AN ABSTRACT FROM THE THESIS OF

Carmen C. Denman for the degree of Honors Baccalaureate of Science in Microbiology presented March 6, 2009. Title: Microbial Abundance and Community Analysis in Devil's Hole, Harrington Sound, Bermuda.

Abstract approved:

S.J. Giovannoni

Microbial activity within elevated CO_2 and oxygen depleted environments changes with density driven stratification overturn in the seasonally anoxic region of Devil's Hole, Bermuda. The temperature gradient developed during the summer months creates a natural laboratory to study bacterial and virus population density and microbial community dynamics in the anoxic layer during stratification and in response to naturally raised pCO_2 levels. Chemical properties and microbial communities were measured and evaluated during stratification and after turnover. The study concludes that there are different bacterial communities present during pre and post turnover conditions, with two unknown bacterial populations accounting for a majority of bacterial diversity. Virus counts fluctuate with depth, while bacterial populations flourish near the sediment layer at the anoxic zone. Organic matter falling through the water column settles in the anoxic layer and sediment, creating a nutrient rich environment for bacteria to metabolize. These anaerobic and elevated pCO_2 conditions give a glimpse into microorganism's nutrient use and production under elevated pCO_2 conditions which are similar or higher than future pCO_2 levels projected according to Intergovernmental Panel for Climate Control.

Key Words: Ocean acidification, stratification, CO_2, anoxic, marine bacteria

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Microbial Abundance and Community Analysis in Devil's Hole, Harrington Sound, Bermuda

by

Carmen C. Denman

A PROJECT

Submitted to

Oregon State University

University Honors College

in partial fulfillment of
the requirements for the degree of

Honors Bachelors of Science in Microbiology (Honors Scholar)

Presented March 6, 2009
Commencement June 2009

Honors Baccalaureate of Science in Microbiology project of Carmen C. Denman presented on March 6, 2009.

APPROVED:

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Carmen C. Denman, Author

Acknowledgements

Thanks to the guidance and project creation and motivation from mentor Rachel Parsons (BIOS Microbial Observatory), sampling cruise trips and life lessons provided by Andreas Andersson, and generosity and hospitality of the staff and researchers at Bermuda Institute of Ocean Sciences. Thanks to John Casey for running flow samples, and Craig Carlson's lab for nutrient and T-RFLP analysis. Thanks to Steve Giovannoni and Kevin Vergin for providing mentoring and the necessary means to continue this project at Oregon State University as my honors thesis.

Thank you to all involved in the teaching, learning, and development of this project. Writing is a process, and revisions and feedback from those more advanced in their scientific careers is essential to create a satisfactory final product.

Thank you to the University Honors College for four and a half years of tireless support and mentoring. Thank you to Dean Arp for agreeing to serve on my thesis committee.

This research was supported by the U.S. National Science Foundation Research Experience for Undergraduates at the Bermuda Institute of Ocean Sciences, the Gordon and Betty Moore Foundation, and the honors excellence fund Chambers memorial fund for environmental research.

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Preface

The following is a detailed account of the input, advising, and references to specific sections of this thesis project.

The introduction of this thesis was written by the author with the aide from the following information sources: personal communication with Rachel Parsons and Andreas Anderson, and the articles by Andersson *et al* (2007 & 2006), Hammond 2008, and Mackenzie (1975).

The materials and methods section of this thesis is mainly made up of pre-existing laboratory protocols distributed and streamlined by Rachel Parsons. The flow cytometry protocol was outlined with help from John Casey (BIOS) the flow cytometry technician at BIOS. Other laboratory, sampling, and analysis protocols were updated and modified for this specific project by the author. The DNA extraction and gel purification protocol came from the lab of Craig Carlson, UCSB.

The Results section of this thesis is work of the author, with help arranging and presenting data by Rachel Parsons, specifically FISH, Shannon Index and T-RFLP graphs were made. She also helped in formulating the most effective way to present microbial abundance data, and pointed out multiple corrections needed to all tables and figures. Tables were made by the author, with the exclusion of Tables 2 and 3, sources being Parsons and Morris, Connon, and Giovannoni from pre-existing protocols.

The analysis and interpretation of results was done by the author, helped by literature articles, and Rachel Parsons (BIOS). Help interpreting specific nuances regarding T-RFLP/Nutrient data came from personal communication with Kevin Vergin

(OSU) and Craig Carlson (UCSB). Analysis of chemical data was supervised and discussed with Dr. Andreas Andersson.

The bibliography section of this thesis was made by the author, using *www.easybib.com* as a web-tool to create an alphabetized list of references, from unpublished papers, textbooks, and research journal articles.

Feedback on formatting and general editing of this thesis manuscript was provided by Rachel Parsons, Kevin Vergin, Dr. Dan Arp, and Dr. Steve Giovannoni.

Microbial Abundance and Community Analysis in Devil's Hole, Harrington Sound, Bermuda

1 Introduction

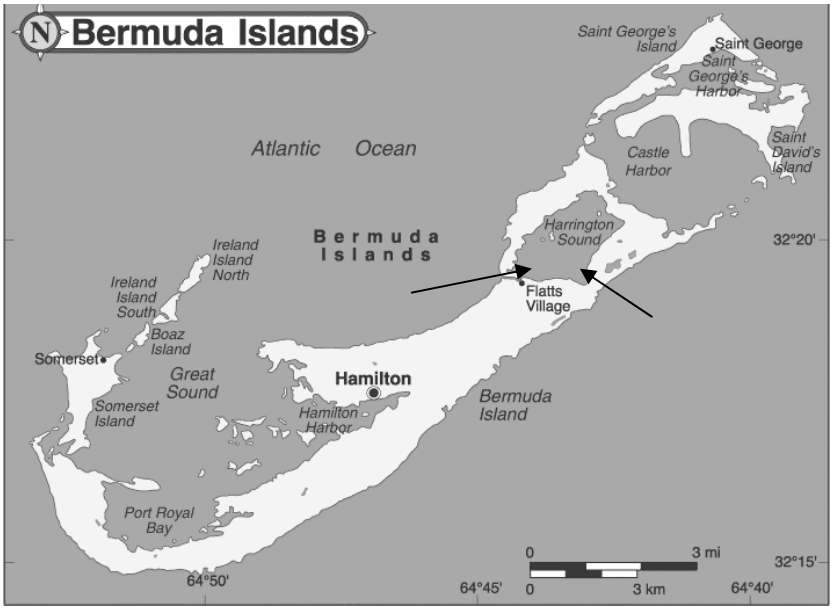
1.1 Devil's Hole (DH) geography.

Ocean acidification arises from the decrease in pH caused by the uptake of CO_2 from the environment. Harrington Sound is one of the most unusual bodies of water in the world, as it is seasonally anoxic and contains areas with elevated pCO_2 levels. Researchers have studied the sound for over forty years (Thorstenson *et al* 1974, Morris 1982, Andersson *et al* 2006, 2007). At the bottom of Harrington Sound are ancient coral reefs. Over 110 million years, rainwater has eroded and dissolved the calcium carbonate reefs to form caves, which have collapsed over time, resulting in the depth of the Harrington sound basin. Devil's Hole (DH) is the deepest part of the sound, a former sink hole lying 24 meters below the surface (Figure 1). It is located in the southeast corner of the sound. During late summer in Devil's Hole, density driven stratification restricts the mixing of the surface and bottom layers. The warmer top layer is separated from the denser cooler layer by the thermocline. Seasonal stratification and turnover of Devil's Hole affects the microbial populations and abundance, but it is not known to what extent. The separate layers create a natural laboratory to study microorganisms in an anoxic deep layer also exhibiting elevated pCO_2 levels. Elevated pCO_2 levels in Devil's Hole arise from microbial decomposition of falling of organic matter and detritus through the water column (Andersson *et al* 2007). This makes Devil's Hole an excellent natural model to study

microbial activity depletes oxygen levels thus creating an anoxic environment. This anoxic event is not unique to Devils' Hole; there are similar seasonally anoxic waters off the Oregon coast (personal communication Steve Giovannoni) and in other parts of the world.

The microbial communities of inshore waters in Bermuda in Devil's Hole can be used as important indicators of ecosystem health and can shed light on possible ways the environmental community adapts in response to elevated pCO_2 levels.

Figure 1. Map of Bermuda. Devil's Hole is located in the Southeast corner of Harrington sound. The only transport of water in and out of the sound is through a narrow space called Flatts Inlet. Left arrow points to Flatts' inlet, narrow opening to the sound, and right arrow points to sampling location Devil's Hole (DH)



1.2 Microbial Diversity and Abundance.

Based on preliminary data from Devil's Hole, it is known that chemical properties change seasonally with the formation and dissipation of the thermocline (Andersson et al 2007). What is unknown is the microbial community composition and abundance in the anoxic layer in response to stratification. Why does the water column post-turnover return to a

uniform bacterial density? The bacterial community affects changes in levels of pCO_2 and pH, and nutrient cycles such as nitrogen and sulfur. The decrease in pH due to the elevated level of pCO_2 affects calcification and thus impacts the ability of calcifying organisms, such as corals, specifically the exoskeleton building process.

In this study, four different bacteria clades were enumerated by Fluorescent in situ Hybridization (FISH): SAR11, *Alteromonas, Cytophoga*, and *Roseobacter*. Total Eubacteria and a negative control were also counted. These probes were chosen based on their performance in testing by Rachel Parsons (BIOS MO). One purpose of this study was to find the percentage of each bacterial clade present in the different depths sampled in Devil's Hole, during and after stratification. If there are different clades or proportions of clades, then a correlation can be made with nutrient and chemical properties, including elevated pCO_2 levels, which may be impacting the bacterial community composition present in Devil's Hole.

Although probing (FISH) is helpful in determining whether a specific number of different bacterium is present within the sample or not, time restricts the bacterial groups that can be analyzed. However, the Terminal Restriction Fragment Length Polymorphism (T-RFLP) technique provides a more conclusive, although qualitative, report of all the bacterial diversity and communities present in the sample. By amplifying the conserved bacterial 16S rDNA gene, this same gene will be amplified for all the bacteria species in the sample. Due to hypervariable regions in the gene, it is possible to distinguish between bacterial groups using a restriction enzyme and a fluorescently labeled PCR primer. By comparing the bacterial community make-up of the different depths in Devil's Hole, we

hope to conclude whether the bacterial communities are changing after the density driven thermocline is created and then dissipated.

2 Material and Methods

2.1 Field work and sample collection.

A single location, Devil's Hole (32 N 19.399', 64 W 43.08') located within Bermuda's Harrington Sound was chosen for collection and analysis of water samples during September and October 2008. As detailed in Table one, six sampling cruises were undertaken.

During each of the six cruises, at least five different depths were sampled. Water samples were collected for bacteria, virus, nutrients, and DNA analyses. Other interns and scientists collaborating on the project sampled for dissolved oxygen, salinity, and salts. The probes used for water monitoring by the Marine Environmental Program lab (MEP lab at BIOS) were manufactured by YSI and used to gather temperature, pH, dissolved oxygen (DO), and salinity. A one liter nisken water sampler was used to collect the water.

Five to eight viral abundance samples were collected per cruise in UV sterilized cryovials (5ml). Five to eight Bacterial abundance samples were collected per cruise in UV sterilized falcon tubes (15ml). The water samples used for DNA extraction were collected using an acid cleaned polycarbonate bottle (1L) and the water samples utilized for bacterial counts were collected in UV sterilized falcon tubes (15ml). Sampling was done wearing gloves to ensure sterility. The water samples were immediately placed on ice in order to preserve the microbial community at the time of sampling.

TABLE 1. 2008 cruise dates and depths (meters) sampled. Devil's Hole (DH) cruises 1-4 were during stratification, turnover started sometime between DH4 and DH5, and turnover was complete at DH6. Some duplicates at the anoxic layer were sampled. Sampling began at 9 a.m. each cruise. The dates listed after the cruise (DH 1-6) is the day of the sampling cruise.

DH1 9/5	DH2 9/11	DH3 9/18	DH4 9/24	DH5 9/30	DH6 10/8
0	0	0	0	0	0
10	10	10	16	16	16
15	16	16	20	22	22
17	20	22	22	25.5*	23
19	21	23	23	25.5*	24
22	22	24	24		
23	23	25*	24.5*		
24	24	25*	24.5*		

*High tide through Flatts' Inlet increased the depth of Devils' Hole by 0.5 to 1.5 meters.

2.2 Virus-like-particle Abundance.

Virus-like-particle (VLP) samples were fixed with 1% 0.02µm filtered formalin then stored at –80°C until processing (within 1 month of all six sampling cruises). 3 ml Samples were filtered onto 0.02µm 25mm filters (ANODISC, 6809-6002 Whatman, UK). Filters were mounted with prolong 20-30µl (P7481 Molecular Probes, OR, USA), and stained with SYBR Green I (S7567 Molecular Probes, OR, USA), for epiflourescence counts (Noble and Fuhrman, 1998). Epifluorescent microscopy is preferred over transmission electron microscopy for obtaining accurate estimates of viral abundances in natural waters (Weinbauer and Suttle, 1997; Chen et al., 2000). Enumeration was done on an Olympus AX70 microscope (Tokyo, Japan) under narrow blue excitation using a Toshiba CCD video camera (IK-TU40A, Tokyo, Japan), image capture Flashpoint 4.0 and Image Pro Plus 4.0 (Media Cybernetics, MD, USA). Twelve images were taken, enhanced and measured for bright field particles in the range of 0.02-0.5 microns. The lowest and highest counts were discarded and the remaining ten counts were averaged.

2.3 Bacterial and Cyanobacterial Enumeration.

Immediately upon arrival, water samples for prokaryotic abundance counts were fixed with 10% 0.2µm filtered formalin and stored at –80°C. Formalin, a derivative of formaldehyde, is used to halt bacterial replication. The remaining 1L water samples were filtered and stored at -80^{o}C for DNA extraction. The bacterial samples were processed in accordance with Porter and Feig's (1980) method amended (Parsons personal communication). Briefly, the water samples were filtered onto Irgalan Black stained 0.2 µM filters under gentle vacuum (~100mm Hg) and stained with 0, 6-diamidino-2-phenyl dihydrochloride (5µg/ml, DAPI, SIGMA-Aldrich, US). The filtration was done in minimal lighting to ensure DAPI stain preservation. Filters were mounted onto slides with Resolve immersion oil (high viscosity) and stored at –20°C. Slides were then enumerated using an AX70 epifluorescent microscope (Olympus, Tokyo, Japan) under ultra violet excitation at 100x magnification. At least 800 cells per slide (twelve fields) were counted.

Cyanobacteria were also enumerated under narrow green excitation at 100x magnification. At least 200 cells per slide were counted as a result of the naturally low cyanobacteria abundance in turbid marine environments such as Harrington Sound.

2.4 Fluorescence *in situ* Hybridization (FISH)—Probing.

The fluorescence *in situ* hybridization (FISH) protocol was followed in accordance with the Morris et al. (2002) procedure and streamlined by Rachel Parsons (personal communication). Briefly, the samples were first fixed with 10% formalin and then filtered through 0.2 µM white polycarbonate 25 mm membrane. The filters were attached

via tough spots to glass slides and stored with desiccant in the dark at –20°C. Probe sequences are reported in Table 3. The following was done in the dark in order to safeguard the light-sensitive probes. The filters were cut into quarters using a sterile razor blade and fixed onto a new slide using tough spots. All filters were probed with 40 µl of 2ng/µl probe in hybridization solution (0.9 M NaCl, appropriate % formamide as detailed in Table 2, 20 mM Tris-HCl (pH 7.4) and 0.01% sodium dodecyl sulphate (SDS) and incubated at the appropriate temperature for 16 hours. The quarter filters were washed twice for 10 mins each in 40ml of washing buffer containing 20 mM Tris-HCl (pH 7.4), NaCl mM (Table 2), 5 mM EDTA and 0.01% SDS. The hybridization wash temperature (Table 2) was predetermined using an empirically derived dissociation curve. Each quarter filter was then air-dried on a kimwipe and the tough spots were removed with a sterile razor blade. The filter is then mounted with 20 µl of 1.67 µg/ml 0, 6-diamidino-2-phenylindole dihydrochloride (DAPI, SIGMA-Aldrich) in citiflour solution (Ted Pella Inc.) and sealed with nail polish. The slides are stored in the dark at -20°C.

TABLE 2. Species probed and accompanying hybridization conditions.

Probe	Hybridization Solution % Formamide	Hybridization Temperature ^{o}C	Hybridization Wash NaCl Concentration	Wash Temperature ^{o}C
Negative 338F	15	37	0.15M	55
Roseobacter 536R-Cy3	35	37	0.07M	52
Alteromonas AC137-Cy3	35	37	0.07M	52
SAR11 152R-Cy3 441R-Cy3 542R-Cy3 732R-Cy3	15	37	0.15M	55
Cytophoga CF319a- Cy3 CF319b- Cy3	35	37	0.07M	48

An epifluorescent microscope as described previously was used to enumerate the specifically probed bacteria under x1000 magnification. Six sets of two pictures were captured using a Toshiba 3CCD Camera and Image Pro Plus version 4.0 software (Media Cybernetics, USA); with one picture under ultra violet excitation to illuminate all the bacteria and with one picture under the CY3 narrow green light excitation to illuminate the specific bacteria probed. The camera has the ability to integrate over multiple frames to ensure that all, even faintly illuminant bacterium are detected. These pairs of raw pictures are then masked (a contrasting black and white picture) and overlapped to ensure that the luminescence detected under CY3 narrow green light corresponds to bacteria specific binding. On average 20-90 bacterial cells were counted per field on the DAPI. A negative control was counted in order to account for autofluorescent bacteria.

TABLE 3. Probe Sequences

Probe	DNA Sequence 5' to 3'	Reference
338F-Cy3[a]	TGAGGATGCCCTCCGTCG	Morris *et al* 2002
152R-Cy3 [b]	ATTAGCACAAGTTTCCYCGTGT	Morris
441R-Cy [3b]	TACAGTCATTTTCTTCCCCGAC	
542R-Cy3[b]	TCCGAACTACGCTAGGTC	
732R-Cy3[b]	GTCAGTAATGATCCAGAAAGYTG	
536R-Cy3[c]	CAACGCTAACCCCCTCCG	Connon (pers. communication)
AC-137R-Cy3[d]	TGTTATCCCCCTCGCAAA	Connon
CF319aR-Cy3[e]	TGGTCCGTGTCTCAGTAC	Connon
CF319bR-Cy3[e]		

a - Negative Control
b - SAR 11
c - *Roseobacte*r
d – *Alteromonas*
e – *Cytophoga*

2.5 DNA Sample Collection.

One liter of water was filtered for DNA samples through a 0.2 µM 47 mm support filter using a gelman rig under gentle vacuum (~100mm Hg). Immediately after the sample filtered through completely, the filter was taken off the filtration rig and placed in a 4 mL cryovial oriented with the cells facing outwards. One mL of 0.2µ filtered sucrose lysis buffer (20mM EDTA, 400mM NaCl, 0.75M sucrose, 50mM Tris-HCl, pH 7.4) was added and stored at –80°C.

2.6 DNA Extraction.

The DNA was extracted using the phenol/chloroform extraction method by Giovannoni (1990), except that lysozyme and RNase were omitted as they lowered purity. Briefly, SDS to 1% and Proteinase K to 200µg/ml were added and incubated at 37^{o}C for 30 minutes then at 55^{o}C for 30 minutes. The lysates (1ml) were extracted twice with an equal volume of phenol: isoamyl alcohol: chloroform (PIC) in a 25:1:24 proportion. Centrifugation was done at room temperature (as opposed to 4^{o}C as done by Giovannoni 1990). The cesium trifluoroacetate purification steps were omitted. The DNA was purified by precipitation using ammonium acetate (7.5M) and isopropanol (100%) in a ratio of 2:1:2 (sample: ammonium acetate: isopropanol) for 1 hour at room temperature and then centrifuged for 30mins at 20,000 x g. The pellet was washed with 80% ethanol, vortexed for 30s and centrifuged at 16,000 x g for 10 mins. The resulting pellet was dried and resuspended in 50µl of TE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA)

2.7 DNA Quantification and Qualification.

The DNA concentration and purity of each pellet was determined using an Agilent 8453 spectrophotometer (260nM). Typically the A_{260}/A_{280} ratios ranged from 1.2-2.2 and averaged 1.6 showing that protein was not contaminating. Samples were stored at -20^{o}C until utilized for T-RFLP analysis.

2.8 T-RFLP and PCR Analysis.

The Terminal Restriction Fragment Length Polymorphism (T-RFLP) technique was used to determine the bacterial communities present in the surface and anoxic layer in the water samples. Ribosomal RNA genes from the mixed communities were amplified as previously described (Morris et al., 2004). The PCR amplification of the 16S rDNA was carried out with 27F-fam (GGRGTTYGATYMTGGCTCAG) and 519R (GWATTACCGCGGCKGCTG) (SIGMA Biosynthesis, USA) primers. The DNA was diluted to 10ng/µl for the reaction and BSA (200ng/µl) was added to each reaction to remove inhibition by humic acids. Acetamide was added to a final concentration of 5% to reduce template DNA G+C content induced bias. A thermal minicycler (Biometra, T-Gradient, Germany) was used for all reactions with the following conditions: 2mins at 94°C, then 35 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 1min followed by a 10 min extension at 72°C. The positive control was DNA extracted from *Alteromonas macleodii* culture and the negative control was UV sterile water. PCR products (5µl) were visualized on a 1% agarose gel stained with ethidium bromide (1µg/ml), visualized on a Kodak Imaging System and compared to a 1kb DNA ladder (Fermentas, MD, USA). Positive results were amplicons in the range of 480 – 530 bp amplicons. The PCR product was then stored at -20^{o}C until cleaning.

After the PCR product was checked, the remaining product was gel purified with Qiagen QiaQuick PCR purification kit to remove both impurities and primers. This step is necessary before the restriction digest to prevent the primers and impurities from incorrectly representing a bacteria species.

PCR products were restricted overnight at 37°under subdued light using *Hae*III enzyme and buffers (NEB, MA, USA). The digest is then inactivated at 80^{o}C for 20 minutes and stored at -20^{o}C. PCR products and digests (5µl) were visualized on a 3% agarose gel stained with ethiduim bromide (1µg/ml), visualized on a Kodak Imaging System and compared to a 1kb DNA ladder (Fermentas, MD, USA). For T-RFLP analysis, the digested fragments were resolved on an ABI 3100 genetic analyzer (Applied Biosystems, Foster City, CA). (Data analysis by Craig Carlson, Rachel Parsons, Kevin Vergin)

2.9 Flow Cytometry (FCM) Analysis.

Samples for picoplankton enumeration by flow cytometry were collected from 0 – 25 m and fixed with paraformaldehyde (0.5% final concentration) at 4°C for 2–6 h before being flash frozen in liquid nitrogen. Samples were sorted and analyzed on a Becton Dickenson (formerly Cytopeia, Inc) high speed jet-in-air InFlux™ Cell Sorter at an average flow rate of 40 µL min^{-1}. Samples were sorted and analyzed for the cyanobacterial populations Prochlorococcus, and Synechococcus. After exclusion of laser noise gated on pulse width and forward scatter (FSC), autotrophic cells were discriminated by chlorophyll (red) fluorescence. Cell enumeration gates were further discriminated with PE (orange fluorescence), granularity (side scatter- SSC), and FSC according to our gating schema. A 70µm nozzle tip was used with a sample pressure of 28.5 PSI (+1.0 PSI over sheath) to optimize speed while maintaining high fluorescent

signal resolution. Sheath fluid was made fresh daily from distilled deionized water (Milli-Q, Millipore Inc., Billerica, MA) and molecular grade NaCl (Mallinckrodt Baker Inc., Phillipsburg, NJ) and was pre-filtered through a 0.2 µm capsule filter (Pall Life Sciences Corp., East Hills, NY). A 100 mW blue (488 nm) excitation laser, run at full power, was used in conjunction with three color and two scatter detectors. Analog signals from red longpass (>650 nm), orange (585/30 nm) and green (530/20 nm) bandpass filters as well as direct laser light from forward and side scatter (FSC, SSC) detectors (Hamamatsu C6270 photomultiplier tubes) were log amplified and converted to digital I/O. Mean coincident abort rates were < 1% and mean recovery from secondary sorts (n = 25) was 97.5 ± 1.1% (data not shown). Spigot™ (Cytopeia Corp., Seattle, WA) was used for data acquisition and FCS Express V3™ (DeNovo Software, Seattle, WA) was used for post acquisition analysis.

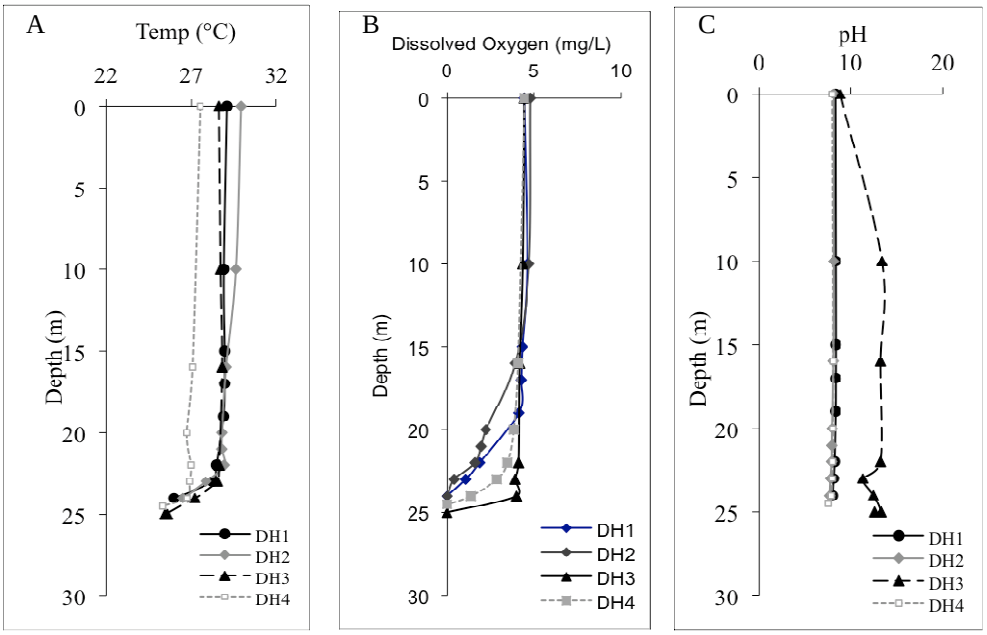
2.10 Nutrient Analysis.

An acid washed bottle was used to collect the water sample after the sample has been filtered through an Anodisk 0.08 µm filter membrane, directly from the nisken sampler. The samples were kept on ice until shore side then frozen at –80°C until time of analysis. Samples were shipped to UCSB for analysis in the Marine Science Institute (MSI) Analytical Laboratory. (http://analab.msi.ucsb.edu/).

3 Results

3.1 General Chemical Properties and Trends.

Devil's Hole has been the subject of oceanographic study since the 1950's. Its chemical properties have been studied (Andersson, *et al.* 2006 & 2007, Thorstenson, *et al.* 1975, Morris, 1982) extensively. Previous studies (Andersson, *et al.*, 2007) estimated the breakups of the thermocline to occur in the late summer or early fall. They also estimated that the thermocline is approximately 17 meters deep. In 2008, the breakup of the thermocline began to occur after tropical storm Kyle's strong winds October 15, 2008 and then completely overturned by early October. The thermocline was at 24 meters (Figure 2). At that depth, temperature, pH, and dissolved oxygen levels all significantly changed, as reported similarly by Andersson et al (2007) in their 2004 study.



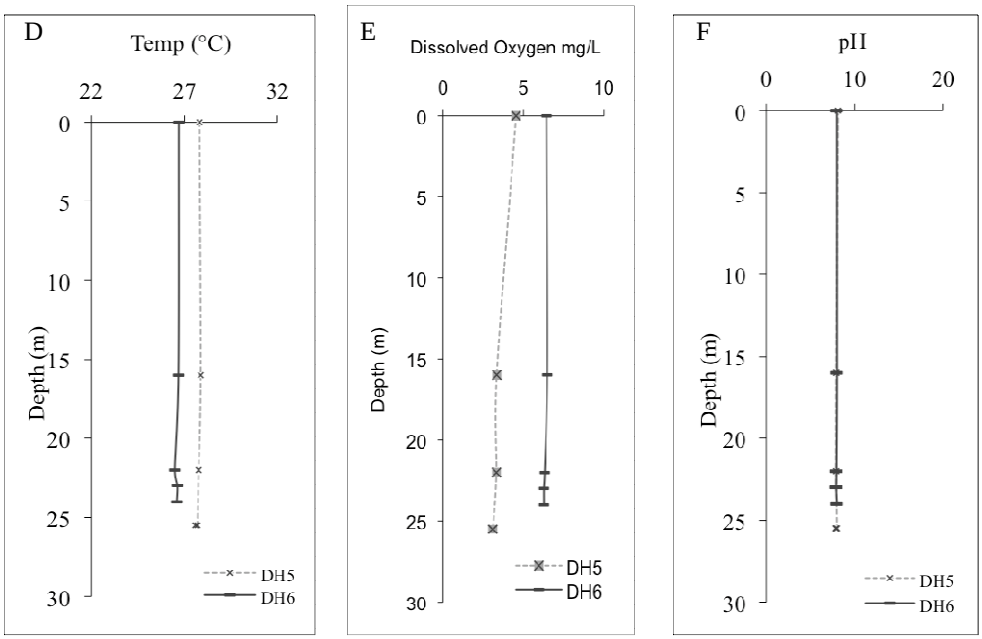


Figure 2: Profiles of A, D) Temperature [°C]; B, E) Dissolved Oxygen [mg/L]; C, F) pH for A-C) Pre-turnover and D-F) Post-turnover from Devil's Hole, Bermuda in September 2008.

3.2 Bacterial, Cyanobacterial and Viral Abundance.

Bacterial cell density (Figure 3) during stratification is uniform through the water column, and then increases by a factor of ten before 22 meters. After turnover, the bacterial abundance returns to uniform concentrations. DAPI counts correlated with Flow Cytometry (FCM) counts. Cyanobacterial counts (Figs 3 B, E) show an overall decrease with depth as light is attenuated through the thermocline towards deeper and organic matter-laden waters. After turnover, Cyanobacterial presence becomes more uniform and overall total counts are lower throughout the water column, as the layers have been mixed together. In Devil's Hole during stratification, surface virus per milliliter ranged from 1 x 10^7 to 4 x 10^7. At anoxic depth, the viral density decreases overall to 2 x 10^6 to 3 x 10^6 virus/mL (Figs 3 C, F).

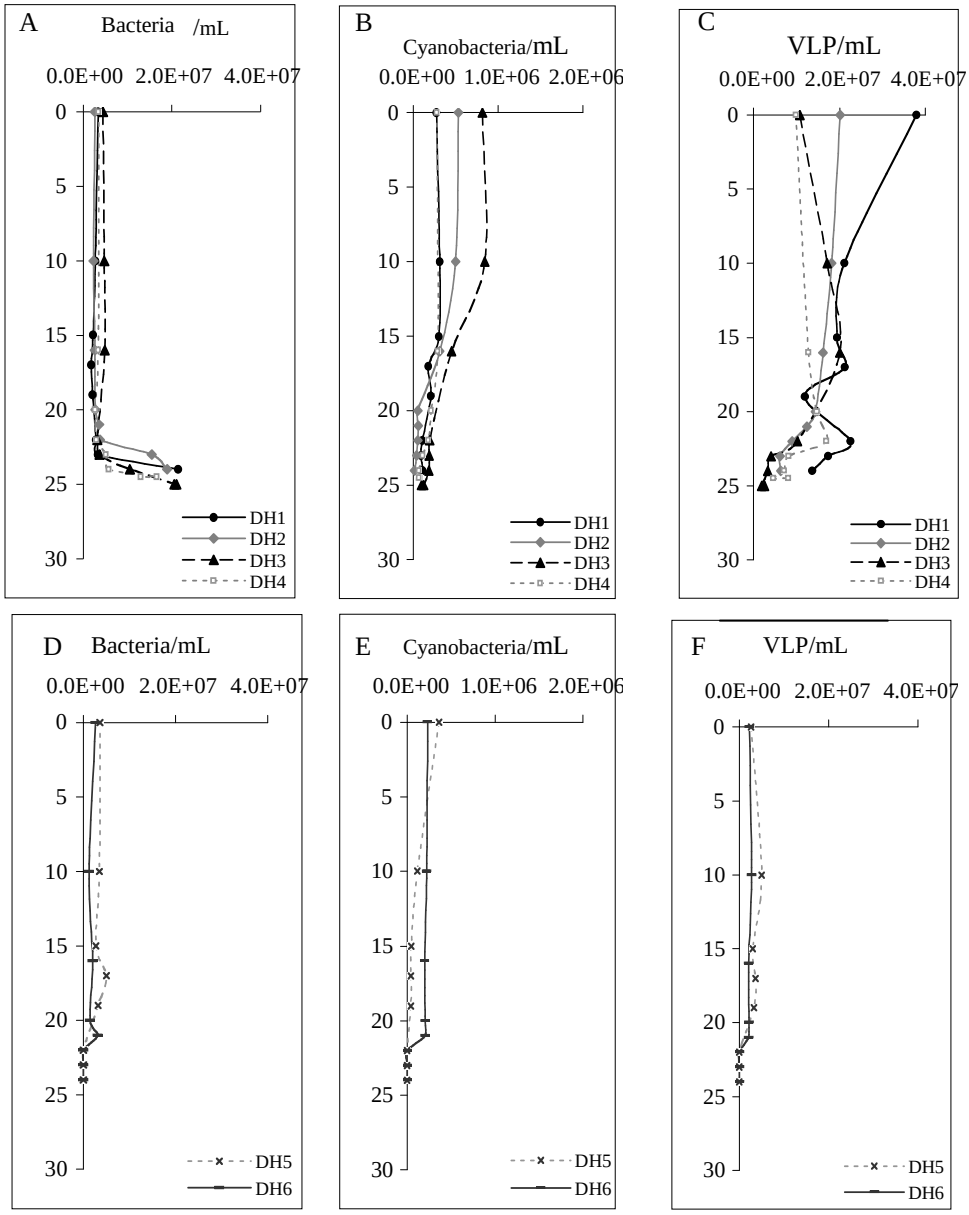


Figure 3: Profiles of A, D) bacterial density B, F) cyanobacteria abundance C, F) virus-like-particles A-C) Pre-turnover and D-F) Post-turnover from Devil's Hole, Bermuda in September 2008.

3.3 Fluorescent *in situ* hybridization (FISH).

Since no previous probe data exists for Devil's Hole, probes chosen were based off previous near shore water sampling sites from around sewage outfalls and clean sites (Fosolino *et al.*, 2008, Sheppard *et al.*, 2008, Amman *et al.*, 2008, Noble, 2006). As shown in figure four A, populations x and y, the most abundant species present except for

the unknown category. These two distinct populations are not a known group of bacteria, but have been identified through flow cytometry (FCM) as significant to the anoxic layer community. The presence of X and y bacterial populations disappear after turnover. SAR11 is the most abundance bacterial clade identified at the surface waters in this study but decreases with depth. *Roseobacter* and *Cytophoga* hits were seen in mid depths, as well as *Alteromonas*. Figure 4 reports total percentage of bacterial species probed of the total cells per mL of water for cruises DH2, DH4, DH5 and DH6 counted by FISH and FCM.

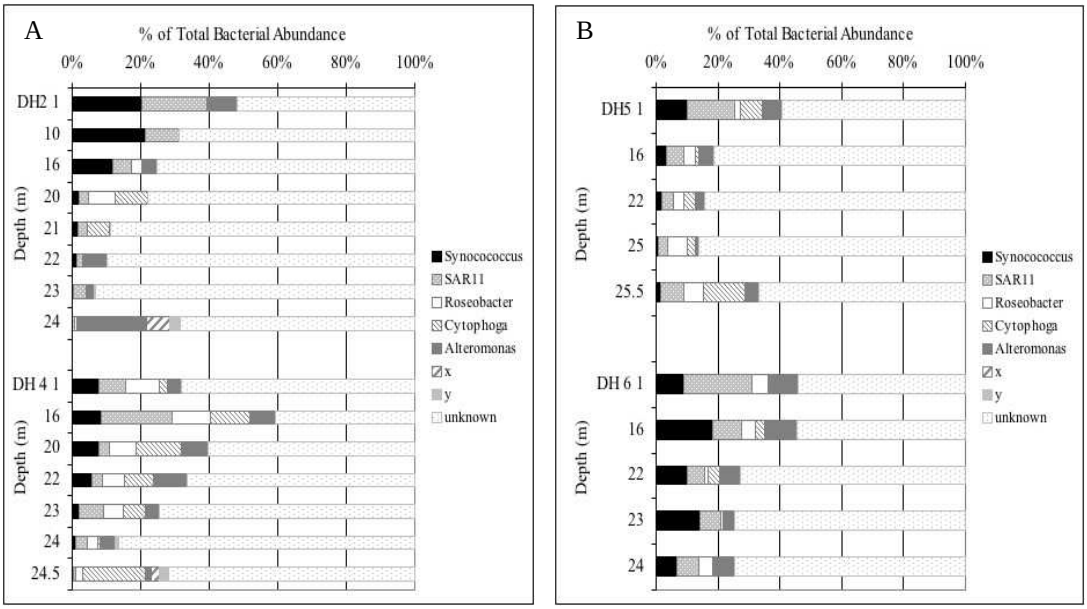


Figure 4. FISH results, by total percentage of bacterial abundance for A) Pre turnover cruises DH2 and DH4 and B) Post turnover cruises DH5 and DH6 from Devil's Hole, Bermuda in September 2008. X and Y are unknown bacterial populations that were identified through flow cytometry (FCM).

3.4 T-RFLP and PCR Analysis.

The Shannon- Weiner Index (*Shannon Diversity Index* 2008) was used to assess the diversity of species found at the surface and anoxic layers in Devil's Hole. The x axis measures the Shannon index value versus depth on the y axis for the pre-turnover

samplings DH2, DH3 and DH4 (Figure 5A), and after-turnover samplings DH5 and DH6 (Figure 5B). The species diversity changes with each cruise. This shows species are in flux with the changes in the thermocline breakdown and dissipation. Even DH5 and DH6, both after turn-over, are different in their species diversity. There is less species diversity, except a 21 meters and overall after turnover.

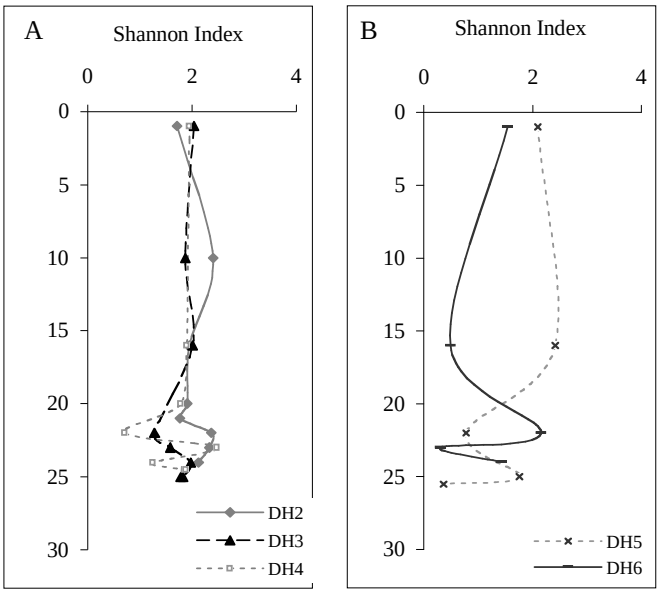


Figure 5. Profiles showing the Shannon index of relative abundance and species richness. A) Pre-turnover and B) Post-turnover from Devil's Hole, Bermuda in September, 2008.

T-RFLP is a high-resolution electrophoretic method used to monitor bacterial community structure shifts. The peaks in a T-RFLP eletropherogram correspond to rDNA genes that have been amplified, restricted with *Hae*III and electrophoretically separated, with fragment length indicated on the horizontal axis and % peak area on the vertical axis (Figure 6). However, peak areas do not quantitatively reflect microbial abundances because of variations in the copy number of rRNA genes in cells and biases in the PCR amplification.

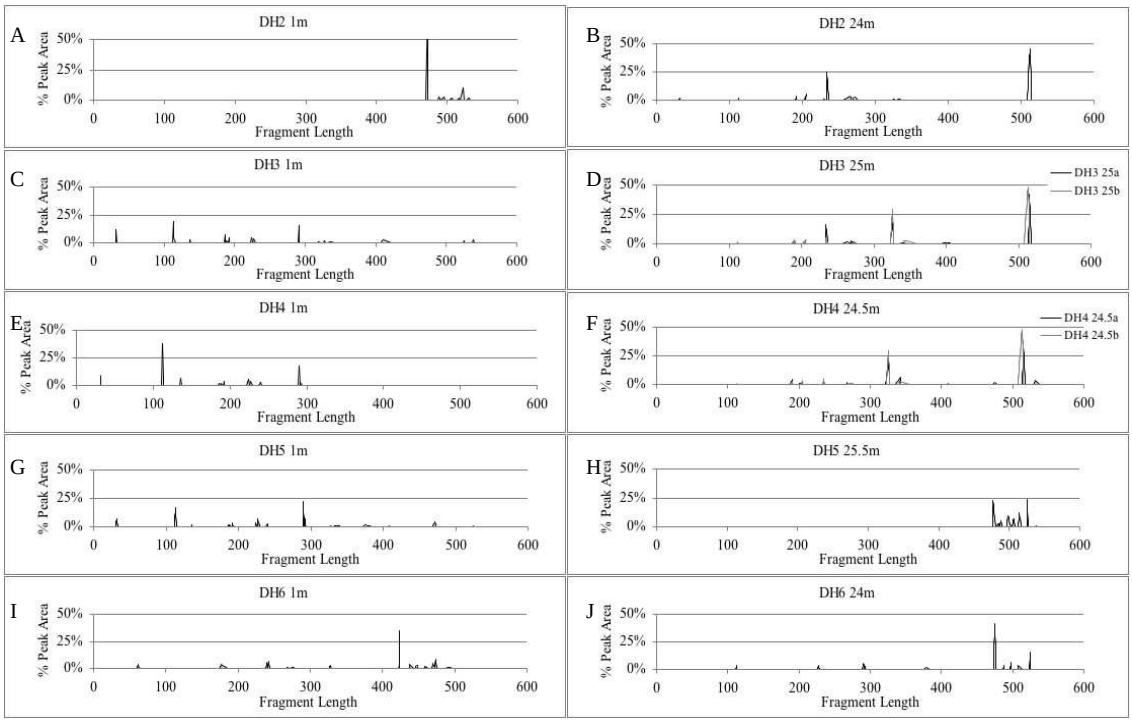


Figure 6. Electropherograms showing DNA fragments amplified by PCR from samples collected for cruises DH2 to DH6 from 1m (left panels A, C, E, G and I) and 24-25.5m (right panels B, D, F, H, and J) from Devil's Hole, Bermuda in September 2008. DH2 (A, B), DH3 (C, D) & DH4 (E, F) are pre-turnover and DH5 (G, H) & DH6 (I, J) are post-turnover. Reproducible peaks from duplicate samples are shown as gray in panels D, and F for the anoxic layer.

3.5 Nutrient analysis.

Nutrients were measured for four cruises, DH2 and DH4, DH5 and DH6. The nutrients measured were nitrite, nitrate and nitrite together, ammonia, and phosphate (Figure 7). The results show a nutrient gradient exists during stratification; nitrite and nitrate are highly concentrated at 21-22 meters in DH2. DH4 there is no spike in levels at 21-22 meters. Ammonia levels in DH4 peak just above the anoxic layer. Ammonia levels are linked to nitrate and nitrite levels and a group of ammonia oxidizing bacteria could exist at this depth and convert ammonia to other forms of nitrogen such as nitrate and nitrite, as part of the ocean nitrogen cycle (Francis *et al.*, 2007). Phosphate is sequestered in high concentrations in the sediments (Karl 2007, Schultz *et al.*, 2005). After turnover, nutrient

concentrations return to uniform and overall decrease is concentration after turnover. Phosphorous still peaks at depth, as it is coming in and out of the sediment layer. There is less overall phosphorous measured at depth. Phosphorite, a mineral that contains high levels of phosphate, is present in sediments with sulfur reducing bacteria. Sulfur reducing bacteria are expected to be present in the anoxic layer in Devil's Hole during stratification because of the strong smell of hydrogen sulfide, the most reduced form of sulfur.

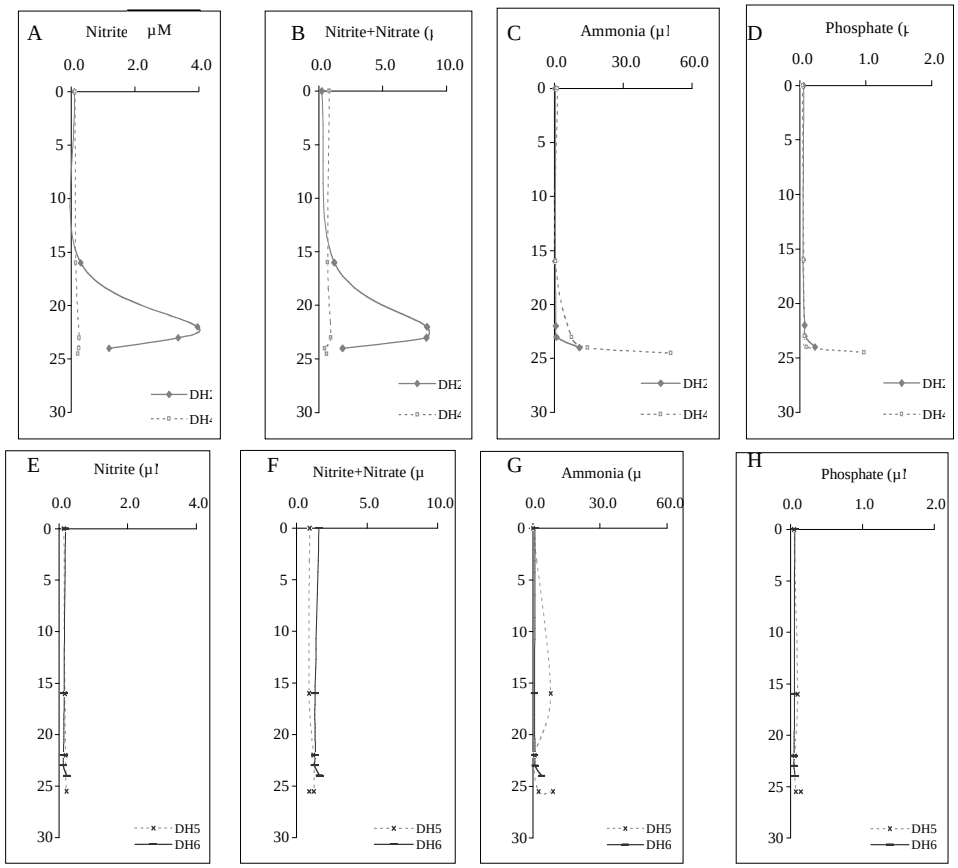


Figure 7: Profiles of A, E) Nitrite B, F) Nitrite+Nitrate C, G) Ammonia D, H) Phosphate concentrations (µM). A- D) Pre-turnover DH2 DH4 and E-H) Post-turnover DH5 DH6 from Devil's Hole, Harrington Sound, Bermuda in September and October 2008.

3.6 DNA sequencing of clone libraries.

16s rRNA genes were cloned into E.coli. Results will be discussed in a follow-up study.

4 Discussion

Chemical data showed when turnover occurred it caused temperature to become more uniform, pH uniformity, and anoxic deep layers to become re-oxygenated. However, microbial analyses using T-RFLP indicated that the chemical change in turnover did not necessarily accompany a microbial community alteration. As clone libraries are processed and reported, more information on microbial composition of Devils' Hole

Cyanobacterial enumeration by FCM showed a wide range of abundance in the surface waters during stratification, but overall decrease with depth as light is attenuated through the thermocline towards deeper and organic matter-laden waters. After turnover, cyanobacterial presence becomes more uniform throughout the water column as the layers are mixed together. Some of these could be dead cells autofluorescing.

Flow cytometry sorting revealed consistent data when measuring abundance of *Synecococcus*, a photosynthetic microorganism. Sampling cruises DH4 and DH6 were sorted to measure cellular abundance, and compared to manual DAPI counts. Manual cell counts and sorting counts for bacterial density were similar to FCM data. Bacterial density fluctuated with depth and the anoxic environment. This study reveals that Devil's Hole bacterial density is much higher at depth when compared to other near-shore water sampling sites around Bermuda in 2007-2008. This is due to the stratification; detritus and organic material fall through the water column creating a nutrient rich environment for bacterial metabolism and anaerobic consumption of carbon, sulfur, and phosphorous from the sediment layer. During stratification, surface bacterial density was ~2 $x10^6$ cells/mL. The anoxic layer contained ten fold more cells, ~3 x 10^7 cells/mL. After the

break up of the thermocline layers, bacterial density was uniform throughout the water column, ranging from 1 x10^6 cells/mL to 3 x 10^6 cells/mL, a significant decrease in bacterial density.

Viral abundance in near-shore waters in Bermuda has been shown to decrease as the bacterial population increases (Sheppard 2008) during late summer. In Devil's Hole during stratification, virus counts at the surface ranged from 1 x 10^7 to 4 x 10^7 per milliliter. At anoxic depths, the viral density decreased to a range of 2 x 10^6 to 3 x 10^6 virus/mL. This could be due to virus living intracellularly at depth.

No previous FISH probe data exists for Devil's Hole. FISH probes were based on previous near shore water sampling sites from around sewage outfalls and clean sites (Fosolino 2008). It was expected that because of Devil's Hole high turbidity and the anoxic layers' high plentiful organic material, there could be similar species as sewage outfall nutrient rich environments. The majority of the bacterial population remained unidentified after FISH probing. Clone library analysis will be necessary to identify bacterial groups associated with the anoxic layer. Populations x, y, the most abundant species present during stratification at depth, identified through flow sorting, are undetected after turnover. The SAR11 clade is the most plentiful bacterial clade identified at the surface waters in the ocean (Morris et al., 2002) but decreases with depth (Figure 4 A, B). *Roseobacter* and *Cytophoga* cells were identified in mid depths (Figure 4 A, B). *Alteromonas* and *Roseobacter* species (Figure 4 A, B) have been identified by previous studies as species able to grow in nutrient rich marine environments, and cells from these species were identified in the mid to deep water column. Roseobacter has been classified as a major marine bacteria group (Buchan *et al.,* 2005, Giovannoni 2000),

and was expected to be present. Anoxic bodies of water around the world contain *Alteromonas macleodii* (Ivars-Martinez *et al.,* 2008), and during DH2 Alteromonas was identified by FISH to and sulfate reducing bacteria to be plentiful at depth (Francis 2008, Yakimov *et al*., 2007).

The Shannon- Weiner Index was used to assess the diversity of species found at the surface and anoxic layers in Devil's Hole. The number of species in a community and the total number of individuals in the sample are included in the calculation. The Shannon-Weiner equation $H= - \sum p_i \ln p_i$ was used to evaluate the diversity at each location. The term p_i stands for S/N (Species richness/Abundance) (Shannon 2008). The Shannon index was computed on the *Hae*III digested PCR products (as described in Section 2.9). Using the known sequences of some bacterial species, we speculate that some of the peaks shown here at depth and at surface represent *Alteromonas* and SAR11. The sampling cruises DH2-DH4 leading up to turn-over show similarities in diversity, including the anoxic layer. Diversity in DH4 is decreased, suggesting the beginning of turnover had disrupted some microbial communities. This analysis, when compared to the T-RFLP and clone library results, demonstrates that the bacterial community is not uniformly mixed following chemical turn-over as hypothesized. Overall, the Shannon indexes show species abundance and diversity increases at depth with the anoxic layer during stratification. The two post turnover cruises show there are still differences through the water column in diversity, but there is less species abundance at depth. The Shannon index is computed using the peak size and peak height from the T-RFLP data.

Nutrients measured for four cruises, two pre and two post-turnover, were expected to yield noticeable changes in nutrient levels during stratification. DH2 and

DH4 pre-turnover, and DH5 and DH6 post-turnover, were analyzed for nitrate + nitrite, nitrate, phosphorous, and ammonia. These samples were filtered and left unfixed, as described in section 2.10. When DH2 and DH4 were tested for nitrate and nitrite content, DH2 had especially high concentrations at 15 and 20 m. DH4 showed uniform concentration until the anoxic layer is reached, at 24 and 25 m. Duplicate samples were taken in DH4 at 25 m. After turnover (DH5 and DH6), a similar gradient as seen in DH4 is reported, with slightly higher values overall for DH6. These differences among the cruises, however slight, convey turn-over is a dynamic process. It is not possible to catch it at the moment turn-over occurs, but sampling weekly enabled us to follow the process as it occurred over several weeks, in part due to tropical storm Kyle (October 15, 2008).

Results from analysis of phosphorous (Figure 7 D, H) pre-turnover show a dramatic increase from 21 to 24 meters upon entering the anoxic layer. After turnover (figure 7H), the phosphorous concentration gradient still increase at depth in DH5 and DH6, but the concentration at depth is less than it was during stratification. Large sulfur bacteria are known to be responsible for the formation of Phosphorite, a mineral that contains high levels of phosphate (Schultz *et al.*, 2007). This could mean anearobic bacteria could be plentiful at the anoxic layer, forming high levels of phosphate, which is normally present in marine sediment. The DH5 and DH6 increase is still present at depth possibly due to sedimentary phosphate. Sediment samples were not taken for this project due to time and equipment constraints.

Nitrate and nitrite do not vary through the upper water column above 21 m (Figure 7B). The cycle of nitrogen is important in relation to all nutrients (Francis *et al.*, 2007). Then, at the anoxic layer in DH2 and DH4, we observed a sharp increase in nitrate

and nitrite levels. This could be due to the microbial populations, dense at the anoxic and sediment layers, cycling organic nitrogen back into these measurable forms, such as high levels of ammonia (Figure 7C).

Ammonia concentrations during stratification increased sharply at DH2 23 meters to the anoxic layer. A bacterial species or clade living at 23m to the anoxic layer of the water column could be (*Francis et al* 2007), cycling nitrogen and reducing the ammonia to elemental nitrogen. Overall, the nutrient levels in the anoxic layer are dictated by the type of microbial metabolism occurring in the sediment and surrounding waters.

Sulfur levels were not tested for in this study. However, the strong smell of the volatile gas hydrogen sulfide from the anoxic layer indicates sulfur reducing microorganisms are present in Devil's Hole during stratification. It is possible that anearobic sulfur reducing bacteria make up the unknown x and y populations identified through FCM. Sulfur transformation and reduction involves several groups of microorganism, including photosynthetic, aerobic and anearobic bacteria (Brock *et al.,* pg 428). Even throughout the water column, sulfur could be present in other forms, and then fall through the water column to the anoxic layer. SAR11 marine bacteria require exogenous reduced sulfur for growth (Tripp *et al.,* 2008). Sulfur in the ocean is an important nutrient, as it is required in its most reduced form to make two essential amine acids cysteine and methionine.

Ongoing research at the Giovannoni lab at Oregon State University will result in sequences of specific bacterial species cloned from extracted Devil's Hole DNA. (Vergin 2001). The unique clones will be genescan analyzed, then compared to T-RFLP peak

results, and isolates with similar peak heights will be fully sequenced and identified through BLAST search. This data will be reported in a follow-up publication.

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