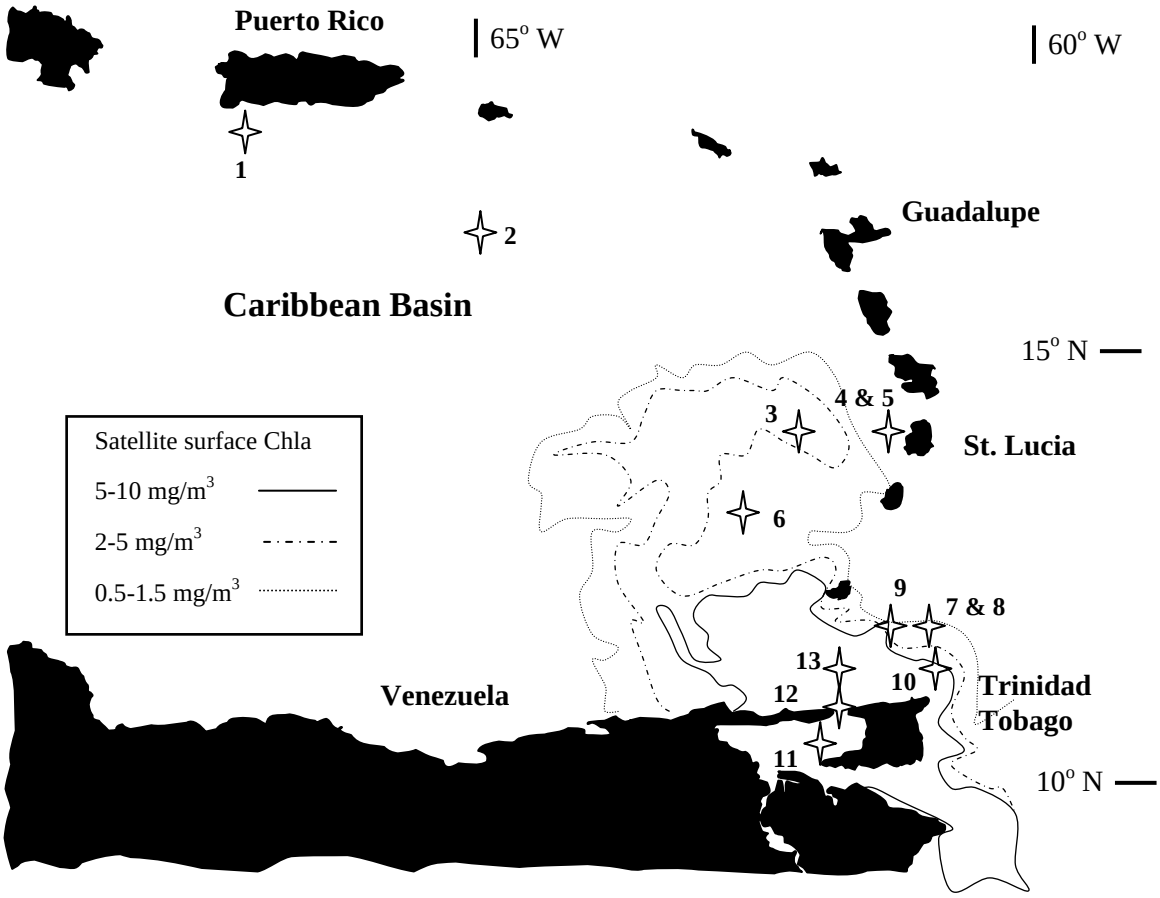


Figure 2.1 Map of the Caribbean with chlorophyll data (dotted lines) and sampling sites (stars) identified

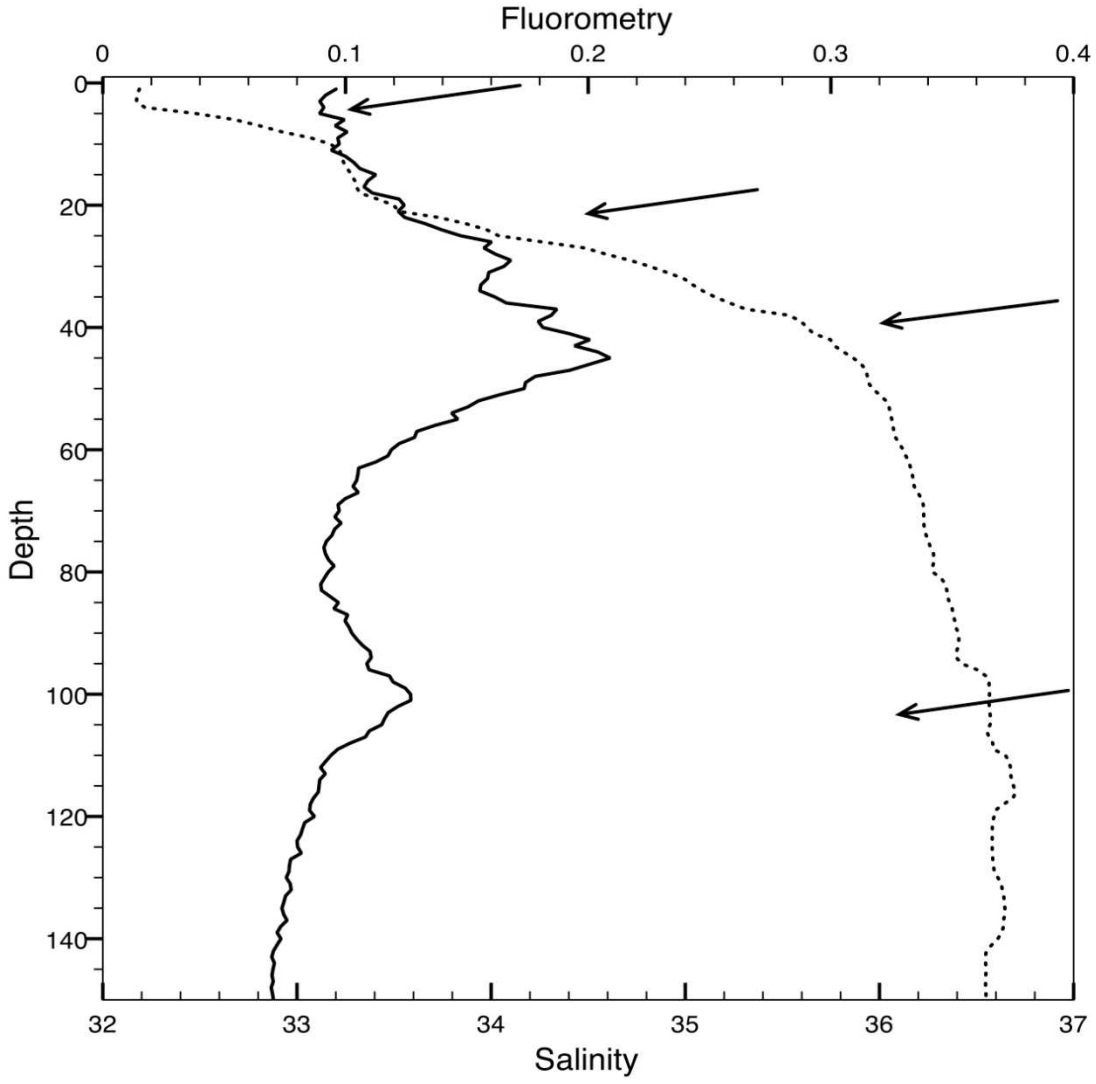


Figure 2.2 Depth profiles of fluorometry and salinity with locations of typical samples indicated by arrows.

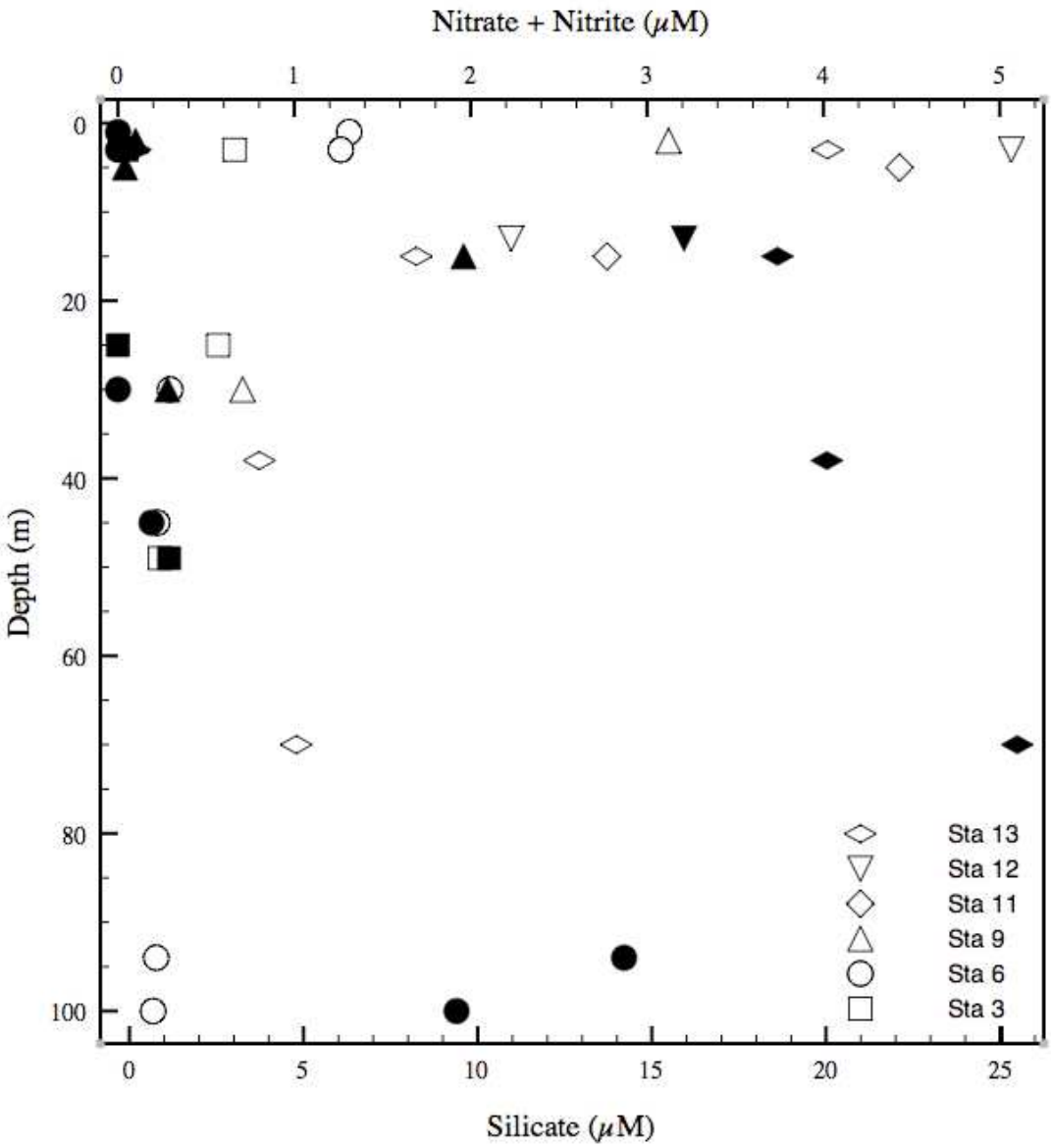


Figure 2.3 Silicate (open circles) and nitrate (closed circles) at six stations

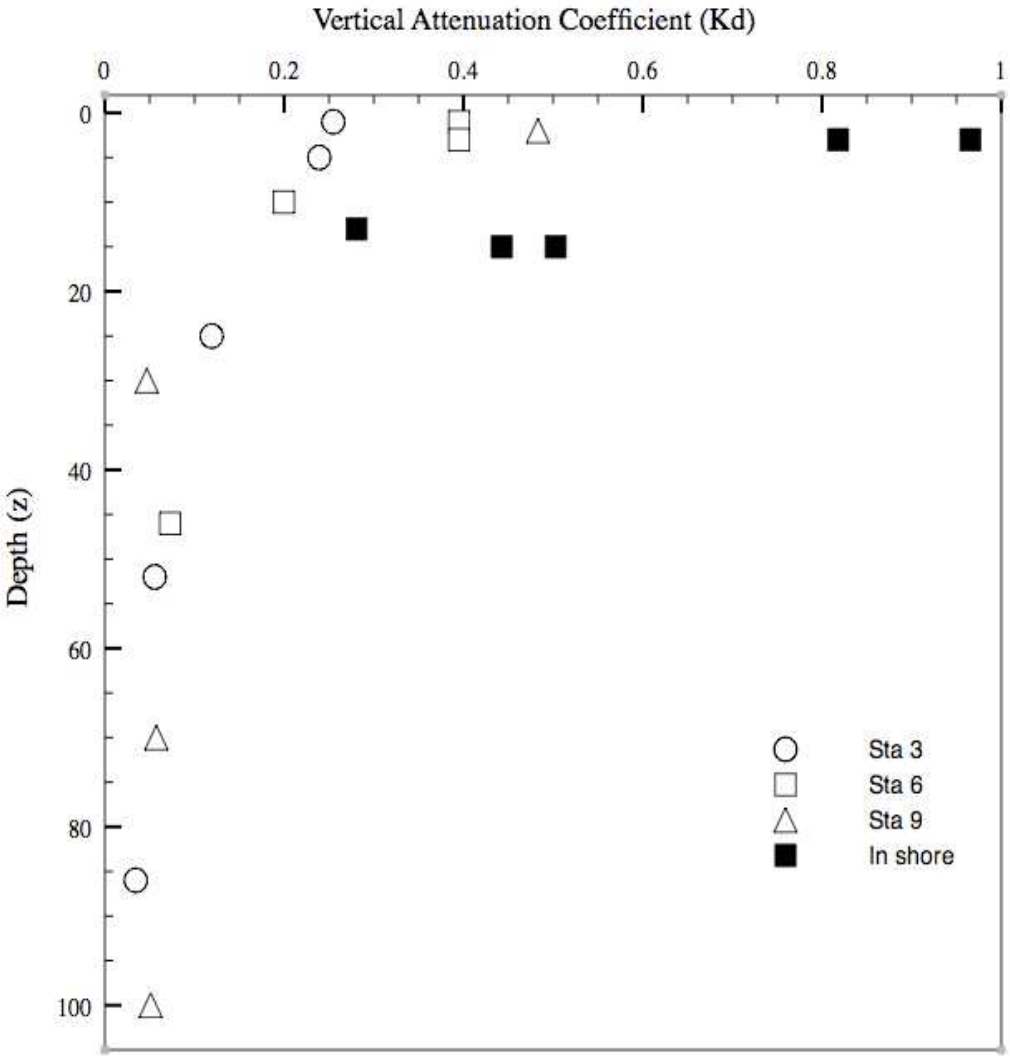


Figure 2.4 Vertical Attenuation Coefficient (Kd) versus depth at four locations (inshore and stations 3,6, and 9).

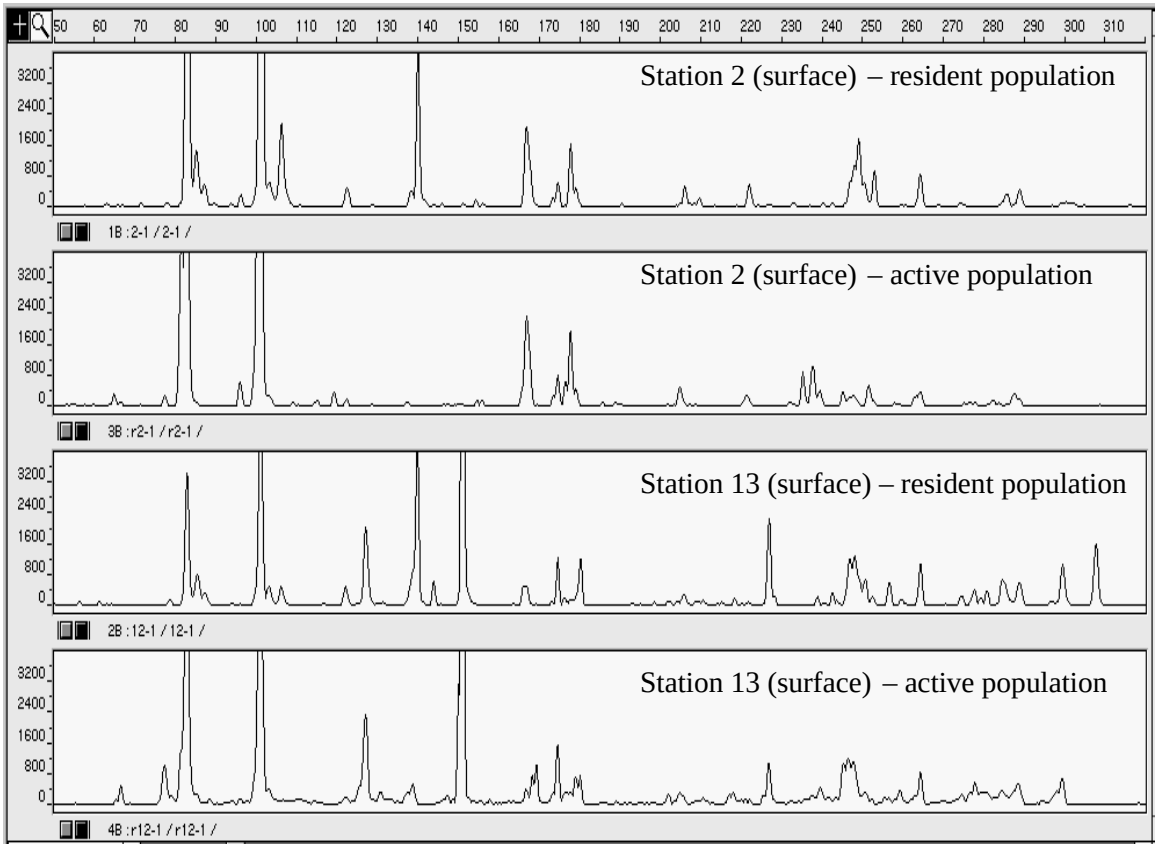


Figure 2.5 Normalized TRFLP profiles of the active and resident populations at the extremes of the transect line

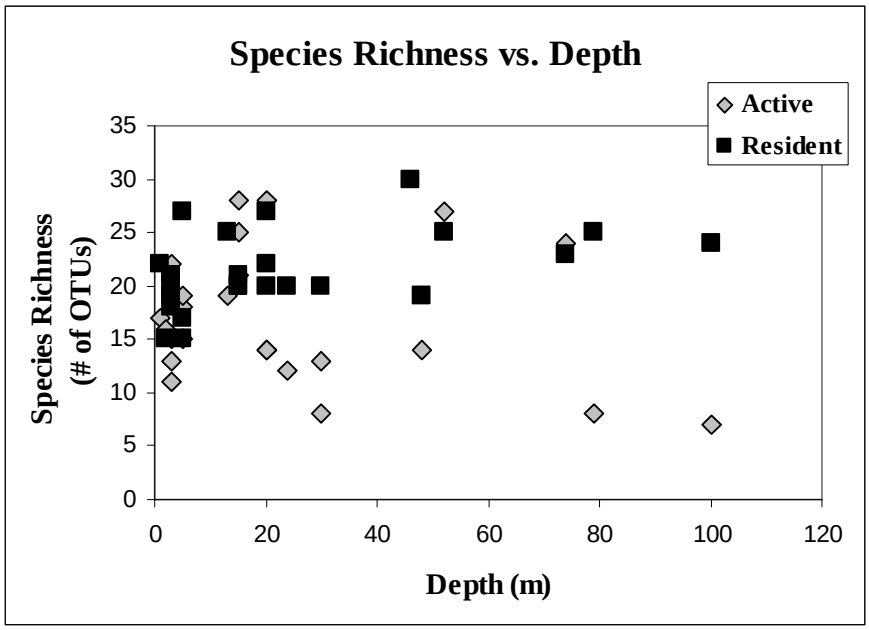


Figure 2.6 Species richness (# of OTUs) in samples from the active and resident populations versus depth.

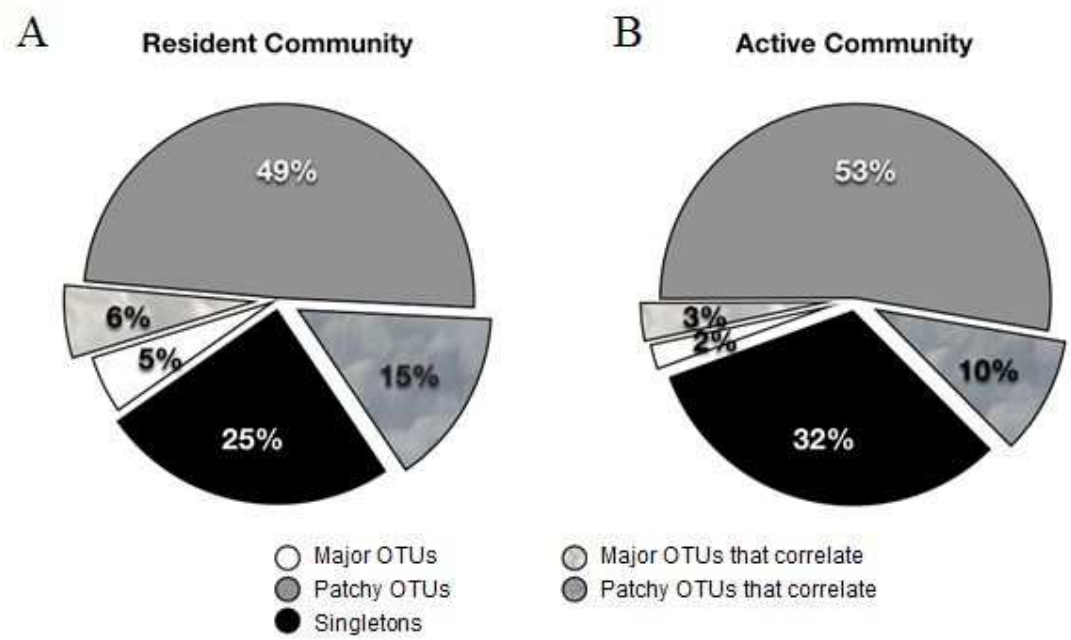
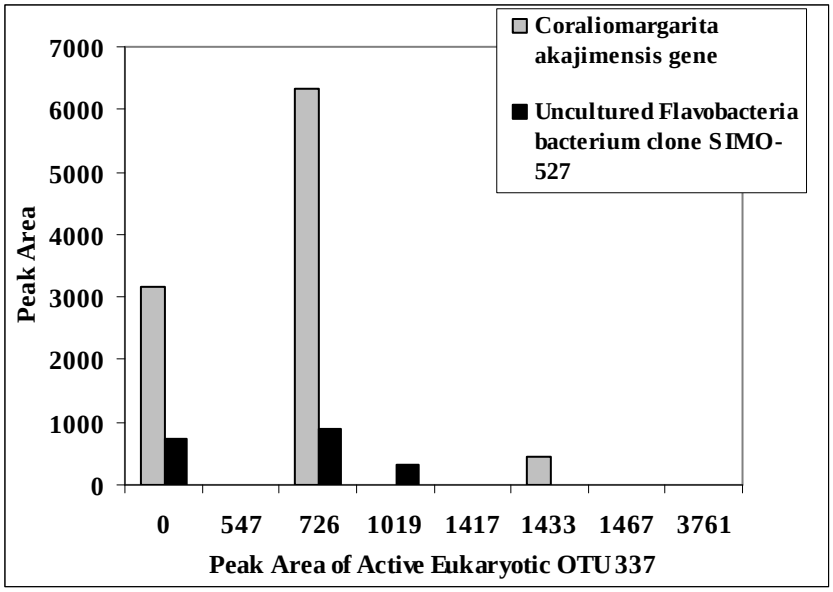


Figure 2.7 Distribution of OTUs in the (A) resident bacterial community (16S rRNA gene) and (B) active bacterial population (16S rRNA). Each slice represents the percentage of major OTUs (those present in 80%+ of the samples or comprising 20% of the total peak area in one sample), patchy OTUs (those that appear on and off throughout the transect), and single OTUs (those found only once) detected in each population. The textured slices represent the proportion of OTUs in each faction that demonstrate a correlation coefficient $r>0.8$.

A



B

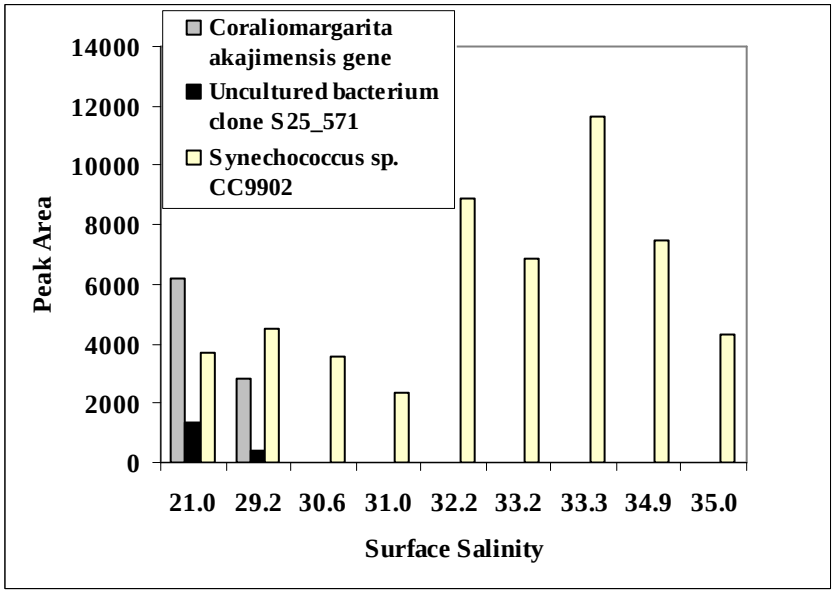


Figure 2.8 Examples of identified OTUs that correlate with the measured paramters. The adjusted peak area for each of these related bacterial OTUs is graphed versus (A) abundance of eukaryotic OTU 337 and (B) surface salinity.

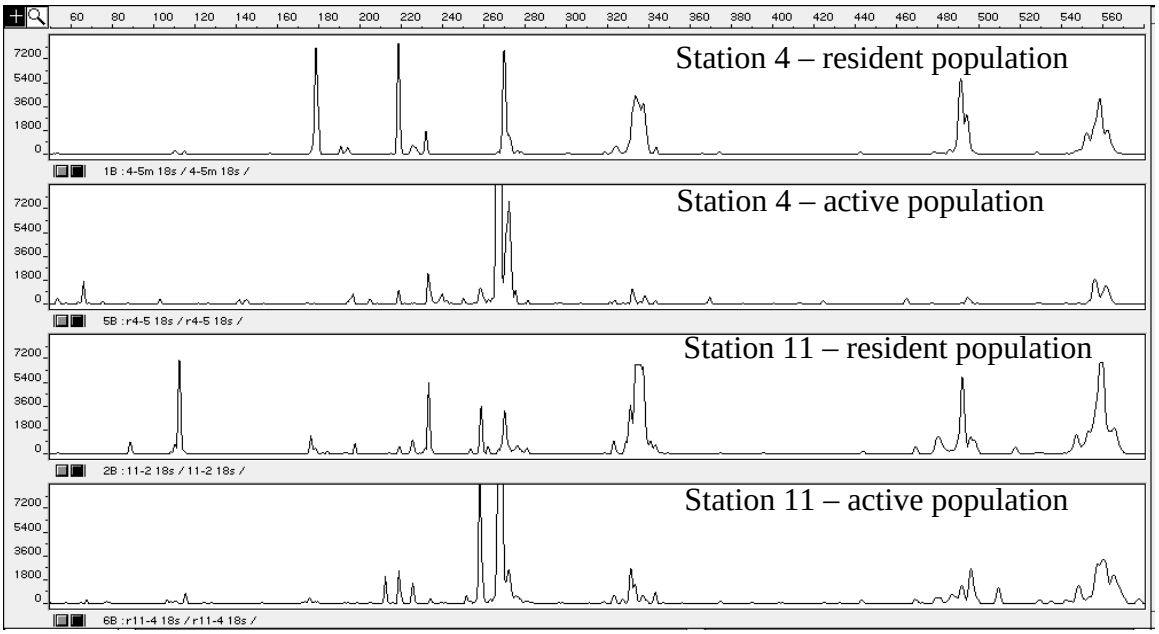


Figure 2.7 Normalized TRFLP profiles of the 18S resident (rRNA gene) and 18S active (intact ribosomes) community obtained from samples collected at two stations.

Station #		CTD Data				Species Richness (# of OTUs)	
	Distance (km)	Depth	Salinity	Fluorescence	PAR	Resident	Active
1	987	3	35.0	0.06	108.8	20	19
2	753	3	34.9	0.07	87.4	21	13
2		20	35.0	0.07	67.0	22	14
2		79	36.6	0.30	6.3	25	8
3	452	3	33.3	0.08	85.3	18	15
3		15	33.3	0.10	24.5	20	21
3		20	33.3	0.10	14.2	20	14
3		52	35.7	0.19	0.9	25	27
4	469	5	34.3	0.06	83.9	15	15
5	468	24	35.4	0.13	0	20	12
5		74	36.3	0.20	0	23	24
6	312	3	32.2	0.09	74.7	19	11
6		30	34.8	0.17	3.5	n/a	8
6		46	35.9	0.20	0.9	30	n/a
7	215	1	33.2	0.08	79.9	22	17
7		48	35.6	0.15	0.3	19	14
7		100	36.4	0.07	0	24	7
8	216	2	31.0	0.09	90.1	15	16
8		30	33.2	0.10	3.8	20	13
9	207	5	30.6	0.14	79.9	17	18
9		20	33.2	0.13	3.1	27	28
10	129	5	29.2	0.28	n/a	27	19
10		15	34.6	0.27	0	20	28
11	44	13	30.2	0.11	2.6	25	19
12	64	3	18.6	0.55	60.2	samples lost	
13	81	3	21.0	0.23	67.7	21	22
13		15	30.0	0.16	2.6	21	25

Table 2.1 Oceanographic and species richness data at each sampling site

		Distance from the Orinoco River mouth	Depth	Salinity	Fluorescence	PAR
Resident	r(AB)	- 0.259	-0.334	-0.187	-0.197	-0.091
	p-value	0.104	< 0.0001*	< 0.0001*	< 0.0001*	0.130
Active	r(AB)	-0.277	-0.124	-0.182	-0.289	-0.136
	p-value	0.081	0.024*	< 0.0001*	< 0.0001*	0.022*

Table 2.2 Results from the Mantel Test comparing genetic distance of the bacterial population to the measured parameters. The significant p-values are marked with an asterisk (*). All values are based upon a significance level $\alpha = 0.05$.

OTU	Correlation	r value (p-value)	# of samples	Phylum	Description	Accession #	Max. Identity
83	Abundance negatively correlates with silicate	0.97 (<0.0001)	9	Cyanobacteria	Synechococcus sp. CC9902	CP000097.1	100%
	Activity positively correlates with salinity	0.92 (0.0005)	9				
101	Abundance negatively correlates with silicate	0.98 (<0.0001)	9	Cyanobacteria	Synechococcus sp. CC9902	CP000097.1	100%
	Activity positively correlates with salinity	0.95 (<0.0001)	25				
140	Abundance correlates negatively with the abundance of eukaryotic OTUs 271 & 491	0.82 (0.0135)	8	Actinobacteria	Uncultured bacterium clone ZA3111c	AF382115	99%
150	Abundance negatively correlates with salinity	0.92 (0.0005)	9	Verrucomicrobia	Coraliomargarita akajimensis gene	AB266750.1	95%
	Activity correlates negatively with the activity of a eukaryotic OTU 337	0.81 (0.0159)	8				
167	Activity correlates with distance from the mouth of the Orinoco	0.92 (0.0005)	9	Cyanobacteria	Synechococcus sp. CC9605	CP000110.1	100%
	Activity correlates with light	0.92 (0.0014)	24				
227	Abundance negatively correlates with salinity	0.87 (0.0021)	9	Planctomycetes	Uncultured bacterium clone S25_571	EF574227.1	100%
248	Abundance negatively correlates with the abundance of a eukaryotic OTUs 271 & 491	0.81 (0.0158)	8	Proteobacteria	Uncultured bacterium clone S23_1458	EF573359.1	99%
284	Activity negatively correlates with the activity of a eukaryotic OTU	0.89 (0.0033)	8	Bacteroidetes	Uncultured Flavobacteria bacterium clone SIMO-527	AY712064.1	94%

Table 2.3 Examples of identified OTUs that correlate with environmental parameters

Chapter 3. Detecting Active Microorganisms Associated with Physical/Chemical Parameters in a New Zealand Fjord

3.1 Abstract

A study was undertaken to track how changes within the active and resident bacterial populations are influenced by alterations in environmental drivers in Doubtful Sound, a New Zealand fjord. This fjord was selected since it is a highly dynamic environment with a stable low salinity layer overlying seawater resulting in sharp salinity and chemical gradients. Samples were collected in September 2006 and June 2007 along the fjord at 3 depths and screened using both prokaryotic and eukaryotic ribosomal RNA genes by Terminal Restriction Fragment Length Polymorphism. High molecular weight rRNA from ribosomes was analyzed in addition to the 16S rRNA genes to discern the active microbial community from the populations that were present, but not growing, in the sample. Results indicate that resident and active population structures are dissimilar, with only about 42% of the operational taxonomic units that are present at a location showing activity. While, salinity, inorganic nitrogen, and specific chromophoric dissolved organic matter compounds may play a major role in the distribution of certain species at this location, overall the measured environmental parameters explain less than 30% of the variability in the species detected.

3.2 Introduction

Understanding the mechanisms controlling diversity and distribution of bacterial species remains one of the overarching themes in aquatic microbiology. However, the diversity of any particular sample may not accurately reflect overall system function or the factors influencing the community. Therefore, it is important to delineate the actively

metabolizing bacteria within any chosen environment from the bacteria that are merely present and to determine the factors affecting this active population. In many environments, the physical/chemical/biological drivers which select or influence microbial diversity and activity are still unclear. General trends of the overall community response to natural forces have been observed and explanations on how particular environmental parameters impact biogeography have been developed (i.e. the linkage between bacterial and primary production [Cole et al., 1988] or phytoplankton dynamics [Kerkhof et al., 1999; Gonzalez et al., 2000]). However, it remains ambiguous how certain species react to environmental stimuli and whether the percentage of metabolically active species correlates to the ambient environmental conditions.

Previous studies have suggested that environmental (i.e. Troussellier et al., 2002; Lami et al, 2009) and/or temporal factors (i.e. Fuhrman et al., 2006; Nelson et al, 2008) may contribute to the variability within microbial assemblages. However, conflicting reports abound within the literature. For instance, del Giorgio & Bouvier (2002), Kirchman et al. (2004), and Crump et al. (2004) all determined that species composition varied along the salinity gradients, while Hewson et al. (2006) claimed that alterations in community structure were not overwhelmingly related to salinity. Likewise, several laboratory investigations (i.e. van Hannen et al 1999; Covert & Moran 2001; Kirchman et al 2004; Judd et al 2006) argue about the importance of dissolved organic matter (DOM) in structuring the bacterial community. Yet, fieldwork done by del Giorgio & Bouvier (2002) and Stepanauskas et al. (2003) found that dissolved organic carbon (DOC) may not be controlling fluctuations within the bacterial population. Finally, Nelson et al., 2008 found a strong seasonal signal structuring Mid-Atlantic Bight

microbial populations, but very little of the community variability between surface and bottom water samples was explained by traditional oceanographic physical/chemical measurements. On the other hand, Fuhrman et al, 2006, investigating surface waters in the San Pedro Channel found strong correlations between many of the physical/chemical measurements ($0.54<r^2>0.72$) for a nearly half of the operational taxonomic units (OTUs).

In an attempt to address these inconsistencies, some researchers have turned their focus towards the active faction of the bacterial population through analysis of the ribosomal RNA (Kerkhof and Ward, 1993; Kemp et al, 1993). Significant differences have been demonstrated between the 16S rRNA gene and the16S rRNA phylotypes found within a sample (i.e. Troussellier et al., 2002; Moeseneder et al., 2005; Gentile et al., 2006; Lami et al., 2009) . Thus, in monitoring both the resident (rRNA gene) and active (rRNA) factions, researchers hope to obtain a clearer understanding of the microbial community and its relationship with the environment.

This study discusses research focused on Doubtful Sound, one of the longest of the fourteen fjords on the southwestern coast of New Zealand's South Island (Figure 1). This site was selected because of the steep environmental gradients in physical or chemical parameters which may affect the resident and active microbial community. Due to extremely high precipitation within the area (> 6 m/yr), extreme freshwater input from the Manapouri hydro-electric power station, and over 150 streams and rivers (Gibbs et al., 2000;Lusseau & Wing 2006), Doubtful Sound has a stable low salinity layer that spans the length of the fjord. The water underneath this low salinity layer (LSL) is marine, originating from the Tasmanian Sea. With the incoming tide, there is a strong

mixing of water throughout the fjord between the first meters of freshwater going out and the seawater entering the fjord (Gibbs et al., 2000). As a result of these forces, a significant salinity and temperature gradient is established on both the horizontal and vertical scales (Figure 2). Likewise, Doubtful Sound has been shown to exhibit a strong chemical gradient. Nitrate and phosphate concentrations at the surface were found to increase with distance from the head of the fjord and at depth (Peake et al., 2001). Moreover, a decrease in the chromophoric dissolved organic matter (CDOM) absorption coefficient descending from 0 to 5m depth has also been observed in this region (Gonsior et al., 2008).

The goal of this study was to evaluate whether any emerging patterns in the resident and active individual bacterial species in Doubtful Sound could be correlated to specific environmental parameters. 16S small subunit ribosomal gene analysis, in conjunction with multivariate statistical approaches, was used to determine if salinity, DOM quality/content, and inorganic nutrients, were linked to changes within the active and resident microbial population. These results agree with previous publications on the importance of utilizing both high molecular weight rRNA from ribosomes and 16S rRNA genes when analyzing an environment (i.e. Troussellier et al., 2002; Moeseneder et al., 2005; Gentile et al., 2006; Lami et al., 2009) and indicate that particular OTUs in our analysis are correlated to changes in salinity, inorganic nitrogen, and CDOM components. Ultimately, the research described here will help to uncover if the environmental factors regulating microbial populations can help to predict the drivers of bacterial diversity.

3.3 Methods

3.3.1 Study Site

Samples were taken in the spring of 2006 at the head of the fjord in Deep Cove (DC), midway through the fjord (DS05), within an arm of the fjord called Crooked Arm (CA02), and towards the mouth of the fjord (DS07) (Figure 1). The following winter in 2007, the same CTD casts, except for DS07 were performed and additional sampling was done on the water entering Doubtful Sound through a pipe into Deep Cove (tailrace) and from a river running into Crooked Arm (CA) (Figure 1). Water was collected at 0m (from the freshwater lens), 3m (the transition zone between the low salinity layer and marine water), and 10m (the marine layer) from each sampling site, except at the tailrace and Crooked Arm river. Four liters of water was filtered through 0.2 µm SUPOR filters to obtain microbial biomass from each depth. Duplicates from all stations and depths were also collected. These filters were stored in the field and transported in a dry shipper at liquid nitrogen temperatures. In the laboratory, they were placed at -80°C prior to extraction.

3.3.2 Chemical Analysis

Nutrients were filtered, preserved with $HgCl_2$ and measured with an Autoanalyzer (Bran and Luebbe) according to seawater standard methods (Kattner and Becker, 1991). For DOC analyses (compare Koch et al., 2005) 15 mL of filtered (GF/F) water was acidified to pH 2 (HCl, suprapur, Merck) and sealed in pre-combusted glass ampoules. DOC was analyzed by high-temperature catalytic oxidation using a Shimadzu TOC/TN analyzer equipped with an infrared and a chemiluminescence detector (gas flow oxygen: 0.6 L min_1). In the autosampler, 6 ml of sample volume in pre-combusted vials were acidified with 0.12 ml HCl (2 M) and sparged with oxygen to remove inorganic carbon. 50 µL sample volume was directly injected on the catalyst (heated to 680°C). Final DOC

concentrations were average values of triplicate measurements. Detection limit (5σ of the blank) was 7 µM C with an accuracy of ±2 M C determined with low carbon water and seawater reference material (DOC-CRM, Hansell Research Lab, University of Miami).

To determine the cDOM components, water was filtered on-site through pre-combusted 0.22 µm GV filters (Durapore, Millipore). The filtrate was then placed in pre-combusted glass bottles with Teflon - lined caps and stored at 4ºC before processing. In the laboratory, EEM measurements and PARAFAC modeling was performed as outlined in Yamashita and Tanoue (2003, 2004).

3.3.3. SSU Gene Fragment Amplification and TRFLP

Total nucleic acids were extracted from biomass samples using a modified phenol chloroform DNA extraction (Kerkhof et al., 1993) for microbial community characterization. Genomic DNA and RNA were used to amplify both the 16S and 18S rRNA genes via PCR (polymerase chain reaction) and RT-PCR (reverse transcriptase PCR), respectively. For the 16S rRNA genes from DNA, 50 µl PCR reactions were set up with 10ng template and 20 pmol of the universal primer 27 Forward (5' AGA GTT TGA TCC TGG CTC AG 3') and the bacterial specific primer 1100 Reverse (5' GGG TTG CGC TCG TTG 3') per reaction. The amplification parameters were as follows: 94ºC for 5 min followed by 25 cycles of 94ºC for 30sec, 57ºC for 30sec, and 72ºC for 1 min and a final extension period of 72ºC for 10 min. The 18S rRNA genes from DNA were similar with 20 pmol of the 18S forward primer cited in Moon-van der Staay et al. (2000) (5' ACC TGG TTG ATC CTG CCA G 3') and 20 pmol of Euk516r (5' ACC AGA CTT GCC CTC C 3') (Amann et al., 1990) under the following conditions: 94ºC

for 5 min followed by 35 cycles of 94ºC for 45sec, 56ºC for 45sec, and 72ºC for 2 min, with a final extension period of 72ºC for 10 min.

To obtain purified RNA for the RT-PCR reactions, total nucleic acid samples were diluted 1/100 and treated with the Turbo DNA-free kit (Applied Biosystems, Foster City, CA) to remove any contaminating DNA. The RNA was further diluted to 10^{-5} times the initial level to provide comparable target molecule concentration as the DNA amplifications. Two microliters of the diluted extract were used with the Titan One Tube RT-PCR kit (Roche, Basal, Switzerland) for amplification of SSU fragments from the most abundant, bacterial ribosomes. The amplification parameters were the same with the exception of the 30 min incubation at 50ºC for reverse transcription. SSU fragment amplification from eukaryotic ribosomes was performed with the high capacity cDNA RT kit (Applied Biosystems, Foster City, CA) as per manufacturer's protocol, followed by the PCR protocol above. Concurrent PCR controls were run without RT to identify any DNA contamination. No amplification was observed in the no-RT controls.

For Terminal Restriction Fragment Length Polymorphism (TRFLP) profiling, all forward primers were labeled with 6-carboxylfluorescein (6-FAM; Applied Biosystems, Foster City, CA). These fluorescently labeled amplicons were run on a 1% agarose gel for quantification via image analysis and 10 ng were digested with the endonucleases *MnlI* for the 16S amplicons and *HaeIII* for the 18S amplicons (New England Biolab, Beverly, MA) at 37ºC for 6 hrs. The 20 µL digestion reactions were then precipitated using 2.3µL of 0.75 M sodium acetate, 5 µg of glycogen, and 37 µl of 95% ethanol. The reactions were then dried briefly and resuspended in 19.7 µL of deionized formamide and 0.3µL of ROX 500 size standard (Applied Biosystems, Foster City, CA) for 15 min.

before analysis. TRFLP profiling was carried out using Genescan software and an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA). TRFLP peak detection was set at 50 arbitrary fluorescent units. All detected peaks, or OTUs, were downloaded into spreadsheets and those peaks representing <0.5% of the total peak area were discarded. The remaining peaks were parsed and normalized to a uniform total profile area to adjust for small loading differences between samples.

In order to identify the major bacterial species, a clone library was established utilizing a TA cloning kit (Invitrogen, Carlsbad, CA) and 120 clones, containing a 1.37kb insert, were screened using TRFLP. Those clones containing a specific OTU were identified using an ABI 310 genetic analyzer along with M13 Forward and Reverse (Invitrogen, Carlsbad, CA) and 519 Reverse primers (Lane, 1991). The resulting sequences were compared with entries in the GenBank database by using Blast (Altschul et al., 1990) and with the Ribosomal Database Project (RDP) classifier (Wang et al., 2007).

3.3.4 Statistical Analyses

The presence or absence of an OTU was used to compare samples with a Sorenson similarity index included within the COMbinatorial Polythetic Agglomerative Hierarchical clustering package, Sim = [2(∑peaks in common between samples x & y)] / (∑peaks in x + ∑peaks in y) (Berardesco et al., 1998)). Similarity matrices were compared to matrices of distance from the tailrace, depth, salinity, fluorescence, temperature, DOC, total dissolved nitrogen (TDN), phosphate, nitrate, nitrite, and silica using a Mantel test (Mantel 1967) and XLStat (Addinsoft). The results are based upon 1000 random distributions and a significance level (α) of 0.05. Principal component

analysis was performed to assess covariance between individual OTUs and distance from the tailrace, depth, salinity, fluorescence, temperature, DOC, TDN, phosphate, nitrate, nitrite, silica, and eukaryotic OTUs. This data reduction technique was conducted utilizing the environmental parameters and the adjusted peak areas from those OTUs that appeared in three or more samples. After determining those environmental parameters that were significantly correlated to the first five principal components, representing ≥85% of the total variance, OTUs were identified that exhibited a p value < 0.05 and an r value > 0.8 in relation to the same principal components.

3.4 Results

3.4.1 Water Column Properties

Due to the influence of the tailrace, enhanced levels of precipitation, and wind stress (Gibbs et al. 2000), Doubtful Sound exhibits a horizontal surface salinity gradient resulting in salinities of approximately 0.6 at the head of the fjord to about 28 at the mouth. Similarly, there is a vertical structure established in the water column with a lower salinity lens at the surface and more saline water at depth (Figure 2). During both seasons, water temperatures ranged from approximately 9 to 12 ºC, with the lowest temperatures at the surface and slightly elevated temperatures below. Chemical analysis indicated nitrite concentrations were elevated at DC and decreased at depth, from 0.40 µM/L at the surface to 0.16 µM/L at 10m, while both DS05 and CA02 displayed the opposite pattern and increased with depth (Table 1). Nitrate values were fairly consistent moving away from the power plant, but increased with depth (3 and 10m). Meanwhile, TDN at the surface ranged from 3.57 and 4.12 µM/L at DC and CA02 to 5.66 µM/L at DS05. These values more than doubled at depth for stations DC and CA02. Phosphate

concentrations at the surface were almost five times higher (1.80 µM/L) at station CA02 than along the main fiord. The vertical profile showed an increase at 10m at station DC and a decrease at the other two stations with depth (Table1). Both silicate and DOC values were quite high in all surface samples and were reduced at depth (Table 1).

Characterization of the CDOM using EEM and PARAFAC analysis isolated five components of the CDOM (Table 2). Components C1 and C4, are roughly classified as terrestrial fulvic acid-type and protein-like compounds, respectively (Coble et al., 1998; Cory and McKnight, 2005; Stedmon and Makager, 2005; Yamashita et al., 2008). These 2 components accounted for more than half of the CDOM at all three locations. The remaining portion is divided between the remaining compounds, which include other fulvic acid-type, terrestrial humic acid – like, protein - like and biologically and/or microbial derived compounds (Coble et al., 1998; Cory and McKnight, 2005; Stedmon and Makager, 2005; Yamashita et al., 2008).

3.4.2 Bacterial Community Structure

A total of 21 samples, 9 from the spring of 2006 and 12 from the following winter in 2007 were subjected to PCR on the genomic DNA, to characterize the resident population, and RT-PCR, on the intact ribosomes to characterize the active faction. After TRFLP analysis and removal of those OTUs with a peak area comprising less than 0.5% of the total sample profile, the 2006 samples contained 98 OTUs in the resident population and 93 OTUs in the active population. In the winter of 2007, there were 144 OTUs detected in the resident population and 128 in the active portion. When samples from the same sites are compared, the total number of OTUs increases by 11 in 2007 for the resident population and 12 in 2007 for the active population. To calculate the active

percentage of the resident microbes within a sample, the TRFLP profiles of the genomic DNA and intact ribosomes were compared. Those OTUs within the active profile which also appeared in the resident profile of that sample were considered the active percentage. Throughout the fjord, the average proportion of active players was 42% with a standard deviation of ± 9%. This value was highly similar between seasons.

All OTUs from both the resident and the active communities were found to belong to three distinct classes. The first group (singletons) is comprised of those OTUs that appear only once within the data set. The next group (abundant) is characterized as those OTUs which are found in more than 80% of the samples analyzed or they comprise more than 20% of the total peak area for one TRFLP fingerprint. Finally, there are OTUs that are detected intermittently throughout the fjord and are considered the patchy community. The resident population in 2006 and both the resident and active factions in 2007 all display the following pattern: approximately 33% singletons, between 56 - 63% patchy OTUs, and from 4 to 8% major OTUs. The only population that differs is the active group from 2006. Here there was a similar proportion of singleton and patchy OTUs, 49% and 43% respectively, and only 8% of the OTUs that can be classified as major OTUs (Figure 3).

3.4.2 Freshwater sources and sinks

To compare the communities of the incoming freshwater sources on the resident and active community within the fjord, winter samples collected at the tailrace, which flows into Deep Cove, and from a small river flowing into Crooked Arm were analyzed. Results of the Sorenson Similarity Index indicated that the resident population found at the tailrace in the winter was highly similar (> 70%) to that residing at the surface of DC

during both seasons. Meanwhile, the active, winter population in the tailrace demonstrated a strong degree of similarity (>70%) only to the active, winter species at the surface of DC in 2007.

In contrast to the relationship between the tailrace and DC, the river flowing into CA contained a community that was quite unique from that of CA02. The similarities between the resident and active populations from the CA River to those from CA at the surface were less than 30 and 35%, respectively. In fact, the samples collected from CA River were extremely different from any of the other samples analyzed, with the highest similarity at approximately 40%.

3.4.4 Location within the Fjord

Samples along the length of the fjord, including the low salinity layer and the water below, were compared to uncover any relationships on horizontal and vertical scales. When the Mantel Test was run on all samples, regardless of depth, the resident community in the spring of 2006 displayed a statistically significant relationship to salinity ($r(AB) = -0.555$, p-value = <0.0001) and temperature ($r(AB) = -0.536$, p-value = 0.000). Meanwhile, in the winter of 2007, the composition of the resident population was related to depth ($r(AB) = -0.264$, p-value = 0.038), salinity ($r(AB) = -0.554$, p-value = <0.0001), temperature ($r(AB) = -0.555$, p-value = 0.030), and nitrite concentrations ($r(AB) = -0.530$, p-value = 0.015) and the active population structure was related to salinity ($r(AB) = -0.365$, p-value = 0.008) and nitrite concentrations ($r(AB) = -0.669$, p-value = <0.0001). From the Mantel Test, it appears that no connections exist between the active community composition from 2006 and any of the measured variables.

Focusing on the horizontal scale only, a significant correlation (r=0.85, p value = 0.007) existed at the surface between the distance from the head of the fjord (i.e. the tailrace) and the percentage of active players within the resident community from both seasons. When samples from the spring were considered alone, there was even a greater relationship (r=0.99, p value = 0.011) between increasing distance and increasing percent activity. However, there appeared to be no relationship between species richness and distance from the tailrace, nor between species richness and salinity. Likewise, according to the results of the Mantel test, no significant relationship existed between the distance from the tailrace and the community composition of either the resident or active populations from both years.

On the vertical scale, there appeared to be no relationship between species richness or percent activity with depth. However, results of the Mantel Test indicated that the resident community composition was related to depth in the winter. An example of this can be seen at DC in 2007. Here, the 3m depth was similar to both the 0m and 10m, yet the 0m and 10m were quite dissimilar with a 38% similarity. It is possible that this was a true transition zone.

3.4.5 Seasonality

The Sorenson Similarity Index implied that there may be quite a bit of similarity between the resident community composition during both seasons, but a high degree of seasonality within the active community composition. With one exception, the active populations at all stations exhibited 70% or greater similarity only to samples collected at stations during their own season. This is not true for the resident community as samples from both seasons showed similarities greater than 70%.

In contrast to these findings, comparison of the OTUs from the spring and winter resident communities suggested that the majority of these OTUs (56%) are detected in only one of these seasons. Meanwhile, only 47% of OTUs from the two active populations appeared in only one season.

3.4.6 Individual Analysis of OTUs

In addition to examining the community as a whole, this study focused on changes in individual OTUs in conjunction with alterations in the physical, chemical, and biological environment by Principal Component Analysis (PCA). Physical/chemical measurements, eukaryotic fingerprints of 18S rRNA genes from genomic DNA and intact ribosomes, and bacterial profiles of 16S rRNA genes (DNA and RNA) were compared to each bacterial OTU appearing more than three times throughout the fjord. The PCA results indicated < 30% of the bacterial community from either season demonstrated a significant correlation ($r>0.8$) with principal components associated with distance, depth, salinity, fluorescence, temperature, DOC, TDN, silicate, phosphate, nitrate, nitrite, eukaryotic OTUs, and the major components of CDOM. In the spring of 2006, only 29% of the resident population and none of the active population showed some relationship with the measured parameters (Figures 3a & 3b). Likewise, in the winter of 2007, 22% of the resident and 19% of the active population correlated with the environmental factors (Figure 3c & 3d). Examples of these correlations are listed in Table 3, while all correlations are detailed in the supplementary material. Of those OTUs which demonstrated significant correlations, a vast majority (75%) from the spring related to salinity and temperature, while ≥80% from the winter correlated with inorganic nitrogen

compound and CDOM components, suggesting the factors driving the microbial community changes over the year.

More specifically, of the identified clones that correlated with an environmental parameter or were major OTUs, the majority belonged to proteobacteria, particularly the alpha proteobacteria subdivision (Table 3). Along with members from the alpha proteobacteria group, the gamma and delta subdivisions of proteobacteria were also highly represented and showed a relationship with a large variety of environmental measurements. In addition to the proteobacteria, single representatives from the phyla Bacteroides and Actinobacteria were identified. These species were both found to have a negative relationship with one particular eukaryotic species.

3.5 Discussion

Determining the various mechanisms that control the overwhelming bacterial diversity that exists within the marine environment is a difficult task. This is particularly true since any microbial community will be comprised of bacteria that are growing at the site and inactive bacteria that have become dormant or are advected/transported into the system. To distinguish these 2 fractions and uncover the influential environmental forces, analyses were conducted using both DNA and RNA as a starting material for TRFLP profiling.

Our analysis indicated that at any point in time or space essentially half (42%) of the total resident bacterial community is active, containing a sufficiently large amount of rRNA to be detected with our molecular methods. Interestingly, only a small percentage of the active bacteria in the winter 2007 samples were correlated with the physical/chemical/biological parameters measured in our field study (<30%). In contrast,

the active 2006 population did not correlate with any measured variables at either the group or individual level. These results suggest that the environmental conditions being monitored were not directly responsible for driving most of the active microbial population in 2006. Conversely, the majority of active OTUs from 2007 that demonstrated a statistically significant relationship with the measured physical/chemical/ biological variables were related to inorganic nitrogen and terrestrial fulvic-type and humic-like components of the CDOM. This finding implies that nitrogen and terrestrial input of humics are very important to a subset of the active microbial community in Doubtful Sound during the winter. These factors appear to have more influence than silica, phosphate, marine based humic-like compounds, or terrestrial fulvic acid-type, protein-like, or microbial derived compounds. Moreover, a number of species, including those relating to nitrogen and CDOM components, co-correlate with two or more variables. This may be a side effect of multiple variables changing in synchrony or potentially a synergistic effect of different compounds working together. It would be helpful to perform single manipulations studies of these various environmental factors to discern the mechanisms inducing active in these winter populations.

Along with nitrogen concentrations and CDOM quality, salinity also appears to be a relevant factor structuring the microbial community. The composition of the active and resident populations in the winter and the resident population in the spring all show statistically significant r values in relation to salinity when subjected to the Mantel Test. Furthermore, the fact that the DC community (salinity = 1.3) only resembles the tailrace community (salinity = 0.6) and that the river into Crooked Arm appears to have little influence on the species composition of the Crooked Arm basin (salinity = 13) indicates

that salinity may have a direct impact on the microbial community. Likewise, the relationship between the percentage of active species and distance from the tailrace, which can be considered a proxy for salinity, supports this finding and has also been seen in previous work (Troussellier et al. 2002). The overwhelming portion of singletons that exist at DC in three of the four populations is further evidence that the species at low salinities cannot thrive at higher salinities. Lastly, the information that the majority of OTUs which exhibit a significant correlation in the spring of 2006, do so with salinity supports this assumption.

More significantly, seasonality may play a role in determining community composition. Individuals may appear throughout the fjord regardless of season, however the active population at each station differs drastically depending upon the time of year. This indicates that species composition at a location may be reliant upon temporal forcing, a concept that has been seen in other locations as well (i.e. Fuhrman et al., 2006; Nelson 2008).

In conclusion, no single parameter appears to drive the microbial diversity of the Doubtful Sound system. Seasonality, salinity, inorganic nitrogen concentrations, and CDOM components are all relevant in shaping both the bacterial community as a whole and at the species level. Contrary to other studies, which indicate that environmental conditions may play a strong role in microbial structure (i.e. del Giorgio & Bouvier, 2002; Crump et al. 2004; Kirchman et al., 2004), our results are more consistent with studies done by Troussellier et al. (2002), Nelson et al. (2008), Ferraro et al. (submitted). These works suggest the percentage of bacterial OTUs that correlate with environmental parameters is quite small. However, all of these studies agree that while patterns can

emerge with regards to specific environmental factors, no single factor appears to dictate the composition or activity of aquatic bacteria in the environment. This argues for the importance of focusing on the bacterial population at the species, not the kingdom level.

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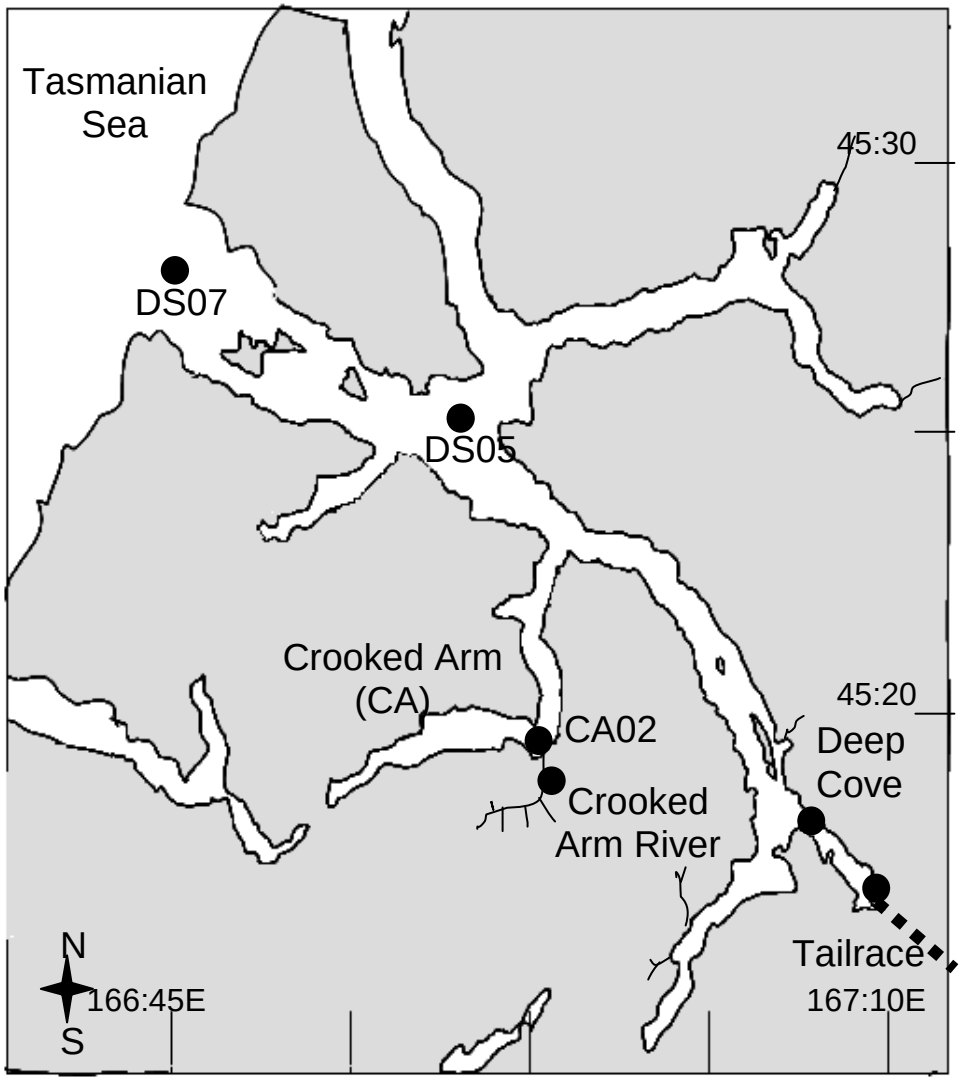

Figure 3.1 Map of Doubtful Sound. Sampling stations are marked by black circles. Rivers are indicated by thin lines.

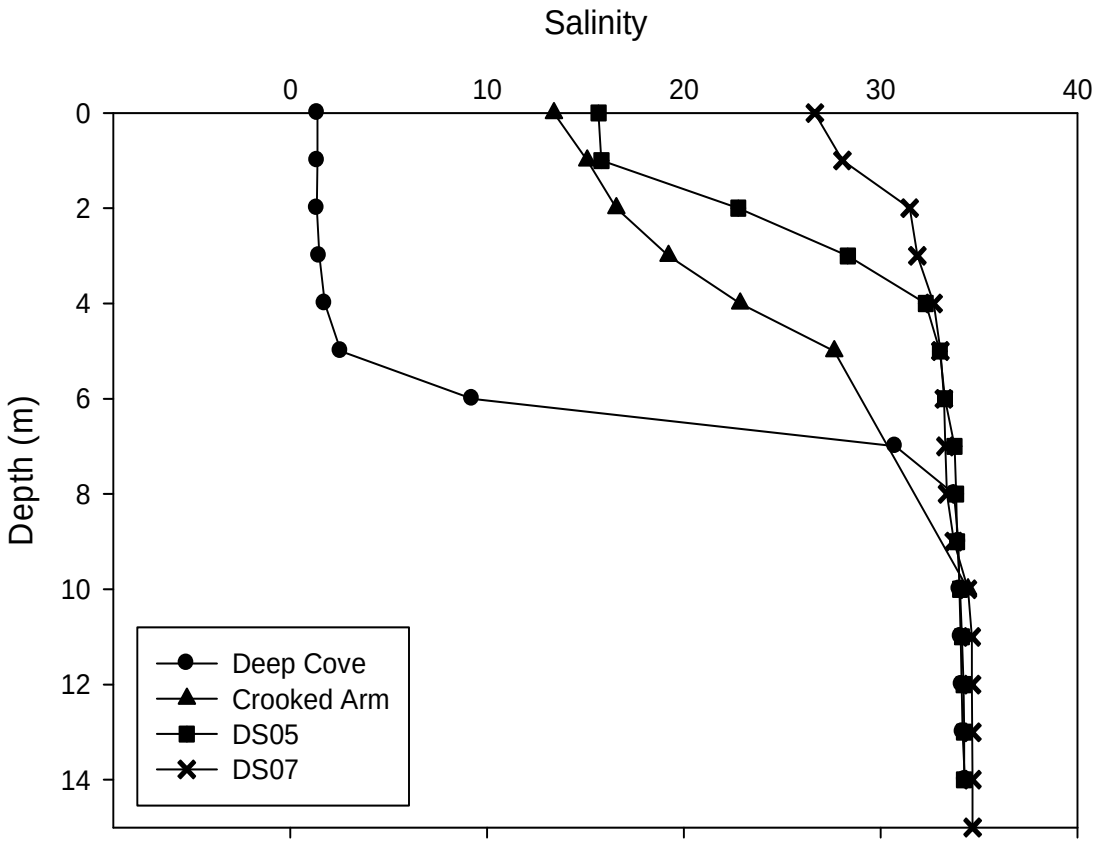

Figure 3.2 Doubtful Sound salinity profiles taken in the spring of 2006 for four stations spanning the length of the fjord.

Sampling Site	Depth (m)	NO2 (μM)	NO3 (μM)	Si (μM)	PO4 (μM)	DOC (μM)	TDN (μM)
Deep Cove (DC)	0	0.40	3.60	49.07	0.33	133.10	4.12
	10	0.16	10.70	6.17	1.16	60.57	10.20
Crooked Arm (CA02)	0	0.08	2.98	30.04	1.80	129.68	3.57
	3	0.15	4.86	15.88	0.51	76.62	9.58
DS05	0	0.16	2.88	41.78	0.27	156.58	5.66
	3	0.20	4.01	12.25	0.39	68.81	4.70

Table 3.1 Various chemical measurements from three stations collected in the fall of 2007.

Relative Abundance

	%C1 Terrestrial qnd autochthonous fulvic-acid type component	%C2 Terrestrial fulvic-acid type component	%C3 Terrestrial humic-like component	%C4 Protein-like component	%C5 Component of biological and/or microbial origin
Deep Cove 0m	33.2	17.9	15.2	18.8	14.9
Deep Cove 10m	30.8	0	8.6	44.2	16.4
Crooked Arm 0m	41.8	8.0	17.8	20	12.4
Crooked Arm 3m	43.6	0.1	13.4	29.0	13.9
DS05 0m	40.1	10.8	16.0	18.1	15.0
DS05 3m	37.4	1.5	11.8	34.9	14.4

Table 3.2 The relative abundance of CDOM components collected at 3 stations in 2007.

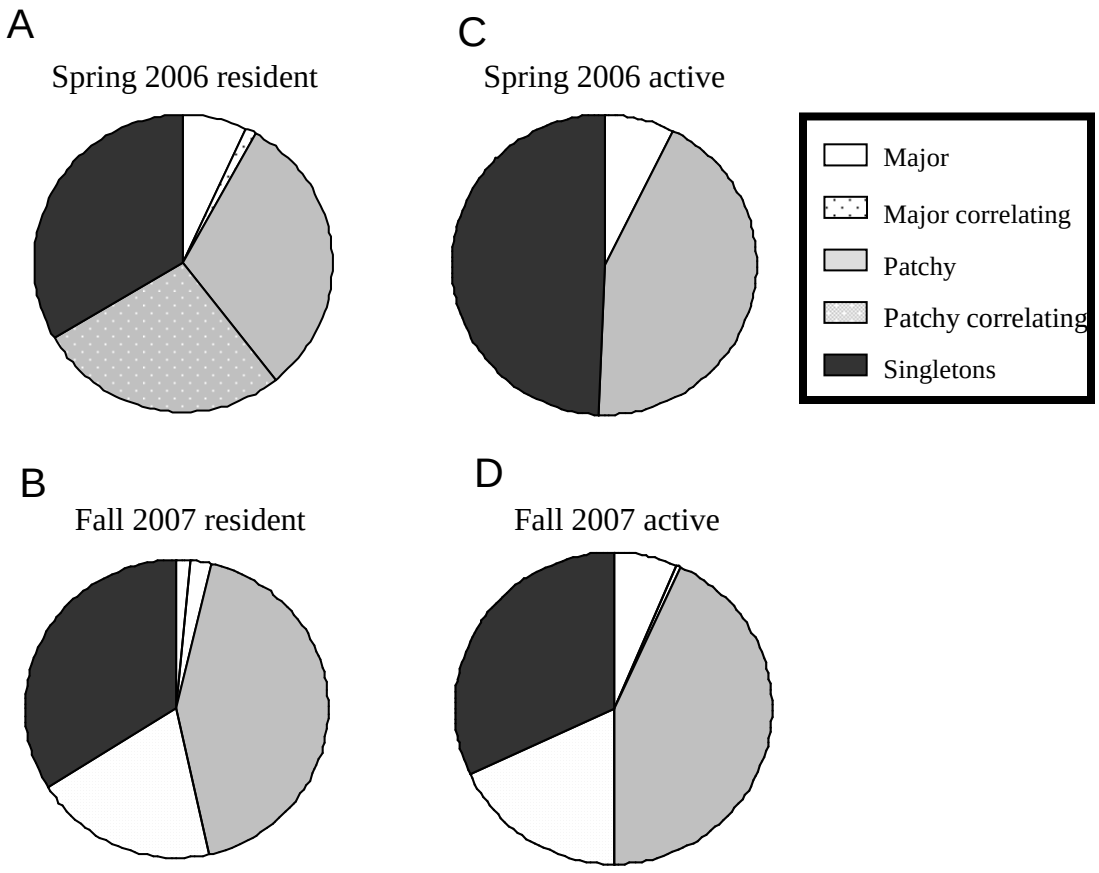

Figure 3.3 Distribution of OTUs in the (A&B) resident bacterial communities (16S rRNA gene) and (C&D) active bacterial populations (intact ribosomes). Each slice represents the percentage OTUs in the following categories: major OTUs,(those present in 80%+ of the samples or comprising 20% of the total peak area in one sample), patchy OTUs (those that appear on and off throughout the transect), and single OTUs (those found only once). The textured slices represent the proportion of OTUs in each faction that demonstrate a correlation coefficient $r>0.8$ to the measured parameters .

OTU	Correlation	Phylum	Description	Accession #	Max Identity
84	Correlates with depth and active eukaryotic species	Proteobacteria (alpha)	Alpha proteobacterium IMCC10407	FJ532494.1	99%
102	Major OTU throughout all populations and seasons	Proteobacteria (alpha)	Alpha proteobacterium IMCC10407	FJ532494.1	99%
107	Correlates with salinity in R07 and nitrate and resident eukaryotic species in A07	Proteobacteria (gamma)	Uncultured bacterium clone N67e_11	EF646131.1	99%
125	Correlates with salinity in R06 and R07. Also, with temperature in R06 and nitrite, component 2 of the cDOM, and resident eukaryotic species in R07.	Proteobacteria (epsilon)	Uncultured bacterium clone AV19F67b	FJ905659.1	98%
130	Correlates with nitrate and active eukaryotic species in A07	Proteobacteria (alpha)	Rhodobacter sp. CR07-44	EU979477.1	98%
131	Correlates with component 3 of the cDOM in R07 and is a major OTU in R06 and A07	Proteobacteria (alpha)	Loktanella vestfoldensis isolate IMCC6033	EU687493.1	100%
167	Correlates with salinity and temperature in R06 and is a major OTU in A06 and A07	Proteobacteria (alpha)	Uncultured alpha proteobacterium clone CL32-G10	GQ204867.1	100%
170	Correlates with resident eukaryotic species in R06 and is a major OTU in A06	Bacteroides	Bacteroidetes bacterium zo35	AF531006.1	100%
173	Correlates with component 3 of the cDOM in R07 and with nitrate and active eukaryotic species in A07. Also, a major OTU in R07	Proteobacteria (gamma)	Uncultured gamma proteobacterium	AM748240.1	99%
179	Correlates with nitrate and active eukaryotic species in A07	Proteobacteria (alpha)	Uncultured alpha proteobacterium clone SHWN_night2_16S_732	FJ745181.1	99%
182	Major OTU in A07	Proteobacteria (delta)	Uncultured marine Nitrospinaceae bacterium clone EB080L20_F04	EF106972.1	100%
185	Correlates with depth resident eukaryotic species in R06 and with nitrate in R07	Actinobacteria	Uncultured actinobacterium clone S1-18	EF491279.1	99%
203	Correlates with salinity and temperature in R06	Proteobacteria (alpha)	Uncultured SAR116 alpha proteobacterium clone EF100-93A06	AY627368.1	99%
208	Correlates with depth and resident eukaryotic species in R06, relates to component 3 of the cDOM in R07, and is a major OTU in R07 and A07.	Proteobacteria (beta)	Polynucleobacter necessarius subsp. asymbioticus QLW-P1DMWA-1	CP000655.1	99%
251	Correlates with salinity and temperature in R06, nitrite and active eukaryotic species in R07, and nitrate in A07. Also, is a major OTU in R06	Proteobacteria (alpha)	Roseobacter sp. 3008	AM110967.1	100%
253	Correlates with active eukaryotic species	Proteobacteria (alpha)	Uncultured Rhodobacter sp. clone YE-1	EF419222.1	99%
254	Correlates with depth and resident eukaryotic species	Proteobacteria (gamma)	Uncultured gamma proteobacterium clone SIMO-899	AY710433.1	98%
274	Correlates with salinity and temperature in R06. In R07, it correlates with salinity, nitrate, active eukaryotic species, and component 2 of the cDOM.	Proteobacteria (delta)	Uncultured delta proteobacterium clone CB22F07	EF471476.1	98%
285	Correlates with nitrate in A07	Proteobacteria (delta)	Uncultured marine Nitrospinaceae bacterium clone EB080L20_F04	EF106972.1	98%

Table 3.3 Specific OTUs, their IDs, and the factors with which they correlate. R06 = resident population from Spring 2006; A06 = active population from Spring 2006; R07 = resident population from Fall 2007; A07 = active population from Fall 2007

Chapter 4. Monitoring the Response of Active Bacteria to Individual Environmental Stimuli

4.1 Abstract

Prior analysis of active bacterial populations and environmental variables has shown correlations with specific OTUs and a measured ecological parameter (such as salinity). However, it is unclear whether the particular environmental parameter is actually the causal agent of microbial community change or if the parameter represents a proxy measure of some other factor that is influencing the community. In order to establish mechanistic evidence for a specific bacterial community forcing factor, a transplant experiment was undertaken to track how changes within the active and resident bacterial populations respond to alterations in salinity and water mass. The microbial community for the experiment was obtained from Doubtful Sound, a New Zealand fjord. The site was selected because it a stable low salinity layer overlying seawater resulting in sharp salinity and chemical gradients. Samples were collected in June 2007 from four sites throughout the fjord and incubated in water of varying salinities and nutrient concentrations. The response of the extant bacterial community was monitored using both prokaryotic and eukaryotic ribosomal RNA genes by TRFLP. Additionally, high molecular weight rRNA from ribosomes was analyzed to discern the active microbial community from the populations that were present, but not growing. Results indicate that while salinity and nutrient modifications had a limited effect on overall bacterial composition, a number of individual species did respond to the constructed fluctuations. Furthermore, the addition of larger organisms (>0.8 μM) to the treatments containing altered nutrient conditions played a minor role in structuring the bacterial community.

Instead, interspecies competition seems to regulate diversity and the abundance and activity of the microbial population.

4.2 Introduction

Bacteria are key players in aquatic food webs and play an essential role in carbon and nutrient cycling. Prior research has indicated that temporal and spatial patterns exist in microbial communities (i.e. Scala and Kerkhof, 2000; Rocap et al. 2003, Perez-Jimenez and Kerkhof 2005, DeLong et al. 2006; Fuhrman et al, 2006; Nelson et al, 2008). These studies in the field have shown that the bacterial community can be related to factors such as salinity (i.e. Crump et al., 1999; del Giorgio and Bouvier, 2002), organic matter (i.e. Gentile et al., 2006; Chapter 3), residence time (i.e. Crump et al., 2004), nutrient levels (Fuhrman et al., 2006), and seasonality (Fuhrman et al., 2006; Kan et al., 2006; Nelson et al, 2008). Unfortunately, inconsistencies between the conclusions drawn from fieldwork also abound within the scientific literature. For instance, Fuhrman et al. (2006) and Kan et al. (2006) claim that much of the bacterial community composition is predictable and related to nutrient conditions, while the results of Stepanauskas et al. (2003) disagree. Likewise, Kirchman et al. (2004) and Hewson et al. (2006) differ on whether salinity structures the resident bacterial population. It is possible that these discrepancies are the result of environmental variables changing in parallel, a synergistic effect of different aspects working together, or represent an artifact resulting from the analysis utilized.

Although the surveys indicate that there are a number of potential influences on community structure, no single feature can be deemed the definitive force regulating biodiversity. A likely explanation for this range of influences is that different species are

responding to different environmental forcing at various scales (Scala and Kerkhof, 2000). For all of these reasons, it is essential to verify that any patterns revealed during environmental monitoring are tested to establish a causal linkage between bacterial community change and the specific variable. Mesocosm or transfer experiments can be established to observe the response of the bacterial community to a single variable and to verify any theorized connections. Although the conditions in these mesocosms may not fully mimic the natural state of an environment, their purpose is to test the observations made in the field. For example, numerous studies in the laboratory have been done to directly link salinity (i.e. Valdes & Albright, 1981), nutrient concentration (i.e. Gasol et al., 2002), and predation (i.e. Jurgens et al., 1999; Simek et al., 1999) to alterations in microbial community composition. Yet, only a few of these studies (i.e. Valdes & Albright, 1981; Gasol et al., 2002; Fisher et al., 2000; Judd et al., 2006) monitor the effects of transplanting bacteria from one set of environment conditions to another set of conditions found to exist at the same location.

Moreover, this prior research focuses solely on the resident bacterial population and does not necessarily reflect the response of the active fraction of the community. One method of delineating the active microbial community utilizes characterization of ribosomal RNA rather than rRNA genes. The relationship between ribosomal RNA content and growth rate has been well established for bacteria (Neidhardt and Magasanik, 1959; Rosset et al., 1966; Schaechter et al., 1958). Later work extended the range of growth rates exhibiting a relationship between rRNA content and growth to environmental realistic doubling times (i.e. Kerkhof and Ward, 1993). Furthermore, evidence has been presented for a global relationship between RNA/DNA ratio and

growth rate under steady-state conditions at doubling times from 0.3-60 hours (Kerkhof and Ward, 1993). The importance of monitoring both the 16S rRNA gene (resident) and the16S rRNA (active) phylotypes within a community has been demonstrated within a sample (i.e. Troussellier et al., 2002; Moeseneder et al., 2005; Gentile et al., 2006; Lami et al., 2009; Ferraro et al. submitted) for a more complete understanding of the mechanics structuring the microbial community.

The purpose of this study was to test specific factors from an environmental study and to measure the leading indicator response (rRNA profiles) by members of the bacterial community. Two transplant experiments were performed to establish how salinity and nutrient concentrations (specifically silica, phosphate, total organic nitrogen, and dissolved organic carbon) regulate the community structure of the local resident and active populations in Doubtful Sound. Using water obtained from this experimental system and replicating similar patterns under laboratory conditions, this work tested the hypothesis that the observed environmental parameters (salinity and nutrient concentration) are associated with changing microbial activity and numbers at the species level. We found that while a few individual species responded to the constructed fluctuations, the overall bacterial composition was generally non-responsive. Interestingly, the factors that seemed to play a larger role in affecting the activity of the bacterial community (as measured by rRNA response) were not those that are routinely measured in environmental studies (e.g., salinity, phosphate concentration, total dissolved nitrogen, etc.), but instead involved interspecific competition among bacterial species.

4.3 Methods

4.3.1 Study Site

Doubtful Sound (Figure 1) is one of fourteen fjords on the southwestern coast of New Zealand's southern island. Due to extremely high precipitation within the area (> 7m/yr), extreme freshwater input from the Manapouri hydro-electric power station (the tailrace), and over 150 streams and rivers (Gibbs et al., 2000; Lusseau & Wing 2006), Doubtful Sound has a very stable low salinity layer that spans the length of the fjord. The water under this low salinity layer (LSL) is marine, originating from the Tasmanian Sea. With the incoming tide, there is a strong mixing of water between the first meters of freshwater going out and the seawater entering the fjord (Gibbs et al., 2000). As a result of these forces, a significant salinity and temperature gradient is established on both the horizontal and vertical scales. Likewise, Doubtful Sound has been shown to exhibit a strong chemical gradient (Peake et al., 2001).

4.3.2 Experimental Set-up

Four sites throughout the fjord (Figure 4.1) were selected based upon previously reported nutrient concentrations and salinity (Chapter 3; Gibbs et al., 2000; Peake et al., 2001; M. Gonsior, personal communication) and transplant experiments were established as follows:

4.3.3 Salinity experiment

Salinity and temperature measurements were taken at the surface of Deep Cove (DC) and from 3m at Thompson Sound (TS). Results of these measurements are listed in Table 1. 12 L of surface water from Deep Cove (DC) and 1.5L of water from Thompson Sound (TS) were then collected in thoroughly washed bottles and transported to shore prior to distribution and filtration. To remove the majority of the biomass, 11 L of DC water was filtered through 0.2 µm SUPOR filters. 0.5 L of this "biomass – free" water

was then distributed into thirteen different 1 L bottles already containing 0.5L of sterile Milli-Q water. An artificial sea salt mixture (0.7g, 5g, 15g, and 26 g; Sigma – Aldridge, St. Louis, MO) was placed in the 1L bottles such that there were three replicate bottles for each volume of salt added (Figure 4.2). Each bottle was well mixed, inoculated with 50mL of either unfiltered DC or TS water, mixed, and incubated in the dark at room temperature. After 24 hours, each bottle was filtered through an individual 0.2 µm SUPOR filter. All filters, including the filters used to sterilize the original DC water, were stored in the field and transported out in a dry shipper at liquid nitrogen temperatures. In the laboratory, they were placed at -80°C prior to extraction. Simultaneous controls were run by filtering the DC and TS water through 0.2 µm SUPOR filters. These bottles were inoculated with 50 mL of unfiltered water, such that the DC bottle contained bacteria obtained from DC and the TS bottle received bacteria from TS. The controls were incubated and filtered alongside the experimental bottles and the filters were stored and shipped as above.

4.3.4 Nutrient Experiment

Water for this experiment was obtained at 2 new stations (DS and CA). While most parameters (i.e. salinity, temperature, and chromophoric dissolved organic matter composition) differed only slightly between water masses, silica, phosphate, and total dissolved nitrogen concentrations showed distinct variations between the two sites (Table 1). 12 L of water from each site was collected and used for the nutrient experiment. Bottles with duplicates were set up as shown in Figure 4.3. 50mL of inoculum from either CA or DS were added, and the bottles were incubated in the dark for 12 hours at room temperature. Simultaneous controls were run as indicated in Figure 4.3. After

incubation, all bottles were filtered through 0.2 µm SUPOR filters, then stored in the field and transported out in a dry shipper at liquid nitrogen temperatures. In the laboratory, they were placed at -80°C prior to extraction.

In this experiment (Figure 4.3), treatment A was established to determine the effects of placing bacteria from both DS and CA together under the new nutrient conditions. The purpose of this was to help uncover any competition between species from different locations. Treatments B and C monitored the response of the bacterial communities from the two locations separately to new nutrient conditions. Treatments D and E were similar to B and C, however larger organisms (including potential grazers) were included to evaluate how predation would influence the microbial community. Lastly, treatments F and G were controls, which allowed for a comparison between how the bacteria responded under the new conditions versus how they naturally change under ambient conditions.

4.3.5 SSU Gene Fragment Amplification and TRFLP

Total nucleic acids were extracted from biomass samples using a modified phenol chloroform DNA extraction (Kerkhof et al., 1993) for microbial community characterization. Genomic DNA and RNA were used to amplify both the 16S and 18S rRNA genes via PCR (polymerase chain reaction) and RT-PCR (reverse transcriptase PCR), respectively. For the 16S rRNA genes from DNA, 50 µl PCR reactions were set up with 10ng template and 20 pmol of the universal primer 27 Forward (5' AGA GTT TGA TCC TGG CTC AG 3') and the bacterial specific primer 1100 Reverse (5' GGG TTG CGC TCG TTG 3') per reaction. The amplification parameters were as follows: 94ºC for 5 min followed by 25 cycles of 94ºC for 30sec, 57ºC for 30sec, and 72ºC for 1

min and finally and extension period of 72ºC for 10 min. The 18S rRNA genes from DNA were similar with 20 pmol of the 18S forward primer cited in Moon-van der Staay et al. 2000 (5' ACC TGG TTG ATC CTG CCA G 3') and 20 pmol of Euk516r (5' ACC AGA CTT GCC CTC C 3') (Amann et al., 1990) under the following conditions: 94ºC for 5 min followed by 35 cycles of 94ºC for 45sec, 56ºC for 45sec, and 72ºC for 2 min, with a final extension period of 72ºC for 10 min.

To obtain purified RNA for the 16S RT-PCR reactions, total nucleic acid samples were diluted 1/100 and treated with the Turbo DNA-free™ kit (Applied Biosystems, Foster City, CA) to remove any contaminating DNA. The RNA was diluted again to a final concentration of 10^{-5} to provide comparable target molecule concentration as the DNA amplifications. Two microliters of the diluted extract were used the high capacity cDNA RT kit (Applied Biosystems, Foster City, CA) for amplification of SSU fragments from the most abundant, bacterial ribosomes. Amplification was performed as per manufacturer's protocol, followed by the PCR protocol above. Concurrent PCR controls were run without RT to identify any DNA contamination. No amplification was observed in the no-RT controls.

For TRFLP profiling, all forward primers were labeled with 6-carboxylfluorescein (6-FAM; Applied Biosystems, Foster City, CA). These fluorescently labeled amplicons were run on a 1% agarose gel for quantification via image analysis and 10 ng were digested with the endonucleases *MnlI* for the 16S amplicons and *HaeIII* for the 18S amplicons (New England Biolab, Beverly, MA) at 37ºC for 6 hours. The 20 µL digestion reactions were then precipitated using 2.3µL of 0.75 M sodium acetate, 5 µg of glycogen, and 37 µl of 95% ethanol. The reactions were then dried briefly and resuspended in 19.7

µL of deionized formamide and 0.3µL of ROX 500 size standard (Applied Biosystems, Foster City, CA) for 15 minutes before analysis. TRFLP profiling was carried out using Genescan software and an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA). TRFLP peak detection was set at 50 arbitrary fluorescent units. All detected peaks, or OTUs (operational taxonomic units), were downloaded into spreadsheets and those peaks representing < 0.5% (for the salinity experiment) and < 1% (for the nutrient experiment) of the total peak area were discarded. The remaining peaks were parsed and normalized to a uniform total profile area to adjust for small loading differences between samples.

In order to identify the major bacterial species, a clone library was established utilizing a TA cloning kit (Invitrogen, Carlsbad, CA) and 120 clones were screened using TRFLP. Those clones containing a specific OTU were identified using an ABI 310 genetic analyzer along with M13 Forward and Reverse (Invitrogen, Carlsbad, CA) and 519 Reverse primers (Lane, 1991). The resulting sequences were compared with entries in the GenBank database by using Blast (Altschul et al., 1990) and with the Ribosomal Database Project (RDP) classifier (Wang et al., 2007).

4.3.6 Statistical Analyses

The presence or absence of an OTU was used to compare samples with a Sorenson similarity index included within the COMbinatorial Polythetic Agglomerative Hierarchical clustering package, Sim = [2(∑peaks in common between samples x & y)] / (∑peaks in x + ∑peaks in y) (Berardesco et al., 1998)). Principal component analysis was used as a data reduction technique and was conducted on the adjusted peak areas from those OTUs that appeared in two or more samples. The first seven principal

components accounted for 100% of the total variance for all experiments. For the salinity experiment, covariance between individual OTUs and salinity was also assessed. After determining which principal component correlated to salinity, OTUs were identified that exhibited a p value less than 0.05 and an r value > 0.8 in relation to the same principal component. For the nutrient experiment, PCA scores for the first five principal components were used in an Analysis of Variance (ANOVA) with Tukey's post-hoc test to determine if any differences existed among treatments A, B, C, and E (Figure 4.3). The OTUs detected within the treatments identified by the ANOVA as unique and found to be correlated with the principal components, were considered related to the effects of the treatments.

4.4 Results

4.4.1 Salinity Experiment - Water Properties

Due to the influence of the tailrace, enhanced levels of precipitation, and wind stress (Gibbs et al. 2000), Deep Cove (DC) exhibits a vertical salinity gradient with a lower salinity lens of approximately 2 psu at the surface and more saline water (about 34 psu at 10m) at depth. Thompson Sound (TS), which is located near the entrance of the fjord, is less susceptible to the freshwater effect of the tailrace and has a shallower low salinity layer except at times of high precipitation (Gibbs et al., 2000; Wing et al., 2003). At the time of collection, the surface water from Deep Cove had a salinity of 2.2 psu, while Thompson Sound at 3m had a salinity of 28.5 psu (Table 4,1).

The experimental systems contained pre-filtered, biomass free water from DC in a diluted state or with salt added. The final salinities of the systems inoculated with

unfiltered DC water were 1.4, 2.3, 8.3, 11.6, and 19.9 psu. In the bottles inoculated with unfiltered TS water salinities of 2.5, 5.8, 12.9, 19, and 28.5 psu were detected.

4.4.2 Salinity Experiment - Bacterial Community Structure

Fourteen samples including initial time points, controls, and experimental systems were subjected to PCR on the genomic DNA, to characterize the resident population, and RT-PCR, on the high molecular weight rRNA from ribosomes, to isolate the active fraction. After TRFLP analysis and removal of those OTUs with a peak area comprising less than 0.5% of the total sample profile, the DC samples contained 26 OTUs in the resident population and 64 OTUs in the active population. In the TS group, there were 24 OTUs detected in the resident population and 46 in the active portion.

Using the profile data, it was possible to create similarity indices between the active and resident profiles using the COMPAH96 software program. Analysis of the DC 16S rRNA and 16S rRNA gene sample pairs indicated 28% +/- 8% similarity between these groups. Likewise, the same analysis applied to the TS samples yielded 41% +/- 10% similarity, suggesting approximately one-third of the resident population appears to be active at any one site. This percentage of active species was not related to salt concentrations. Species richness, as measured by number of OTUs in a sample, also did not display a relationship to salinity.

Like the proportion of active individuals and the species richness, community similarity does not appear to be linked to salinity. Results of a test comparing the various datasets (Mantel 1967) at a significance level of 95%, indicated that neither the overall resident ($r(AB) = -0.178$, p-value = 0.147) nor active ($r(AB) = -0.113$, p-value = 0.369) community compositions were significantly related to changes in salinity. When only

samples receiving the same inoculum (DC or TS) were compared, the Mantel Test yielded similar results (results not shown).

In order to uncover any potential associations between specific OTUs and the environmental parameters, PCA was performed using the peak areas for each OTU in the resident and the active populations detected in two or more samples. This analysis was run in conjunction with salinity to establish a connection between the variation in salinity and the variation in the OTU. Once the OTUs that demonstrated a connection to salinity were found, 120 clones were screened in an attempt to identify these OTUs.

PCA results indicated that in the bottles inoculated with TS water, the total peak area (abundance) of eleven OTUs was correlated with salt concentration. *Alpha proteobacterium IMCC10407*, *Rhodobacter sp. CR07-44*, *Roseobacter sp. 3008*, and unidentified OTUs 87, 166, 247, and 463 all increased in abundance as salinity increased (Figure 4.4). Meanwhile, *Polynucleobacter necessarius subsp. asymbioticus QLW-P1DMWA-1* and unidentified OTUs 63 and 238 showed a negative relationship with salinity. In contrast, the bottles inoculated with DC water and the active faction of the TS inoculated samples contained no OTUs that demonstrated a significant change in abundance or activity with increasing salinity.

4.4.3 Nutrient Experiment - Water Properties

Concentrations of total dissolved nitrogen (TDN), silicate, phosphate, and dissolved organic carbon (DOC) for station DS at 1m and CA at 0m are listed in Table 1. While the DOC values and CDOM (chromophoric dissolved organic matter) component (Chapter 3) were similar at both sites, silicate and TDN concentrations were slightly higher at DS. However, phosphate levels were 10-fold higher at CA than at DS.

4.4.4 Nutrient Experiment - Bacterial Community Structure

This study consisted of 13 samples: 5 from the experimental systems, 4 from the controls, and 4 samples which were replicates of the experimental systems (Figure 4.3). All samples were analyzed as above. TRFLP analysis was carried out and those OTUs with a peak area comprising less than 1% of the total sample profile were removed. The percentage of active players (34% +/-9%) and species richness of the resident (17 +/- 3) and active populations (14+/- 2) did not appear to change despite the alteration in nutrient concentration, even when DS and CA populations were mixed together and when organisms greater than 0.8μM were added.

The Sorenson Similarity Index supports the results that there was little difference in the overall resident populations regardless of changes in the nutrient concentration or the addition of organisms greater than 0.8μM. The resident DS samples under new conditions were highly similar (>70%) to the controls and the CA resident population, while slightly less similar to its corresponding control (about 60%), was also not overwhelmingly different. However, results of the same analysis on the active faction show that while the DS samples unfiltered (presumably containing eukaryotic predators) and filtered through a 0.8 μm filter were very much alike, they were both very different from the control, demonstrating only ~30% similarity. This change in overall active community structure was not true for the CA samples, which contained an active population that was highly similar (>70%) to the CA controls.

The negligible influence of adding larger organisms (> 0.8μM) to the systems under the new nutrient conditions had is also evident through the analysis of the 18S rRNA gene profiles. In these profiles, only seven OTUs, two in the CA bottles and five

in the DS bottles, appeared in the systems which were set up with unfiltered water. However, these eukaryotic OTUs compose less than 4% of the total peak area in the profiles and as such appear to be minor players. Furthermore, PCA (Figure 4.5a and 4.5b) and ANOVA results for both the active and resident factions of the DS inoculated samples indicated that adding the larger sized organisms to these treatments is not a significant factor influencing the individual species changes. No ANOVA data was available to test this for the CA inoculated bottles due to a lack of replicates for CA treatments that included the unfiltered water.

While the overall population may not have changed significantly due to alterations in the nutrient conditions or the inclusion of larger organisms, individual OTUs appeared and disappeared in the treatments. Examples of these OTUs can be seen in Figure 4.6. Of the 37 resident OTUs in the bottles inoculated with DS water, 20 OTUs were not found in the controls but appeared in the treatments (7 under new nutrient conditions and 13 under the new conditions with larger organisms present). Similarly, 13 out of the 30 resident OTUs from CA were detected in the treatments only, 4 OTUs with and 9 OTUs without the larger organisms included. In the active population, 6 of the 17 OTUs from the DS bottles and 5 of the 18 OTUs in the CA bottles were not in the controls, but in the treatments. Of these OTUs, 14 of the DS and 2 of the CA appeared when placed in the new conditions with the 0.8μM filtered water. In all of these treatments, when only the nutrient concentrations were changed or larger organisms were included under these conditions, these OTUs grew or became more active and thus may represent species that thrive in this environment.

Unlike changing the nutrient conditions for one group or adding larger organisms, inoculating the nutrient altered water with the DS and CA populations together does appear to affect the population. The systems in which the populations were blended together were less than 70% similar to any of the other treatments or controls. Likewise, according to the ANOVA output, adding the populations together under new conditions is a significant factor accounting for changes in the population from both the DS and CA samples. More specifically, this analysis in combination with the PCA suggests that in the resident population, OTUs from DS demonstrated both and increase (OTUs 72, 77, 233,238, and 278) and decrease (OTUs 175, 250, 276, and 280) in abundance when combined with the CA population under new conditions (Figure 5a). Meanwhile, in both the DS and CA active populations this mixing may have caused a decrease in activity of Loktanella vestfoldensis and an increase in the activity of a species of alpha proteobacteria and OTUs 331. Furthermore, in the CA active population only, OTUs 123, 133, 135, and 250 showed a statistically significant increase in activity as a result of this combination (Figure 5b).

4.5 Discussion

Numerous field and laboratory studies have been performed to ascertain whether environmental parameters, as measured by traditional oceanographic methods, influence bacterial diversity. However, from the conflicting observations that abound within these studies, it becomes clear that alternative methods are needed to identify the true structuring mechanisms. One alternative technique that was attempted was to partition out the actively growing component of the community. Though this method may be effective, the simultaneous variation of factors within the field may obscure individual

relationships. To reveal the connections between individual species and single variables, this study monitored the resident and active bacterial populations and evaluated if salinity and/or nutrients influenced abundance and/or growth.

The results of this study concur with those of Painchaud (1995) and Hewson (2006) in that salinity appears to have little effect on controlling the overall resident population. Perhaps the susceptibility to mixing along/across the horizontal or vertical salinity gradient has adapted these species to become more halotolerant. This finding is contrary to studies performed within Doubtful Sound and in the Caribbean Basin (Chapters 2 and 3) that showed strong correlations between the microbial community and salinity. One potential explanation for this is that the analysis in previous work grouped a variety of populations from a number of locations and depths. Although a connection between the bacteria and salinity may exist at a number of sites, this is not true for all locations. As such this may be an overgeneralization, obscuring samples in which this relationship does not exist. Moreover, salinity can act as a proxy for other drivers, thus the true relationships between bacteria and the environmental factor may not be readily apparent.

Despite these observations, individual species exhibited a response to the removal or introduction of salt. Among the species whose abundance coincided with salinity were *Alpha proteobacterium IMCC10407*, *Roseobacter sp. 3008*, and *Polynucleobacter necessarius subsp. asymbioticus QLW-P1DMWA-1*. The positive relationship between the alpha proteobacteria subphylum and salinity has well documented in works focusing on Georgia (Gonzalez and Moran, 1997) and Maryland (Bouvier and del Giorgio, 2002) estuaries. *Roseobacter sp. 3008*, which also seems to thrive at higher salinities, has also

been shown to increase in abundance in Doubtful Sound as the salinity increased (Chapter 3). Meanwhile, *Polynucleobacter necessarius subsp. asymbioticus QLW-P1DMWA-1*, which demonstrated a negative relationship with salinity, has been identified by Hahn et al. (2009) as a freshwater species. The existence of these species whose presence appears linked to salinity further demonstrates the need to examine both entire communities and individual species.

As with salinity, the alteration in nutrient concentration appeared to have a limited affect on the overall resident community structure of the microbial population originating from sampling sites CA and DS. However, the new nutrient conditions did appear to influence the structure of the DS active community, as evidenced by the less than 30% similarity between the treatments and the controls. This divergence is due to the fact that of 17 total OTUs found active in the treatments and controls combined, 71% were detected in either the controls or treatments only. While these species did not appear to change in abundance, as evidenced by the 16S rRNA gene profiles, this may have been a result of the shorter incubation time. Gasol et al. (2002) found that incubations done under different nutrient regimes for 12 hours versus those for 24 hours yielded different populations, theorizing that perhaps the bacteria needed more than 12 hours to adapt to the new environment. Thus, it is possible that given more time, these species would have become more dominant.

Under the new nutrient conditions, neither the DS nor CA communities changed significantly when larger organisms, such as grazers or phytoplankton, were incorporated. One explanation for this is that the incubation time and light conditions were not optimal for the growth of photosynthetic organisms. Therefore, the

phytoplankton were most likely not very active and would not be a major influence on the microbial population. Moreover, this study differs from others that indicate that predators have a profound impact on microbial populations (Jurgens et al., 1999; Simek et al., 1999) because these mesocosms contained a limited number of eukaryotes, as shown in the 18S rRNA gene fingerprints. Of the eukaryotic OTUs detected, the majority was present in all treatments and controls and would potentially have a similar impact on the bacterial population in all mesocosms.

The one factor that may appear to play a role in controlling the activity and abundance of both the DS and CA populations under the new nutrient regime was the addition of outside competitors. When the two populations were combined, changes occurred that fostered the growth of some species, while hindering others. While further testing is necessary, these results suggest that interspecies competition plays an important role in structuring the bacterial community.

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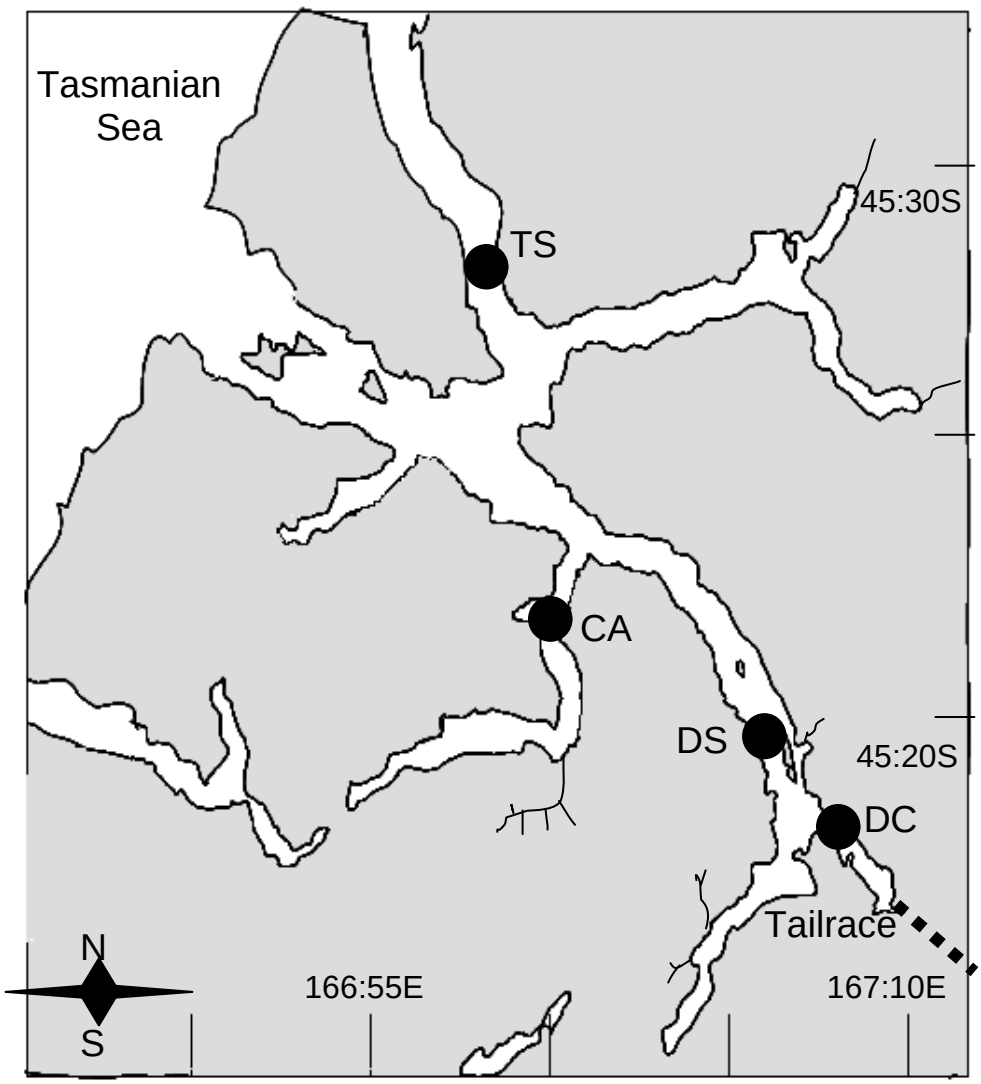

Figure 4.1 Map of Doubtful Sound. Sampling stations are marked by black circles. Rivers are indicated by thin lines.

Sample	Depth (m)	Salinity	Temp. (°C)	Si (mM/ L)	PO4 (mM / L)	Total Dissolved Nitrogen (mM / L)	Dissolved Organic Carbon (mM / L)	Dissolved Organic Carbon / Total Dissolved Nitrogen
Deep Cove (DC)	0	2.2	n/a	n/a	n/a	n/a	n/a	n/a
Thompson Sound (TS)	3	28.5	n/a	n/a	n/a	n/a	n/a	n/a
Crooked Arm (CA)	0.2	17.0	8.0	38.9	4.7	3.2	105.4	32.9
Middle of Fjord (DS)	1	21.5	9.9	44.9	0.4	6.2	101.4	16.4

Table 4.1 Chemical measurements from the four sites at which the experiments were performed. Only salinity values were collected for the salinity transplant experiment.

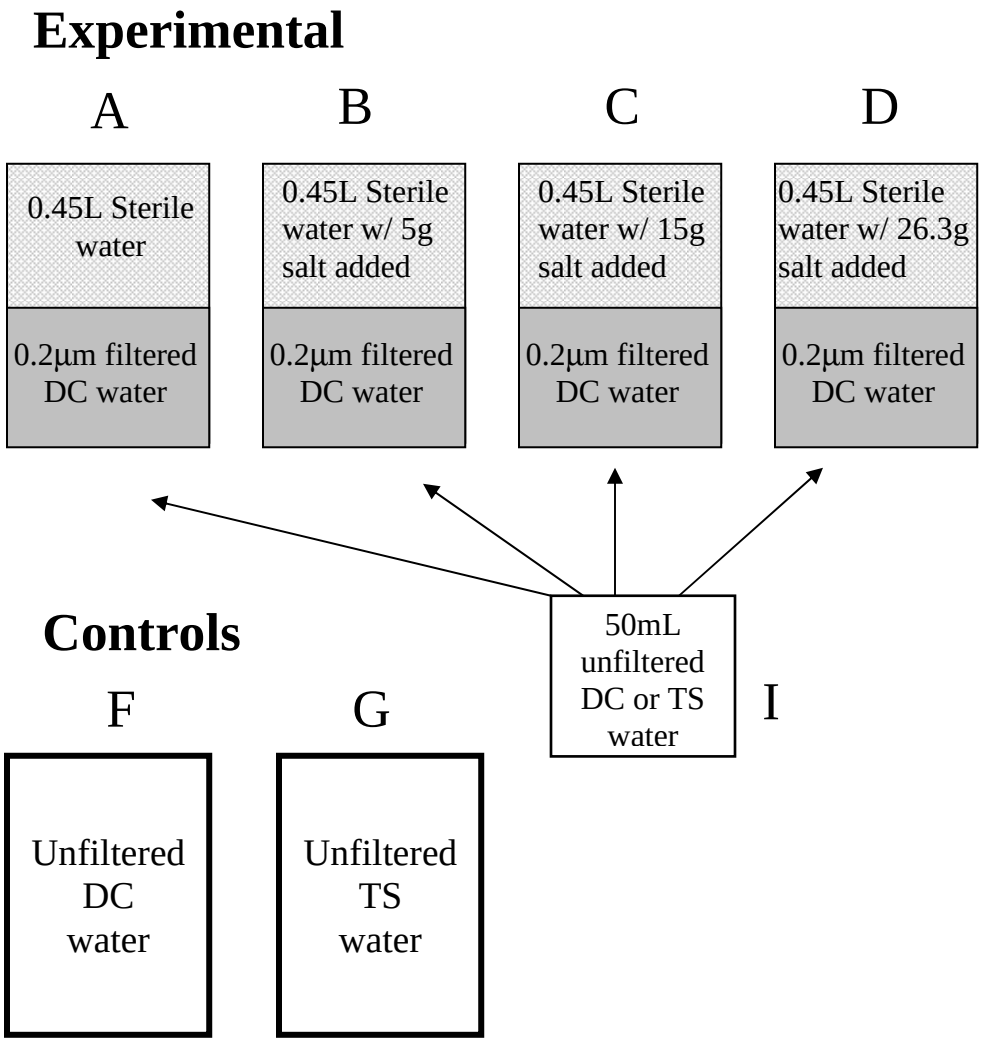

Figure 4.2
Set-up of the salinity transplant experiment. Treatments A – D monitored the effect of altering salinity on freshwater and marine bacteria. Treatments F – G measure the bottle effects on the communities.

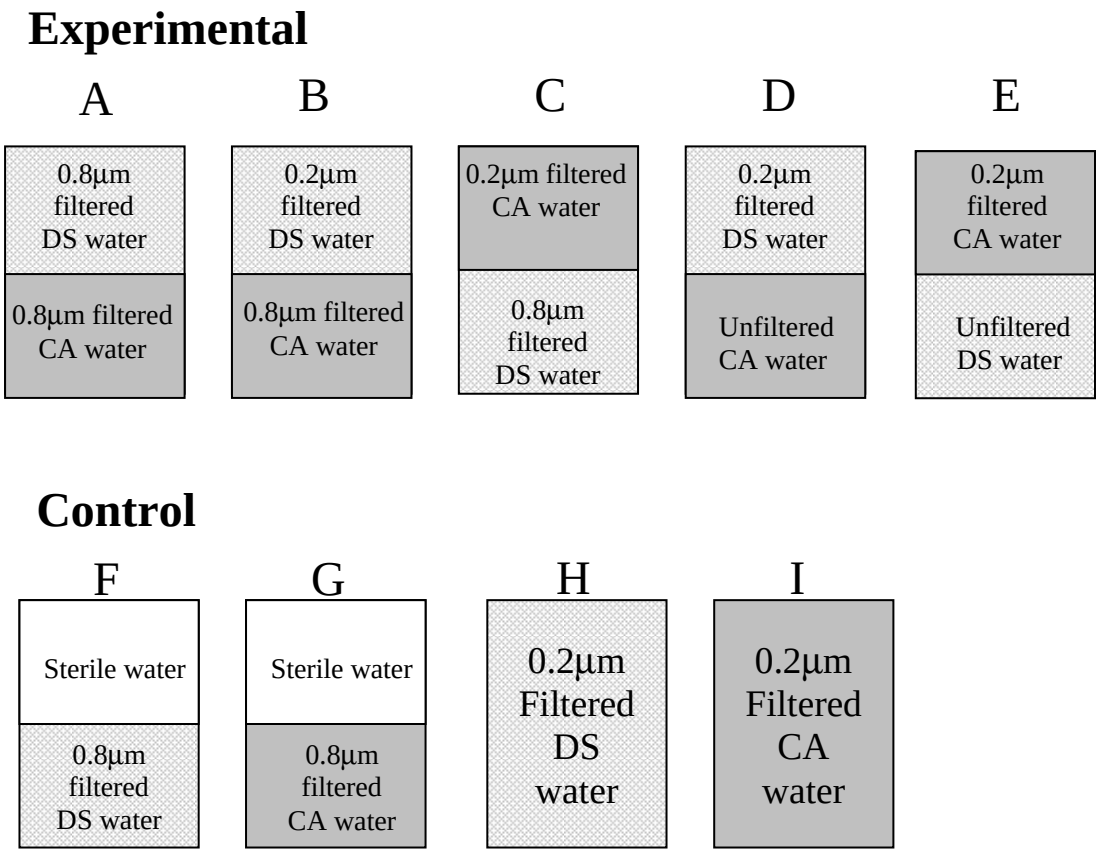

Figure 4.3
Set-up of the nutrient transplant experiment

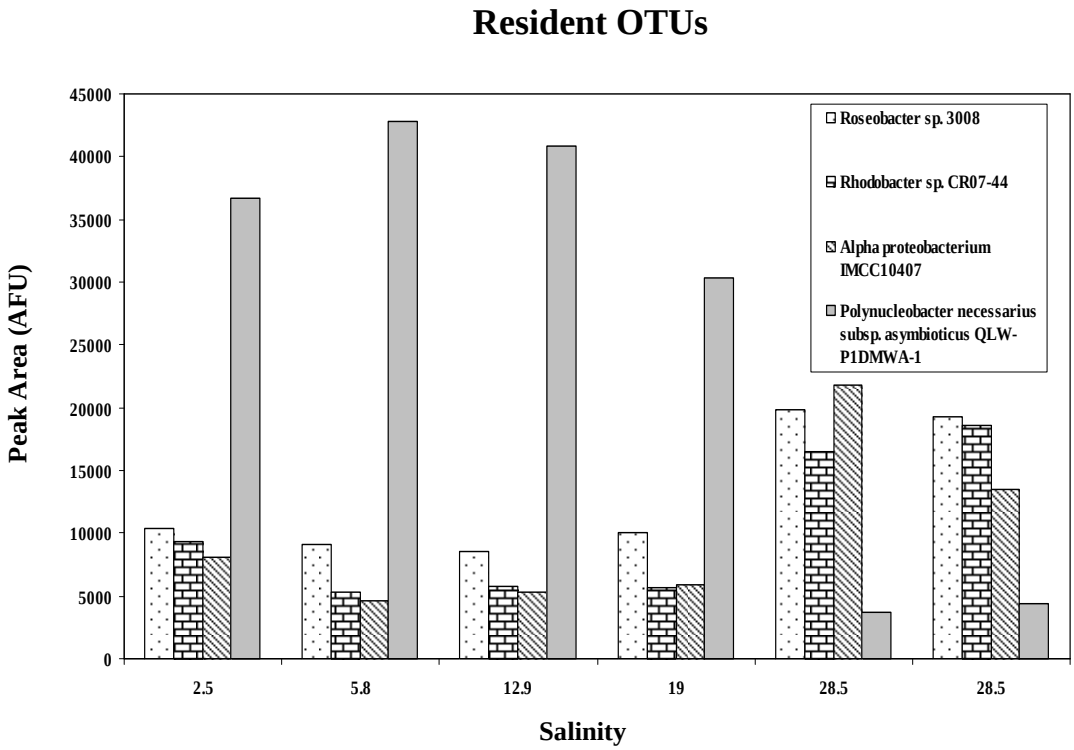

Figure 4.4 Examples of resident species that correlate with salinity and their abundance (based upon peak area) at different salinities.

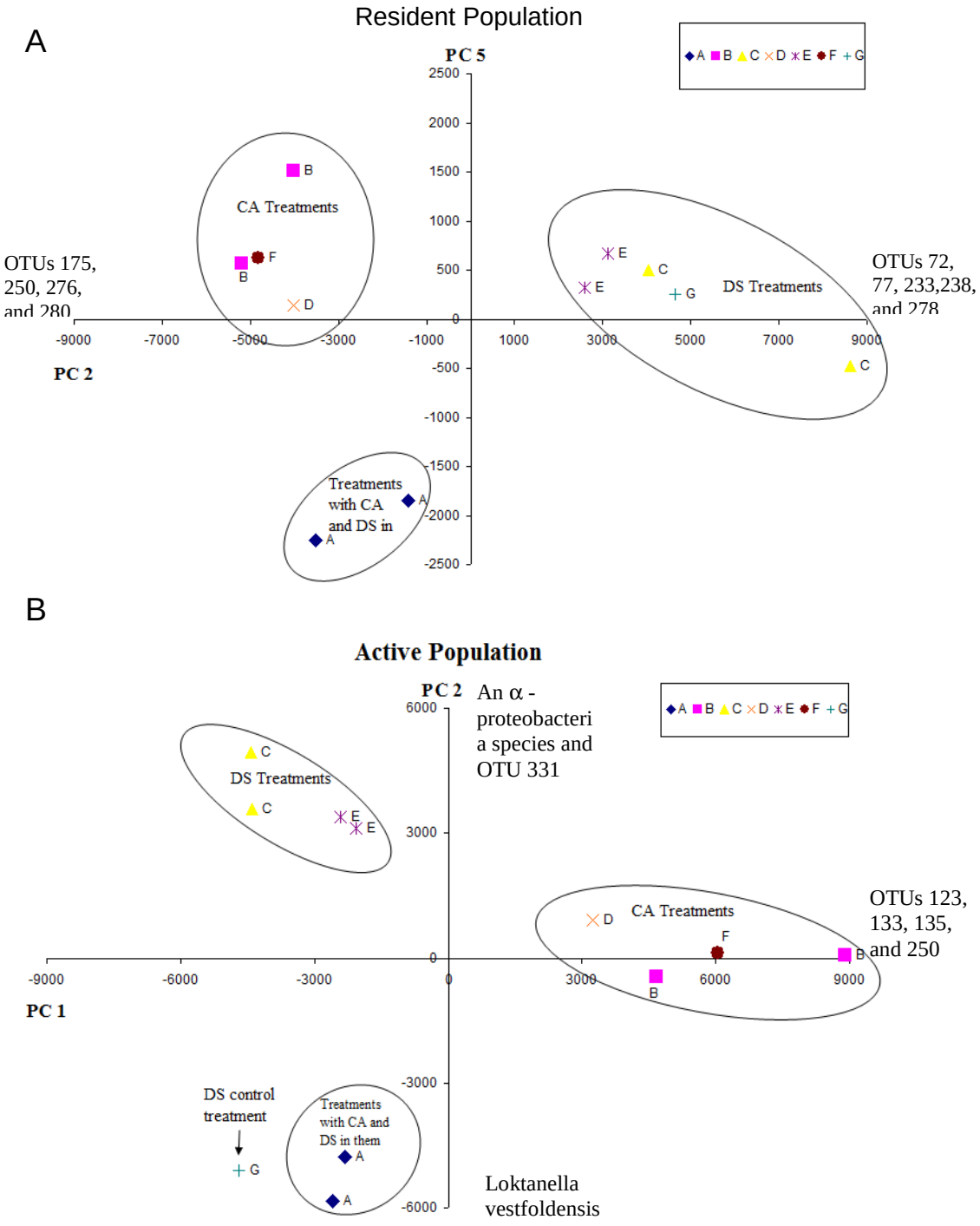

Figure 4.5 Plots of the significantly relevant Principal Components (PC) according to the ANOVA and identified species or unidentified OTUs which correlate (>0.8) with these PCs. (A) A plot of PC 5 vs. PC 2 for the resident population. (B) PC 2 vs. PC1 for the active population. The symbols with letters correspond to the treatments in Figure 4.3. Each inoculum source is shown to group separately.

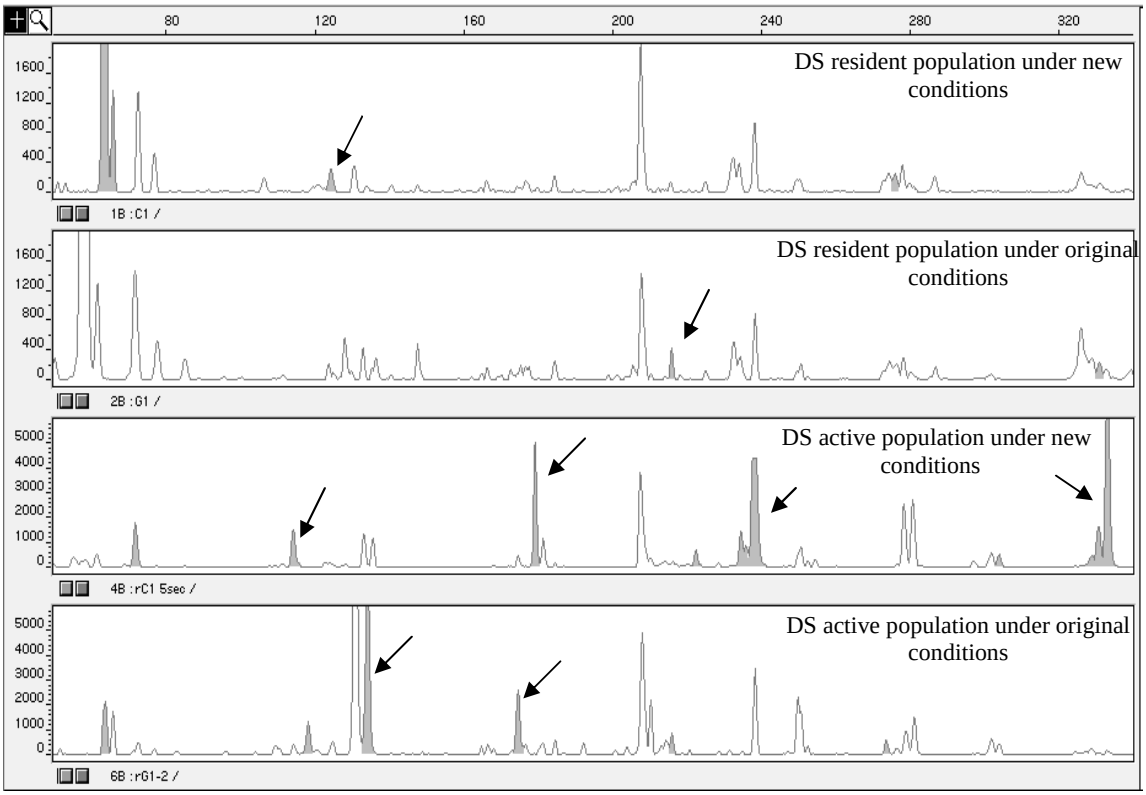

Figure 4.6 Appearance and disappearance of OTUs in treatments versus controls in the resident and active populations of DS. The gray highlighted OTUs with arrows pointing towards them are those that either only appear in the controls or only appear in the treatments.

Chapter 5. Dissertation Conclusions and Future Work

The overall goal of my research is to work towards elucidating the forces that enhance marine microbial diversity and understanding how microbial community structure relates to community function. To achieve this objective, three main questions can be posed: (1) what are the driving factors behind the diversity found within each environment and how does this relate to the proportion of the community that is actively involved within an environment (2) what are the metabolic capabilities of the active members of the community, and (3) what are the consequences of altering the controlling parameters within a system (i.e. through glacial melting and nutrient discharge). This dissertation addresses the first of these driving questions, while the future work section outlines questions two and three in more detail and describes the processes I would employ to find the answers.

5.1 Dissertation Overview

The diversity of a single sample may not be an accurate evaluation of how any system functions. Therefore in order to effectively analyze an environment, it is crucial to uncover "who" is actively metabolizing within the chosen environment and what factors control this population. To distinguish the members of the bacterial community that are growing from those that are dormant or have been advected/transported into the system, this work analyzed high molecular weight rRNA from ribosomes in addition to the 16S rRNA genes. Measuring 16S and 18S rRNA can act as a surrogate for ribosomal quantification, which in turn relates to protein synthesis and cell metabolism. The results of this thesis work (Chapters 2,3, and 4) and previous studies (i.e. Troussellier et al., 2002; Moeseneder et al., 2005; Gentile et al., 2006; Lami et al., 2009) indicate that

significant differences exist between the active (rRNA) and resident (rRNA gene) bacterial populations. In fact, our analysis indicated that at any point in time or space essentially half of the bacterial community is active, containing a sufficiently large amount of rRNA. This percentage had a very narrow range (30 – 60%) and barring one exception in the Doubtful Sound did not appear to fluctuate despite the variety of environmental conditions.

In addition to stressing that it is essential to isolate the actively metabolizing population from the inactive faction, this thesis demonstrated the value of focusing on individual members rather than an entire community. Chapter 2, 3, and 4 of this thesis established that while the entire bacterial community may not correlate with alterations in the physical, chemical, and biological environment, individual species may be responding in abundance or activity. For example, in Doubtful Sound, the composition of the overall active population is not related to nitrate concentrations, yet 21 species or Operational Taxonomic Units (OTUs) within this group have a significant relationship to fluctuations within the nitrate values (Chapter 3). These results further prove that grouping the population together obscures any variability within the system and prevents a comprehensive understanding of how changes within the microbial community impact ecosystem function.

While individual members of the microbial community demonstrated fluctuations in abundance and activity in conjunction with an environmental parameter, this work determined that no single parameter appears to drive the microbial diversity and that the proportion of individuals that do respond to these parameters is minor. Within the Orinoco River Plume and Doubtful Sound no more than 30% of either the active or

resident communities show changes related to their surroundings. This suggests either that a number of the factors that were eliciting change were not monitored or that forces other than the environment are constraining distribution and growth.

Lastly, this work reflects the need to isolate individual variables when studying the community response to environmental fluctuations. Along the chosen gradients a number of conditions were changing simultaneously. Therefore, some OTUs co-correlated with a few measured parameters. In Chapter 4, single environmental variables were manipulated and fewer species were found to respond to the selected factors than in the field studies. This argues that it is necessary to evaluate any connections between individuals and environmental parameters.

5.2 Future Work

Through this PhD research it became clear that the abundance and activity of certain species can be correlated with a number of measured parameters (i.e. phytoplankton species, nutrients, and light) and that less than half of the bacteria in a sample are actually metabolically active. Expanding the knowledge on how this percentage and individual species are influenced by the ambient conditions and on the mechanisms by which the "inactive" species come to exist and persist in these environments (i.e. what are their origins and how do they survive) would give a more complete picture of microbial diversity within dynamic systems. To do this, it is necessary to explore additional environments and uncover the proportion of active species, the origin of both inactive and active species, and the mechanisms by which inactive species exist/survive.

In addition to determining which species are actively growing, to gain real insight into an environment it is crucial to assess what these species are doing. The physiological traits and activity of the microbial population can provide further insight into the biogeochemical cycling of an environment. Previous studies have indicated that depth and spatial patterns exist in carbon and energy metabolism (i.e. Rocap et al. 2003, Perez-Jimenez and Kerkhof 2005, DeLong et al. 2006). It is possible to expand upon this knowledge by focusing on gene expression by tracking mRNA. This process will provide insight into the metabolic processes occurring under different environmental conditions. For example, examinations into the *nifH* gene (Zehr and McReynolds 1989, Zehr and Paerl 1998), involved in nitrogen fixation, can convey information on the introduction of fixed nitrogen into an area, while the *phoX* gene can indicate the patterns of phosphorus metabolism in members of the bacterial community (Majumdar et al. 2005, Monds 2006, von Kruger 2006, van Mourik 2008, Sebastian and Ammerman). In the future, one could expand upon this previous work and look into additional functional genes to uncover the metabolic capabilities of microbial population.

Finally, one major application of identifying the relationships between bacteria and their environment is its use in predicting how alterations in the ecosystem will influence the biogeochemical cycling on earth. To do this it is necessary to continue to studying how anthropogenic processes (i.e. pollution, climate change) influence the microbial population and the overall function of an ecosystem. The results from these studies will not only give insight into how the environment changes the diversity of resident populations but could also provide a number of biomarkers that could trace changes in the environment and predict possible consequences. After performing field

and laboratory experiments, the next step would be to create basic models. Constructing models will help characterize the ecological applications of this work and, perhaps more importantly, could lead to predictions on the implications of anthropogenic actions in the environment.

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Appendix A: Supplemental Information for Principal Component Analyses in Chapter 3

Appendix A1

Statistical Information for Doubtful Sound Study

Specific OTUs, their IDs, the factors with which they correlate, their correlation coefficient (r value), and p - value

OTU	Description	Correlation	Year	r value	p - value	Number of samples
84	Alpha proteobacterium IMCC10407	Abundance and depth	2006	0.83	0.013	8
		Abundance and resident eukaryotic species	2006	0.83	0.0103	8
107	Uncultured bacterium clone N67e_11	Abundance and salinity	2007	0.81	0.0045	11
		Activity and nitrate	2007	0.97	0.0017	6
		Activity and resident eukaryotic species	2007	0.9	0.011	11
125	Uncultured bacterium clone AV19F67b	Abundance and salinity	2006	0.89	0.0033	8
		Abundance and temperature	2006	0.89	0.0033	8
		Abundance and salinity	2007	0.94	<0.0001	11
		Abundance and nitrite	2007	0.9	0.0142	6
		Abundance and component 2 of the cDOM	2007	0.9	0.0142	6
		Abundance and active eukaryotic species	2007	0.94	0.0001	11
130	Rhodobacter sp. CR07-44	Activity and nitrate	2007	0.97	0.0017	6
		Activity and active eukaryotic species	2007	0.88	0.0019	11
131	Loktanella vestfoldensis isolate IMCC6033	Abundance and component 3 of the cDOM	2007	0.91	0.019	6
167	Uncultured alpha proteobacterium clone CL32-G10	Abundance and salinity	2006	0.94	0.0004	8
		Abundance and temperature	2006	0.94	0.0004	8
170	Bacteroidetes bacterium zo35	Abundance and resident eukaryotic species	2006	0.89	0.0034	8
173	Uncultured gamma proteobacterium	Abundance and component 3 of the cDOM	2007	0.82	0.0436	6
		Activity and nitrate	2007	0.97	0.0017	6
		Activity and active eukaryotic species	2007	0.83	0.0052	11
179	Uncultured alpha proteobacterium clone SHWN_night2_16S_732	Activity and nitrate	2007	0.97	0.0015	6
		Activity and active eukaryotic species	2007	0.9	0.0008	11
185	Uncultured actinobacterium clone S1-18	Abundance and depth	2006	0.85	0.008	8
		Abundance and resident eukaryotic species	2006	0.85	0.008	8
		Abundance and component 3 of the cDOM	2007	0.89	0.0164	6
203	Uncultured SAR116 alpha proteobacterium clone EF100-93A06	Abundance and salinity	2006	0.92	0.0012	8
		Abundance and temperature	2006	0.92	0.0012	8
208	Polynucleobacter necessarius subsp. asymbioticus QLW-P1DMWA-1	Abundance and depth	2006	0.89	0.0031	8
		Abundance and resident eukaryotic species	2006	0.89	0.0031	8
		Abundance and component 3 of the cDOM	2007	0.99	0.0003	6

OTU	Description	Correlation	Year	r value	p - value	Number of samples
251	Roseobacter sp. 3008	Abundance and salinity	2006	0.89	0.0029	8
		Abundance and temperature	2006	0.89	0.0029	8
		Abundance and nitrite	2007	0.97	0.0011	6
		Abundance and active eukaryotic species	2007	0.82	0.0067	11
		Activity and nitrate	2007	0.89	0.019	6
253	Uncultured Rhodobacter sp. clone YE-1	Activity and active eukaryotic species	2007	0.81	0.0085	11
254	Uncultured gamma proteobacterium clone SIMO-899	Abundance and depth	2006	0.8	0.0161	8
		Abundance and resident eukaryotic species	2006	0.8	0.0161	8
274	Uncultured delta proteobacterium clone CB22F07	Abundance and salinity	2006	0.97	<0.0001	8
		Abundance and temperature	2006	0.97	<0.0001	8
		Abundance and salinity	2007	0.84	0.0011	11
		Abundance and nitrite	2007	0.99	<0.0001	6
		Abundance and active eukaryotic species	2007	0.91	0.0006	11
		Abundance and component 2 of the cDOM	2007	0.99	<0.0001	6
285	Uncultured marine Nitrospinaceae bacterium clone EB080L20_F04	Activity and nitrate	2007	0.86	0.0266	6

Curriculum Vita

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