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Elucidation of the Microbial Consortium of the Ubiquitous Shore Sponge, Tedania ignis

Clarisse Sullivan
University of Rhode Island, rcsullivan@uri.edu

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Elucidation of the microbial consortium of the ubiquitous shore sponge, *Tedania ignis*

Clarisse Sullivan
College of the Environment and Life Sciences, Department of Biological Science,
University of Rhode Island, Kingston RI 02881
Email: rcsullivan@my.uri.edu

Abstract

Sponges are filter-feeding organisms that contain a dense and diverse microbial community. These bacteria and archaea can comprise about 40% of the sponges' total biomass often exceeding the microbial biomass of seawater by two to three orders of magnitude. The presence of bacteria during the reproductive stages of the sponge is an indicator of symbiosis. This study used culture-independent techniques to investigate the microbial community of *Tedania ignis*; an abundant marine sponge in the inshore coral reef environments around Bermuda. Sponge and water samples were collected from Ferry Reach, Helena's Bay and Bailey's Bay in Bermuda. Epifluorescent microscopy was used to quantify the microbial abundance from within the sponge tissue and compare it to that of the surrounding water. *T. ignis* was categorized as an high microbial abundant sponge. Two extraction methods were used to isolate bacterial and archaeal DNA for clone library analysis of the microbial community composition. The Mo Bio UltraClean Soil DNA Isolation Kit was more successful at amplifying bacterial DNA while the CTAB method had higher DNA yields and purity but residual phenol may have led to PCR inhibition. Clone sequence analysis showed inconclusive results when the 27F primer sequence did not align well with the sample sequences. *T. ignis* was determined to harbor *Synechococcus sp., Actinosynnema mirum, Collimonas fungivorans, Rhodococcus opacus* and *Candidatus Puniceispirillum marinum* based on the BLAST sequence alignment. Water samples contained *Actinosynnema mirum,* and *Paenibacillus graminis* based on the BLAST sequence alignment.

Introduction

Sponges are diverse sessile animals with a relatively simple body plan (Wulff, 2006) and among the oldest metazoans dating back to 580 million years ago (Tamilselvan & David. 2012). They are filter-feeding organisms that have associated microbes within their tissues, which comprise about 40% of their total biomass often exceeding the microbial biomass of seawater by two to three orders of magnitude (Hentshel *et al* 2002). The presence of bacteria in the reproductive stages of the sponge is an indicator of symbiosis (Hentshel *et al* 2002). This symbiotic relationship is important because of the role of cohabitant microbes in sponge skeleton stabilization, metabolic waste processing, nutrient acquisition and secondary metabolite production (Hentshel *et al* 2002; Taylor *et al* 2007). Additionally, sponge-derived secondary metabolites are of interest in research due to the potential for development of pharmacological compounds, which exhibit tumor-inhibiting and antimicrobial properties (Tamilselvan & David. 2012).

Sponges are also classified as either high microbial abundant sponges (HMA) or low microbial abundant sponges (LMA). HMA sponges have 10^8-10^{10} bacterial cells per mL or g of tissue while LMA sponges have 10^5-10^6 bacterial cells per mL or g of tissue that is more similar to natural seawater (Giles *et al* 2012). These microbial abundances may have an influence on sponge morphology and physiology with HMA sponges having a more complex aquiferous system than LMA sponges.

In a study by Hentshel *et al* (2002), it was shown that the microbial communities from two different sponge species were both uniform and phylogenetically complex. Also, the cohabiting microbial groups phylogenetic signature was found to be distinct from that of the surrounding seawater. Additionally, 68% of all the sponge-derived 16S rDNA sequences showed less than 90% homology to their nearest sequence relatives from non-sponge sources. This means that sponges establish a strong selective pressure on the microbial community that live within their tissues.

Hentshel *et al* (2006) showed that sponge-specific lineages exist between marine sponges and microbes. Cyanobacteria are found in the outer sponge surfaces and are the most abundant photosynthetic organism on earth with the *Synechococcus* clade dominating the world's oceans (Hentshel *et al* 2006). The *Synechococcus* clade has been confirmed in 26 Demospongiae families (Hentshel *et al* 2006). Microorganisms that are typically found in the sponge inner core are archaea, *proteobacteria* and *actinobacteria* ((Hentshel *et al* 2006). Sponge archaea were first discovered through 16s rRNA gene sequencing and identified as *Crenarchaeum symbiosum* (Preston *et al* 1996). It resembles free-living marine crenarcheote group 1 that are taken up by filtration. Similar *C. symbiosum* phylotypes were found in Australian, Mediterranean and Korean sponge species (Webster *et al*, 2001; Margot *et al*, 2002; Lee *et al*, 2003). In *proteobacteria,* two thirds of deepwater and boreal sponges are dominated by *alpha* and *gammaproteobacteria* (Hentshel *et al* 2006). The MBIC3368 *alphaproteobacteria* strain was the most dominant in culture collections and has bioactivity that prevents phagocytosis by the host cells (Thiel & Imhoff 2003). *Actinobacteria* are known for their profuse secondary metabolite production and have been recovered from *Rhopaloeides odorabile* via culture-dependent and independent techniques (Webster *et al*, 2001). Sponge-specific actinobacteria specifically belong to *Acidimicrobiae*, who are closely related to *Microthrix parvicella* and *Acidimicrobium ferreoxidans* (Hentshel *et al* 2006).

Culture-dependent molecular techniques used to elucidate microbial communities limit cultivation of bacterial diversity to an estimated 0.1-1% (Hardoim *et al* 2012). Thus, the research described herein attempted to use culture-independent techniques that rely on DNA "fingerprinting" methods to allow for a wider and more complete range of microbial identification. Terminal restriction fragment length polymorphism (T-RFLP) offers a certain, although qualitative, rapid profiling of bacterial community and diversity structure in different ecosystems (Liu *et al* 1997). However, this technique relies on PCR amplification that can demonstrate bias when based on microbial density differences. This bias can lead to erroneous results where there is underrepresentation of minor bacterial types in the T-RFLP profile (Liu *et al* 1997). Additionally, T-RFLP can only identify changes in the microbial community of a particular sample through changes in the presence/absence of resulting restriction fragment lengths specific to each bacteria. In

order to identify a specific bacterium, however, clone libraries of a bacterial fragment length can be made and phylogenetically analyzed through sequence determination and subsequent annotation. The limitation to this method is the required number of clones or PCR products available for sequencing because samples may require over 40,000 sequencing reactions to document 50% of the richness of the sample (DeSantiz *et al*, 2007). This method is, therefore, laborious, costly and time-consuming to complete on one clone library analysis. Despite these limitations, clone library analysis is still done because it provides the greatest estimate of diversity within a sample (DeSantiz *et al*, 2007) and is useful for determining the dominant bacteria in the study (Janssen 2006)

In a study by Bibbings (2013), T-RFLP analysis of the microbial community in *Tediania ignis* revealed that fragment 74 contributed 5.65% (+/- 1.40%) of the total number of fragments and 189 contributed 0.43% (+/- 0.23%) of the total number of fragments. But the most abundant restriction fragment in the *T. ignis* sponge samples was fragment 336 contributing 46.36% (+/- 6.01%) of the total number of fragments. This is a strain with a restriction fragment made of 336 base pairs. Restriction fragments can serve as a species-specific molecular markers (NCBI, 2014) and therefore, serve as a means of identification via fingerprinting In light of this, the current project was designed to use new fire sponge samples collected in Bermuda. *Tedania ignis* is the most abundant marine sponge in Caribbean mangroves (Wulff, 2009) but is specifically found in more protected inshore coral reef environments in Bermuda. Sponges were collected and clone libraries were produced to determine the dominant bacteria in the hopes of identifying the 336-restriction fragment.

Methods

Sample Collection and Processing

Four fire sponge samples were taken from each of three sites in Bermuda: Ferry Reach, Bailey's Bay and Helena's Bay, on June 30th, July 1st and July 3rd respectively. The sponges were collected with gloves and placed in separate ziplock bags whilst underwater in order to prevent air exposure. Two 1L bottles were used to collect representative water samples from each site as well. There were a total of 12 sponge samples and 6 water samples collected for the study.

One gram of sponge was measured out from the sponge samples and ground up using a mortar and pestle with 10mL of 0.2μm filtered seawater. The sponge samples were placed in 15mL falcon tubes then centrifuged at 3381rcf for 5 minutes. The supernatant containing the suspended microbes was transferred to a clean and properly labeled 15mL falcon tube and the pellet was discarded. The supernatant was then filtered through a 500μm Nitex mesh using a pump connected to four 500mL Gelman filtration units to collect the liquid resulting in the removal of larger sponge cellular debris. This was followed by filtration through a 3μm filter to remove any remaining cellular debris. The supernatant for each of the sponge samples was processed to determine microbial abundance and isolate total DNA.

Two hundred microliters from each of the sponge extracts was diluted with 10mL of filtered seawater and fixed with 1mL of formalin (10% final concentration in order to keep the bacteria from multiplying and to preserve them in their original state. These tubes were stored at -80°C freezer for further analysis. 5mL of the remaining supernatant was taken from the 4 sponge stock solutions, filtered through a 0.2μm filter under gentle vacuum and stored in 1mL of sucrose lysis buffer at -80°C for DNA analysis. Previous studies (Giles *et al* 2012; Friedrich *et al* 1999) have used the slurry method in order liberate microbes that may only occur at certain sections within the sponge and may not be equally distributed throughout the mesohyl.

Ten millimeters was removed from the 1L seawater sample, fixed with 1ml of formalin (10% final concentration) and stored at -80°C until processing for bacterial abundance. The remaining two 1L seawater samples were separately filtered through a 0.2μm filter under gentle vacuum and stored in 1mL of sucrose lysis buffer at -80°C for DNA analysis.

Microbial and Cyanobacterial Abundance

The samples used to determine microbial abundance were thawed, and 5mL of seawater and 1mL of diluted sponge supernatant was filtered onto 0.2μm filters prestained with Irgalan Black (0.2g in 2% acetic acid) under gentle vacuum (~100 mmHg). They were then stained with 0,6-diamidino-2-phenyl dihydrochloride (5μg/ml, DAPI, SIGMA-Aldrich, St. Louis, MO, USA) (Porter and Feig, 1980; Parsons et al 2014). The DAPI slides were enumerated for total microbial abundance using an AX70 epifluorescent microscope (Olympus, Tokyo, Japan) under ultra violet light (330-385 excitation) at 100× magnification. The DAPI slides were then enumerated for cyanobacterial abundance using an AX70 epifluorescent microscope (Olympus, Tokyo, Japan) under narrow green light (545 excitation) at 100× magnification.

DNA Extractions using CTAB (Cetyltrimethylammonium Bromide)

The DNA samples from the three sites (18 in total) were thawed. DNA was extracted using the phenol-chloroform method (Giovannoni *et al* 1990) modified with a CTAB step. Initially, the bacteria cell membrane was lysed with 100μL of 10% sodium dodecyl sulfate (SDS) and 10μL of 20mg/ml Proteinase K (0.2mg/ml) at 37°C for 30 minutes and 55°C for 30 minutes using the Hybridiser oven (HB-1D, Bio-Techne, Minneapolis, MN, USA). Then, 500μL of the sponge and water samples was pipetted into clean 2mL tubes. Then 1μL of mercapthoethanol was added per 1mL of CTAB. Then 150μL of CTAB solution and 150μL of 5M NaCl were added to remove the orange pigmentation found in the sponge samples as well as polysaccharide contaminants, that may affect DNA purity (Murray and Thompson, 1980). Then 800μL of PIC (Phenol Isoamyl Alcohol Chloroform) was added to each tube. The 6 tubes were manually inverted for 5 minutes and were then centrifuged at 9391rcf for 10 minutes to remove larger particulates.

Once the tubes were removed from the centrifuge, the upper aqueous layer was

pipetted out and transferred into new 2mL tubes, respectively. The lower organic layer was discarded. Then 800µL of IC (Isoamyl Alcohol, Chloroform) was added to each of the samples. The tubes were then manually inverted for 5 min and centrifuged at 2348 rcf for 5 minutes. The upper aqueous was pipetted out and transferred into new 2mL tubes again. The lower layer was discarded and the addition of the IC, manual inversion and centrifugation was repeated. The aqueous layers were again isolated from the samples and placed in new 2mL tubes. The DNA was precipitated using 50 µL of 3M NaOAc (Sodium acetate) and 1mL of 100% Isopropanol in a -20°C freezer overnight. The resulting DNA was pelleted by centrifugation at 24041 rcf for 30 minutes. The pellets were washed with 1mL of 70% ethanol, vortexed for 30sec to remove salts and centrifuged again at 24041 rcf for 10 minutes. The supernatant was decanted and the pellet dried at 37°C for 10-20 minutes. Afterwards, the DNA was resuspended in 50µL of Tris EDTA (TE) buffer solution and quantified via the Quant-iT™ High-Sensitivity DNA Assay Kit (Oregon, USA). DNA Purity was also measured using the photospectrometer (Model No. HP8453, Agilent Technologies,Waldbronn, Germany).

DNA Extraction using the MO Bio UltraClean^{TM} Soil DNA Isolation Kit

Sponge and water samples were also extracted using the MO Bio UltraClean^{TM} Soil DNA Isolation Kit (MO BIO Laboratories Inc. CA, USA) with the following modifications. The bead solution tubes and solution 1 were not used. Instead, sodium dodecyl sulfate (SDS) to 1% and proteinase K to 200µg/ml were added to the sample and incubated at 37°C for 30 minutes and then at 55°C for 30 minutes. The IRS solution was added and then the tubes were vortexed for 1 minute. The tubes were inverted for 5 minutes prior to centrifuging. After centrifuging, the supernatant was transferred to a new tube and the manufacturer's protocol followed until the elution step. After solution 4 was added and centrifuged, the samples were then left to sit open for 10 minutes at 25°C. Finally 25µl of solution 5 was added and the samples were left to sit for 10 minutes with the lids closed. The samples were then centrifuged for 30 seconds. This step was repeated twice so that an end volume of 50µl was obtained.

Polymerase Chain Reaction (PCR) and Gel Extraction

The resulting DNA from both extraction methods was diluted down to 10ng/µl of DNA for the DNA amplification using polymerase chain reaction (PCR).

For the PCR, the 18 samples (6 from each location) were amplified using the NEB High fidelity PCR kit following the manufacturer's instructions. Primers 27F-FAM (5'FAM-AGRGTTYGATYMTGGCTCAG) and 519R (GWATTACCGCGGCKGCTG) (SIGMA Biosynthesis, St. Louis, MO, USA) were used to amplify the 16S ribosomal RNAfrom bacterial DNA (Morris et al 2005). The PCR reaction consisted of 25µl of master mix, 2.5µl of primers (10µM conc), 0.5-2µl of genomic DNA and made up to 50µl using sterile water. The positive control tube had 1µl of *Alteromonas* culture while the negative control consisted of 1µl sterile water. The 20 tubes were placed in the thermocycler (Biometra® TPersonal 48,Goettingen, Germany) and was set to run using a 59°C annealing temperature and the following thermocycle: 94°C for 2 minutes, followed

by 30 cycles of 94°C for 30 seconds, 59°C for 30 seconds, and 72°C for 1 minute. The reaction was held at 72°C for 10 minutes.

After DNA amplification, the PCR products (5µl) were visualized on a 1% gel (1g agarose and 100mL of 1xTAE buffer) using 100bp ladder (5µl) stained for 30 minutes in ethidium bromide, washed for 15 minutes in sterile q-water and imaged using the Kodak Image Station 4,000R. The resulting amplicon was expected to be 492 base pairs (bp). PCR products (40µl) at the 500bp mark were gel extracted using a 1% agarose gel (1.5g agarose and 150mL of TBE buffer) and 5µl of 100bp ladder. The gel was stained for 30 minutes in ethidium bromide and then left for 20 in sterile q-water to get rid of excess ethidium bromide. The gel was then placed on a UV illuminator and a razor was then used to manually cut out bands that were located on the 500bp mark. The excised band was purified using the MO Bio Gel extraction kit as per the manufacturer's instructions with the following modifications. Prior to elution the tubes were left open for 10 minutes to evaporate out the ethanol.

Ligation of PCR Product

The MO Bio extracted PCR product (5µl) was visualized using a 1% gel (1g agarose; 100ml TBE buffer) to see the quality of the extracted DNA. The bands were analyzed to see which samples would be ligated. The chosen samples were then ligated into an expression vector using the pGEM-T easy Vector kit (Promega WI, USA). The ligation reaction consisted of 3µl of PCR product, 5µl of 2x rapid ligation buffer, 1µl of pGEM-T easy vector and 1µl of T4 DNA ligase. The solution was mixed by pipetting and incubated overnight at 4°C.

Transformation

2µl of each ligation solutions was pipetted into labeled tubes and incubated on ice. 50µl of JM109 component bacteria cells were added to each of the tubes and mixed gently. The tubes were incubated on ice for 20 minutes, then heat shocked at 42°C for 45 seconds and then placed back on ice for 2 minutes. After the 2 minutes, 950µl of SOC medium were pipetted into the tubes, and placed in the shaking incubator at 37°C for 1.5 hours. During the incubation, the LB ampicillin plates had 100µl of 0.1M Isopropyl β-D-1-thiogalactopyranoside and 25µl of 50mg/ml x-gal added evenly across the plate. The plates were incubated for 30 minutes at 37°C. Afterwards, 25µl of the incubated samples were plated onto the prepared LB ampicillin plates and incubated overnight at 37°C. Blue and white colonies are expected to grow on the plates such that white colonies characterize plasmids with the DNA inserted into multiple cloning sites. The blue colonies would either have no insert or very small inserts that did not cause a disruption on the beta-galactosidase gene.

Clone Culture Growth

Five white colonies and one blue colony acting as the negative control, were obtained using a toothpick and grown up separately in 3mL of LB ampicillin liquid medium. The cultures were incubated overnight at 37°C in the shaking incubator.

Plasmid Extraction

One milliliter of the incubated clone cultures was transferred to a labeled 2 ml centrifuge tube. The tubes were centrifuged at 9391 rcf for 5 minutes at room temperature. The supernatant was discarded. The procedure was repeated for a second milliliter of clone culture. The plasmid DNA was extracted using the Promega Wizard Plus SV Miniprep DNA Purification System protocol with the following modifications. During the clear lysate production protocol bacterial lysate was centrifuged at 21130 rcf for 10 minutes at room temperature. In the centrifugation step the supernatant was re-spun at 30427 rcf for 5 minutes. Lastly, 30µl of nuclease-free water was added onto the spin columns instead of 100µl.

Digestion of Extracted Plasmids

The DNA concentrations of the extracted plasmids were determined using the Quant-iT™ Broad Range DNA Assay Kit (Oregon, USA). Once the concentration was known, 1000 ng of the plasmid was digested using the New England BioLabs *EcoR1* enzyme (MA, USA). The enzyme will cut the insert and the plasmid at the following palindromic sequence sites: 5' G↓AATTC 3' and 3' CTTAA↓G 5'. For every 1000ng of plasmid, 2µl of 10x buffer and 1 ul of enzyme (10 U/ul) was used followed by the addition of QH$_2$O for a total reaction volume of 20µl. The samples were digested at 37°C for 20 hours. The enzyme was deactivated by exposing the samples to 60°C for 20 minutes to avoid excess cutting. Afterwards the digested products (5µl) were visualized on a 1% gel (1g agarose and 100 mL of 1xTAE buffer) using NEB 2log ladder (1µl) and NEB 50 kb ladder (1µl) stained for 40 minutes in ethidium bromide and imaged using the Kodak Image Station 4000R.

The *EcoR*1 digested products (20 µl) at the 500 bp mark were gel extracted using a 1% agarose gel (1g agarose and 100 mL of 1xTBE buffer) and 5µl of NEB 2log ladder. The gel was stained for 40 minutes in ethidium bromide and then left for 20 minutes sterile q-water to get rid of excess ethidium bromide. The gel was then placed on a UV illuminator and a razor was used to manually cut out bands that were located on the 500 bp region and 300 bp region. The excised band was purified using the MiniElute® Gel Extraction Kit, Qiagen, Germantown, Maryland, USA per the manufacturer's instructions with the following modifications. Prior to elution the tubes were left open for 10 minutes to allow for the evaporation of the ethanol.

The 500 bp gel extracts were then digested with NEB *HaeIII* restriction enzyme that cuts in the palindromic sequence 5' GG↓CC 3' and 3' GG↓CC 5'. This was done to remove extra bases and isolate the 336 fragment.

Microbial abundance statistical analysis

The microbial abundances were analyzed using a two-tailed t-test (Microsoft Excel 2011). A p=0.05 was used as the significance level. Standard error is shown in error bars.

Sequence analysis

The sequences were analyzed and trimmed off of the plasmid sequence using Serial Cloner 2.6 (Developer: Serial Basics, 2012). Serial Cloner 2.6 was also used to perform a virtual *HaeIII* digestion on the sequences. The BLASTN 2.2.31+ was optimized for somewhat similar sequences (BLAST) (Zhang et al 2000) and was used to determine the identification of microbes based on sequence analysis. The sequence search in BLAST is shown in Table 2.

Results

Total Microbial and Cyanobacteria Abundance

The microbial abundance of the sponge samples from Ferry Reach, Bailey's Bay and Helena's Bay were compared to those of the water samples. The averages of microbial abundance were plotted on a logarithmic scale (Figure 1). The microbial cell abundance was ~3 orders of magnitude higher in the sponge tissue than that of the surrounding water (Figure 1). Microbial cell abundance averaged 2.37×10^9 cells g^{-1} (+/- 7.70×10^8) in the sponge tissue and 1.21×10^6 cells ml^{-1} (+/-2.74×10^5) in the water. The microbial cell abundance in the sponge tissue from all samples was significantly different from the surrounding seawater samples (p<0.001; sponge n=12; water n=6). The microbial abundance within sponge tissue from all three sites was significantly higher than the microbial abundance found in the surrounding seawater (Reach p-value: 0.019; Bailey's Bay p-value: 0.021; Helena's Bay p-value: 0.035).
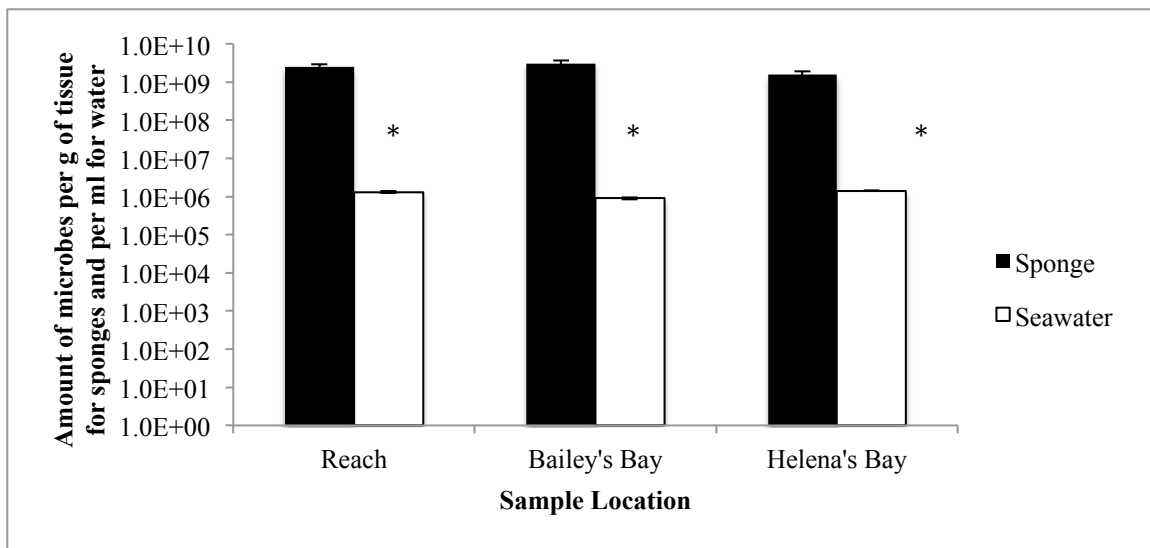

Figure 1. Graph showing microbial abundance for sponge and water samples from

Ferry Reach, Bailey's Bay and Helena's Bay sampled in 2014. The star indicates a significant difference (p=0.05). Both sponge and water microbial abundances are plotted in logarithmic scale so both results could be visualized on the same graph. Standard error is shown in error bars.

In Figure 2, The Cyanobacterial abundance of the sponge samples from Ferry Reach, Bailey's Bay and Helena's Bay were compared to those of the water samples. *Cyanobacteria* include all autoflourescent prokaryotic cells and for this study, the majority of *Cyanobacteria* are *Synechococcus* (Parsons et al 2014). The averages of Cyanobacterial abundance were plotted on a logarithmic scale as depicted in Figure 1. The Cyanobacterial cell abundance was ~1-2 orders of magnitude higher in the sponge tissue than that of the surrounding water (Figure 2). Cyanobacterial cell abundance averaged 3.96×10^6 cells g^{-1} ($+/-5.86 \times 10^6$) in the sponge tissue and 1.42×10^5 cells ml^{-1} ($+/-5.65 \times 10^4$) in the water. However, *Cyanobacteria* only contributed to 0.13% +/- 0.06% of the microbial community in the sponge tissue samples while contributing 11.65% +/- 1.23% to the microbial community in the surrounding water. The Cyanobacterial cell abundance in the Reach sponge tissue was significantly different from the surrounding seawater samples (p=0.0028; sponge n=4; water n=2). There was no significant difference between sponge and water Cyanobacterial abundance in Bailey's Bay samples (p=0.58). *Cyanobacteria* were not found in Helena's Bay sponge samples but were detected in water samples and so its water samples had significantly more *Cyanobacteria* than sponge samples (p<0.001). The Cyanobacterial abundance within sponge tissue from all three sites had varying abundances compared to those in in the surrounding seawater (Reach p-value: 0.0028; Bailey's Bay p-value: 0.58; Helena's Bay p-value: 6.79×10^{-5}).
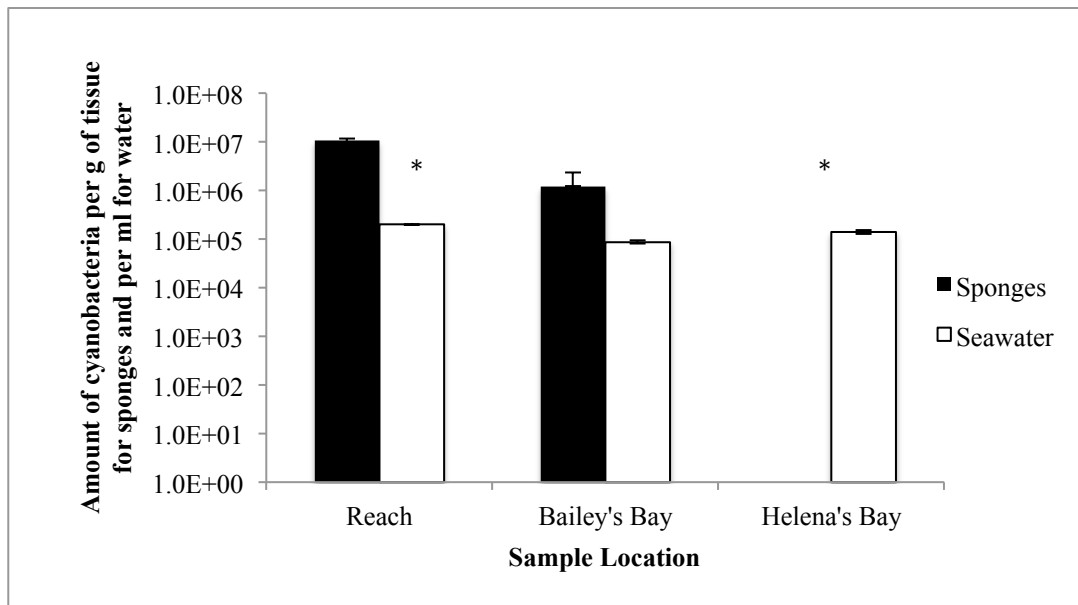

Figure 2. Graph showing Cyanobacterial abundance for sponge and water samples from Ferry Reach, Bailey's Bay and Helena's Bay sampled in 2014. The star indicates a significant difference (p=0.05). Both sponge and water microbial abundances are

plotted in logarithmic scale so both results could be visualized on the same graph. Standard error is shown in error bars. Note: No Cyanobacteria were seen in Helena's Bay sponge samples.

DNA Extraction Comparison

DNA was extracted from the water and sponge samples from the Reach, Bailey's Bay and Helena's Bay, using CTAB and the Mo Bio Kit. The DNA concentrations were measured using a spectrophotometer. The CTAB extracted DNA had a higher average concentration of 15.27 ng μL^{-1} while the Mo Bio Kit had a lower average concentration of 0.178 ng μL^{-1}. The CTAB extracted DNA had a higher average purity of 1.42 while the Mo Bio Kit had a lower average purity of 1.06 (Table 1). Note: a A_{260}/A_{280} ratio of 1.8 is equivalent to 100% DNA in the sample. A ratio below 1.8 means that the sample is contaminated with proteins while a ratio above 1.8 means that the sample has RNA contamination.

Table 1. Table showing the Comparison of DNA Concentrations ($\mu g\ mL^{-1}$) and DNA Purity (A_{260}/A_{280} ratio), extracted using either CTAB vs. MO Bio UltraClean^{TM} Soil DNA Isolation Kit.

Samples	DNA Concentrations (ug/mL)		A_{260}/A_{280} ratio	
	CTAB	Mo Bio	CTAB	Mo Bio
Reach Water 2	3.01	0.34	1.49	1.19
Reach Sponge 1	28.9	0.34	2.06	1.08
Reach Sponge 2	27.6	0.27	2.01	1.13
Reach Sponge 3	25.9	0.38	1.76	1.38
Reach Sponge 4	30.1	0.061	1.96	1.10
Bailey's Bay Water 1	2.26	0.050	1.03	1.03
Bailey's Bay Water 2	2.97	0.050	1.04	0.96
Bailey's Bay Sponge 1	17.6	0.13	1.43	1.01
Bailey's Bay Sponge 2	9.16	0.070	1.29	0.95
Bailey's Bay Sponge 3	8.74	0.20	1.29	1.00
Bailey's Bay Sponge 4	6.83	0.18	1.33	0.99
Helena's Bay Water 1	1.19	0.050	1.02	0.94
Helena's Bay Water 2	5.76	0.050	1.11	0.96
Helena's Bay Sponge 1	20.9	0.150	1.26	1.01
Helena's Bay Sponge 2	9.5	0.16	1.32	1.12
Helena's Bay Sponge 3	23.6	0.48	1.36	1.03
Helena's Bay Sponge 4	35.5	0.073	1.41	1.07
Average	15.27	0.178	1.42	1.06

Polymerase Chain Reaction (PCR) and Gel Extraction

DNA extracted using both methods was amplified using PCR. The DNA extracted using the MO Bio UltraClean^{TM} Soil DNA Isolation Kit amplified more

successfully that the DNA extracted using the CTAB method.

Clone Library Formation

       Cloning of Bailey's Bay and Helena's Bay samples were not done because its samples did not amplify well unlike those of Ferry Reach (data not shown). CTAB extracted DNA from Reach sponge 1 and 2 and from Reach water 2 had 6 plasmids made for each sample (18 total). Mo Bio extracted DNA in the 500bp from Reach Sponge 1, 2, 3, 4 and Reach water 2 had 5 plasmids made each (25 total). The Mo Bio extracted DNA in the 300bp from Reach Sponge 1, 2 and 3 had 4 clones made each and Reach sponge 4 had 5 plasmids made (17 total). A total of 60 clones were made.

Digestion of Extracted Plasmids

       ECO R1 enzyme was used to digest the plasmids and cut the insert and the plasmid at the following sequence sites: 5' G↓AATTC 3' and 3' CTTAA↓G 5'. Bands on the 500bp were digested with NEB *HaeIII* restriction enzyme that cuts in the 5' GG↓CC 3' and 3' GG↓CC 5'. This was done to cut off extra bases and isolate the 336 fragment. This digestion did not work. A second 300bp product was seen. The plasmid extracts of the 500bp and 300bp from the *EcoR1* digest were sent for sequencing at the Beckman Coulter Genomics lab in Massachusetts, USA. 17 clones had 300bp inserts and 43 clones had 500bp inserts. 6 out of the 17 clones and 19 out of the 43 were sent for sequencing
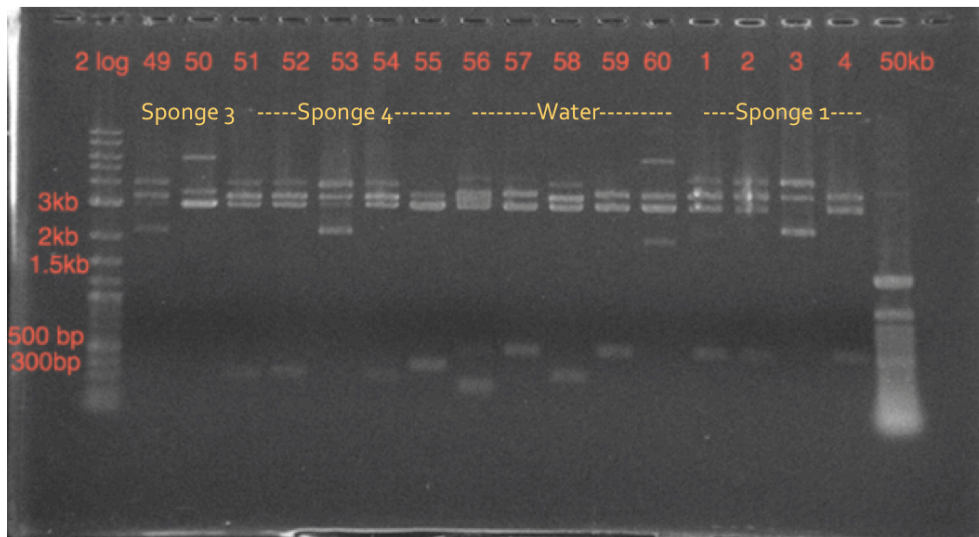

Figure 3. Eletrophoresis Gel images showing the cleaned-up plasmids digested with Eco R1 from the Ferry Reach sponge and water samples used for clone library analysis. A 2 log ladder (NEB) is shown on the left and a 50kb ladder (NEB) is shown on the right. The desired insert was 500bp in length.

Sequence analysis

       Table 2 shows the trimmed T7 sequences from Mo Bio plasmids. The complete 519R primer binding site sequence is double underlined. The 27F primer sequence is

shown in italics. The 27F of the given samples have bases in bold, which indicate its similarity to the original 27F sequence. The underlined sequence is the product of virtual *HaeIII* digestion (done via Serial Cloner). The sample description column shows the 500bp clone insert sequences of Reach sponge 1, 2, 3 and 4 and Reach water 2. It also shows the 300bp clone insert sequence of Reach sponge 4 only (Note: * denotes clone with 300bp gel extracted insert). The BLAST results contain the microbe identification and Gen Bank number. The alignment results are given as percentages.

Table 2. BLAST results of the Mo Bio 500bp and 300bp gel extracted clone inserts

Sample Description	T7 Sequences	*HaeIII* Digested T7 Sequences with sequence length count	BLAST Results and Alignment %
19_S1_1_T7 Reach Sponge 1 Clone 1	*ATGAGACGGAGCTGGCGCAG* TGGCGCGCGGGCTTCAACAGC ACGGTGGTGGTGCGCGGGGTT GACCTGGTGCACGGCATGCCG AATGCGGCGGTGGTGGAGGAG GCGCTGGAGCAGGTGGACACG GTGGTGTATGTGGGCGGCTTCA TGGATGACACGGCGCAGATGG CGGACCTGGTGCTGCCGGAGG CCACGTTCCTGGAGAGCTGGG GCACGGGCGTGCCGGATCCGG GGCCGGGCTATCCGGTGCTGA CTTTCCAGCCGCCGCGGTAATA CA	*ATGAGACGGAGCTGGCGCAG* TGGCGCGCGGGCTTCAA CAGCACGGTGGTGGTGC GCGGGGTTGACCTGGTG CACGGCATGCCGAATGC GGCGGTGGTGGAGGAGG CGCTGGAGCAGGTGGAC ACGGTGGTGTATGTGGG CGGCTTCATGGATGACA CGGCGCAGATGGCGGAC CTGGTGCTGCCGGAGG **189**	*Actinosynnema mirum* DSM 43827, complete genome Sequence ID: gb|CP001630.1| 87%
20_S1_2_T7 Reach Sponge 1 Clone 2	*ATGAGACGGAGCTGGCGCAG* TGGCGCGCGGGCTTCAACAGC ACGGTGTGGTGCGCGGGGTTG ACCTGGTGCACGGCATGCCGA ATGCGGCGGTGGTGGAGGAGG CGCTGGAGCAGGTGGACACGG TGGTGTATGTGGGCGGCTTCAT GGATGACACGGCGCAGATGGC GGACTTGGTGCTGCCGGAGGC CACGTTCCTGGAGAGCTGGGG CACGGGCGTGCCGGATCCAGG GCCGGGCTATCCGGTGCTGACT TTCCAGCAGCCGCGGTAATTCA	*ATGAGACGGAGCTGGCGCAG* TGGCGCGCGGGCTTCAA CAGCACGGTGTGGTGCG CGGGGTTGACCTGGTGC ACGGCATGCCGAATGCG GCGGTGGTGGAGGAGGC GCTGGAGCAGGTGGACA CGGTGGTGTATGTGGGC GGCTTCATGGATGACAC GGCGCAGATGGCGGACT TGGTGCTGCCGGAGG **189**	*Actinosynnema mirum* DSM 43827, complete genome Sequence ID: gb|CP001630.1| 87%

26_S2_3_T7 Reach Sponge 2 Clone 3	*ACT**GTCTGC**TCCT**T**GCCCAA* GAGGCGCGCAACAAGCACACT TAGTCCAATGAACTGCCACGC CGCCGCCGCGCCGCCCAGCAA TACCGTCGCCCACCATAACGG CGACGGCTGCCTGTAGAACAG CATCAGCACCAGCAGGGCAAG CGTGATGACAAGCACTGCTGC CGTGCCCAGAACCTCTCCCAA GGAAGTGCCAGCCTTCATCGC CCCCGCCCCTTGCCGGGGAGC AACTCAATGCCCAAGAGGCGC GTTGCCAGCACCAGCGCGAGG ATGCCGACCATAAGGACGATG CCCCAGATCAGCCAGCCGTATT CGCCTGTCAGCAAGCCGGCAA TCACCATCCAGCCGCCGCGGT AATAC<u>A</u>	*ACT**GTCTGC**TCCT**TGCCCAA* GAGGCGCGCAACAAGCA CACTTAGTCCAATGAAC TGCCACGCCGCCGCCGC GCCGCCCAGCAATACCG TCGCCCACCATAACGGC GACGGCTGCCTGTAGAA CAGCATCAGCACCAGCA GGGCAAGCGTGATGACA AGCACTGCTGCCGTGCC CAGAACCTCTCCCAAGG AAGTGCCAGCCTTCATC GCCCCGCCCCTTGCCG GGGAGCAACTCAATGCC CAAGAGGCGCGTTGCCA GCACCAGCGCGAGGATG CCGACCATAAGGACGAT GCCCCAGATCAGCCAGC CGTATTCGCCTGTCAGCA AGCCGGCAATCACCATC **344**	*Collimonas fungivorans* Ter331, complete genome Sequence ID: gb|CP002745.1| **86%**
28_S2_5_T7 Reach Sponge 2 Clone 5 500bp gel extract	*ACG**CAC**G**CATCATGG**GCGCCC* CGCTTCCGACGCCGGCAACGG AGGCCCAGGAGGAGGCAAACC GTGAGTGGGACCGAATAGTGG CCGCTGAGGAGCCCGCAATGG TGGCGGATGCCGGTGCCGCTT ACGCAACCCGCACTGACTCCG CCGCGCCCGCACTCCAGCGCCT CGCACTCCGCCGACCC<u>CAGCC</u> <u>GCCGCGGTAATTC</u>A	*ACG**CAC**G**CATCATGG**GCGCC C* CGCTTCCGACGCCGGCA ACGGAGGCCCAGGAGGA GGCAAACCGTGAGTGGG ACCGAATAGTGG **86**	*Rhodococcus opacus* strain R7 sequence Sequence ID: gb|CP008947.1| **78%**
30_S3_2_T7 Reach Sponge 3 Clone 2	*ATGAGA**CG**GAG**CTGGCGCAG*** TGGCGCGCGGGCTTCAACAGC ACGGTGGTGGTGCGTGGGGTT GACCTGGTGCATGGCATGCCG AATGCGGCGGTGGTGGAGGAG GCGCTGGAGCAGGTGGACACG GTGGTGTATGTGGGCGGCTTCA TGGATGACACGGCGCAGATGG CGGACCTGGTGCTGCCGGAGG CCACGTTCCTGGAGAGCTGGG GCACGGGCGTGCCGGATCCGG GGCCGGGCTATCCGGTGCTGA CTTTC<u>CAGCAGCCGCGGTAATA</u> <u>C</u>A	*ATGAGA**CG**GAG**CTGGCGCAG*** TGGCGCGCGGGCTTCAA CAGCACGGTGGTGGTGC GTGGGGTTGACCTGGTG CATGGCATGCCGAATGC GGCGGTGGTGGAGGAGG CGCTGGAGCAGGTGGAC ACGGTGGTGTATGTGGG CGGCTTCATGGATGACA CGGCGCAGATGGCGGAC CTGGTGCTGCCGGAGG **189**	*Actinosynnema mirum* DSM 43827, complete genome Sequence ID: gb|CP001630.1| 87%

34_S4_1_T7 Reach Sponge 4 Clone 1	*TGCCC**TGATCA**G**GGCAG**GC* GGATCAGATCCACATCCCAGC GTCCGTTCCGGCTCACCTGCAC CGCCAGCCGCCGCGGTAATTC A	*TGCCC**TGATCA**G**GGCAG**GC* GGATCAGATCCACATCCC AGCGTCCGTTCCGGCTC ACCTGCACCGC **65**	*Synechococcus sp.* CC9605, complete genome Sequence ID: gb|CP000110.1| **100%**
38_S4_5_T7 Reach Sponge 4 Clone 5 500bp gel extract	*CGCGGTA **ATCATTGCT**GTC* GCGATAGCACAGATGCGGAAT GTCTGTGCCGTGCCGTGCCGCT GCCTGCCAGATGGTCTCACCCG GCCCCGCATCGACCTGCCGGC CATCCAGCGTGAAGGTGACTG TCTTGCTGATTGAATCAGGCAT TGTTACTGCCTCCCACATCCTC GGGAAAATGTTTCATTACGCTG ATCAGCGGGTCTGAAGCCGCC TGGCCCAGCCCGCAGATACTT GCATCCGCCATAGCCACTGAC AGCTCACCCAGCAGCCGCGGT AATACA	*CGCGGTA **ATCATTGCT**GTC* GCGATAGCACAGATGCG GAATGTCTGTGCCGTGCC GTGCCGCTGCCTGCCAGA TGGTCTCACCCGG **85**	*Candidatus* *Puniceispirillum* *marinum* IMCC1322, complete genome Sequence ID: gb|CP001751.1| **82%**
37_S4_4_T7 Reach Sponge 4 Clone 4	*ACC**G**AGC**A**T**GGCCAG**GC* GTCCGTTCCAGACCTCGGAACT GTTGTTCCAACCCCATTCCCAT TTTTCCTGGGGGTAGAGCTTCA CTTTGGTGGGCAGTTCAGCCGC CGCGGTAATACA	*ACC**G**AGC**A**T**GGCCAG**GC* GTCCGTTCCAGACCTCGG AACTGTTGTTCCAACCCC ATTCCCATTTTTCCTGGG GGTAGAGCTTCACTTTG GTGGGCAGTT **98**	*Synechococcus sp.* WH8102 complete genome; segment 5/7 Sequence ID: emb|BX569693. 1| **98%**
56_W1_T7 Reach Water 2 Clone 1	*C**GG**AG**TTGAACACGG**AA**ACG*** GTGCTGAATTCTGCCTCCAGCG GCTCGGAAAACACACCACCAC ACGCTCCGGGGCCGCCTCCAG CGCCGCCTCCGGGGCAGGGGC AGAGCGCACTTGGTTGGCATG CGCGTCCGCCGGGGCGCCCGC CGCTGACCACAGCGCCGCCGC CATCGCCGCTATAATAATGAA GGGCAGCGCACGCATCGCCAA TTAGGCGGGAGGGTCGTCACC GCCGCTGTCGCCGTCAGCCGCC	*C**GG**AG**TTGAACACGG**AA**ACG*** GTGCTGAATTCTGCCTCC AGCGGCTCGGAAAACAC ACCACCACACGCTCCGG GG **74**	*Paenibacillus* *graminis* strain DSM 15220, complete genome Sequence ID: gb|CP009287.1| **86%**

	<u>GCGGTAATAC</u>A		
57_W2_T7 Reach Water 2 Clone 2	***ATGAGACG**GAG**CTGGCGCAG*** <u>TGGCGCGCGGGCTTCAACAGC</u> <u>ACGGTGGTGGTGCGCGGGGTT</u> <u>GACCTGGTGCACGGCATGCCG</u> <u>AATGCGGCGGTGGTGGAGGAG</u> <u>GCGCTGGAGCAGGTGGACACG</u> <u>GTGGTGTATGTGGGCGGCTTCA</u> <u>TGGATGACACGGCGCAGATGG</u> <u>CGGACCTGGTGCTGCCGGAGG</u> <u>CCACGTTCCTGGAGAGCTGGG</u> <u>GCACGGGCGTGCCGGATCCGG</u> <u>GGCCGGGCTATCCGGTGCTGA</u> CTTTC<u>CAGCCGCCGCGGTAATA</u> <u>C</u>A	***ATGAGACG**GAG**CTGGCGC** ***AG*** <u>TGGCGCGCGGGCTTCAA</u> <u>CAGCACGGTGGTGGTGC</u> <u>GCGGGGTTGACCTGGTG</u> <u>CACGGCATGCCGAATGC</u> <u>GGCGGTGGTGGAGGAGG</u> <u>CGCTGGAGCAGGTGGAC</u> <u>ACGGTGGTGTATGTGGG</u> <u>CGGCTTCATGGATGACA</u> <u>CGGCGCAGATGGCGGAC</u> <u>CTGGTGCTGCCGGAGG</u> **189**	*Actinosynnema mirum* DSM 43827, complete genome Sequence ID: gb|CP001630.1| **87%**
54_S4_4_T7 Reach Sponge 4 Clone 4 *	***TGG**CT**CCCACAT**CAA**TGCT**C* <u>CAAGTGGTGTTGGTAATCGTTG</u> <u>CCCCCTTCGCGATCACCCGCAT</u> <u>CGCCTCAGCGGTGGCGAACTG</u> <u>TTCGGACGGCCCTTGGGCTGTG</u> <u>GCCCGCTTGCCACCGGCAAGA</u> <u>GCCATGGATCCCAGAAGGAGG</u> <u>CTCGCCATCAACAGCGCCGCG</u> <u>TTTCGCATTGTTAGGGCCCGCG</u> <u>ATGGGGTTGCTTCTCCTTTCGG</u> <u>TTTACACAGAACGCTTCCAGGT</u> <u>GACAGCCGTCCTGTGCTCAA</u><u>C</u> <u>AGCCGCCGCGGTAATAC</u>A	***TGG**CT**CCCACAT**CAA**TGCT***C <u>CAAGTGGTGTTGGTAATC</u> <u>GTTGCCCCCTTCGCGATC</u> <u>ACCCGCATCGCCTCAGC</u> <u>GGTGGCGAACTGTTCGG</u> <u>ACGG</u> **97**	*Synechococcus sp.* WH 8109, complete genome Sequence ID: gb|CP006882.1| **91%**

Discussion

High Microbial Abundance Sponges with Cyanobacteria

The microbial abundance within sponge tissue was about three orders of magnitude higher than the microbial abundance of the surrounding seawater. Thus, *Tedania ignis* can be considered as an HMA sponge. This is consistent with previous findings (Jouett, 2012) but in contrast to another study (Gloeckner *et al* 2014), which categorized *T. ignis* as an LMA sponge. This study is from a different location from that

of Gloeckner *et al* (2014). In that study, *T. ignis* samples were collected from reefs in Florida (3-5 samples per site). The fire sponges in that location may have lower abundances than those in Bermuda due to other factors that may be affecting *T. ignis'* microbial abundance in that location. Furthermore, Gloeckner *et al* (2014) used TEM microscopy for all the different sponge species (56 total) and did a separate fluorescent microscopy with DAPI staining for certain sponge species samples (15 total). *T. ignis* was not included in the latter analysis. This study enumerated 12 sponge replicates and seawater samples using DAPI staining and compared those. Cell counts for the sponge samples were significantly higher than those of water and exhibiting an HMA range of 10^8-10^{10} cells/g. According to Giles *et al* (2012) bacteria can be missed in TEM surveys of sponges because bacteria may only occur at certain sections within the sponge and may not be equally distributed throughout the mesohyl. And so Giles. *et al* used the slurry method to liberate the microbes that are in certain portions within the sponge mesohyl. Similarly, this study used the slurry method to look at all the associated microbes in sponge tissue.

Nevertheless, Weisz *et al* (2008), found that HMA sponges had denser mesohyls and lower pumping rates compared to LMA sponges. According to Schläppy *et al* (2010), the sponge's microbial abundance and anatomy is attributed to its nutritional strategy such that those with reduced aquiferous systems host more microbes thereby having denser mesohyls. The sponges are believed to be utilizing the microbes as a food source, which is a process called "microbial farming". This same process may be present in the *T. ignis* due to the observed microbial abundance. However, this study did not investigate the mesohyl density and aquiferous system of the *T. ignis*. Therefore, "microbial farming", while plausible, cannot be definitely confirmed in *T. ignis*.

The *Cyanobacteria* abundance between sponge and water samples was not consistent among the 3 sites. Sponge samples had significantly more *Cyanobacteria* than water samples from Ferry Reach (p=0.0028). In Bailey's Bay samples, sponges have more *Cyanobacteria* than the water but were not statistically significant (p=0.58). *Cyanobacteria* was only significantly abundant in water samples than sponge samples from Helena's Bay, which is due to not identifying any *Cyanobacteria* as autoflourescent cells using epiflourescent microscopy in any of the Helena's sponge samples. With Helena's Bay having almost half the number of associated bacteria in the sponge tissue when compared to Bailey's Bay, it is possible that there could still be *Cyanobacteria* in Helena's Bay sponges but it was just not observed in the sample loading used for epiflourescent microscopy.

Nevertheless, *Cyanobacteria* are present in most of the sponge samples (n=5) and in all of the water samples (n=6). According to Hentschel et al (2006), they are probably the most abundant photosynthetic organisms on Earth that are responsible for sponge-host coloration and changes in phycobiliprotein ratios in sponges. Additionally, in the *Cyanobacteria* phylum the most abundant clades are *Synecoccocus sp.* and *Perchlorococcus* (Hentschel *et al* 2006). *Cyanobacteria* are typically found in the outer surfaces of sponges in order to efficiently absorb light but they can also be found in the inner core of the sponge (Hentschel *et al* 2006). Furthermore, *Cyanobacteria* are the only photosynthetic prokaryotes able to perform oxygenic photosynthesis (Nelson, Pers.

Comm.), which suggest that *T. ignis* must be in areas with sufficient O_2 to be able to support the *Cyanobacteria*.

DNA Extraction Comparison

The CTAB method was able to both extract DNA with higher yields and purity than the Mo Bio kit. Nevertheless, CTAB extracted DNA did not amplify well during the PCR when compared to DNA extracted using the MO Bio Kit. Both methods used SDS and Proteinase K, known PCR inhibitors (Rossen *et al*, 1992), to disrupt the cell membrane during the lysis step. However, the Mo Bio UltraClean^{TM} Soil DNA Isolation Kit had an inhibitor removal solution (IRS) that helped with the PCR amplification. Furthermore, the CTAB method required the use of phenol, which is used for protein removal and is also a PCR inhibitor (Rossen *et al*, 1992). Based on this, the chemicals required for CTAB such as phenol allowed for purer DNA but had inhibited the proper amplification of the DNA because it lacked an IRS, which was present in the Mo Bio Kit.

Simonelli *et al* (2009) had investigated the effectiveness of DNA extraction of algal cultures using commercially available kits and two methods based on hexadecyl-trimethyl-ammonium bromide (CTAB). When these methods analyzed algae inside copepods the Mo Bio Kit's vigorous lysis process was suggested to have caused DNA shearing thus reducing the amount of purified recoverable DNA. This would then explain the lower DNA yields and A_{260}/A_{280} ratios that were observed with the Mo Bio extracted sponge and water DNA. However, Mo Bio was capable of isolating PCR amplifiable DNA for both free-living and non free-living algae but did so more efficiently with the free-living algae. CTAB-based protocols gave little or no amplicons when free-living algae were analyzed. The CTAB-based protocols were not used for analyzing algae in copepods. This study suggests that commercially available kits may be better in extracting DNA from algal cultures than CTAB-based methods. This observation is similar to this study's finding although the Mo Bio Kit's effectiveness may be attributed to the chemicals used in the IRS.

In another study, Taylor *et al* (2004) investigated the host specificity of marine sponge-associated bacteria and had utilized a CTAB method for DNA extraction. Taylor et al successfully amplified the bacterial DNA extracts from the following sponge species: *Cymbastela concentrica*, *Callyspongia* sp. and *Stylinos* sp. However, the extraction method Taylor et al had used differed from this study's method. The overnight precipitation step in the 2004 Taylor study used an incubation temperature of 4°C while this study had used -20°C. Furthermore, phenol was not used but the chloroform and isoamyl alcohol was used. In light of this, Chen *et al* (2010) found that for all the extraction methods used (CTAB SDS, DNAzolH, PuregeneH and DNeasyH) to isolate DNA from *Diabrotica virgifera virgifera* samples, overnight precipitation at 4°C increased DNA yield when compared to -80°C or -20°C.

Additionally, the use of phenol in this study could have been a factor in reducing PCR amplification since phenol is a PCR inhibitor (Rossen *et al*, 1992). However, most

studies that have used the phenol-chloroform method to investigate bacteria in marine sponges have had PCR amplifiable DNA extracts (Zuppa *et al*, 2014; Simister *et al*, 2011; de Paula *et al*, 2012; Hajdu *et al*, 2013). Thus, the poor amplification of the CTAB extracted DNA could be due to traces of phenol left during extraction or it could be attributed to increased inhibitors within the sponge matrix that are more effectively removed by the IRS solution in the MO Bio Kit than the phenol in the CTAB method.

Clone Sequences and Upstream PCR Errors

The *EcoR1* separated the plasmids from the inserts (Figure 3). An insert of 300bp PCR product was seen suggesting that there was DNA shearing or contamination from a second smaller amplicon during the cloning process. In order to determine whether the 336 fragment was present in any of the cloned inserts, a *HaeIII* digest was done on the 500bp bands. This digestion was not sucessful. The plasmid extracts of the 500bp and 300bp from the *EcoR1* digest were sent for sequencing at the Beckman Coulter Genomics lab in Massachusetts, USA.

In the sequence analysis the 519R parallel and antiparallel sequence, aligned for each of the sample sequences (Table 2). However, only a partial 27F sequence was seen for each of the sequences (Table 2). This could have been caused by the *E. coli*'s (JM109 bacterial cells) response to unstable inserts created during the transformation (Promega, n.d). According to Promega (https://www.promega.com/~/media/files/resources/ product%20guides/subcloning%20notebook/screening_recombinants_row.pdf?la=en), the insert may have been a substrate for recombination by recombinases thus resulting in *E. coli* strains being deficient in multiple recombinases apart from *recA*. This deficiency creates unstable inserts in the *E. coli* cells. However, other studies that have used the JM109 for cloning sponge microbes have had successful clone sequence yields with no reports of recombinase deficiency in transformed JM109 cells (Hardoim *et al* 2009; Dealtry *et al* 2014). Recombinase deficiency may be a possibility in this study but it is more likely that the 27F has been sheared off during the cloning steps or, during the clone formation parts of the vector may have been extended rather than the actual insert (Parsons, Pers. Comm.).

Table 2 also shows the BLAST results of the Mo Bio clone sequences only. The sequences of sponge 4 (clone 1 and 4) and sponge 4 (clone 4*) were most similar to those of *Synechococcus sp.* These clones had percent alignments to *Synechococcus sp* of 100%, 98% and 91% respectively. The rest of the sequences in Table 2 had percent alignments in the 80's. All clones with 300bp inserts gave inconclusive results when blasted due to low to no percent alignment except for sponge 4 clone 4. Low to no percent alignment was also observed in all CTAB clones and some Mo Bio clones (data not shown).

In light of this sequence cloning may have been affected by upstream PCR errors of the Mo Bio and CTAB extracted DNA. Vargas *et al* (2012) demonstrated that sponge DNA extracts are complex mixtures of sponges' holobiont meta-genome. Thus, co-amplification of non-target microbes is likely to occur and is difficult to resolve because impurities within sponge tissues are difficult to completely isolate from target specimens. Vargas et al also noted that the existence of secondary metabolites in sponge species may

cause PCR inhibition. *T. ignis* is known to have secondary metabolites (Muricy *et al* 1993) such as tedanol (Costantino *et al* 2009) and tedanolid (Schmitz *et al* 1984), which may have caused the lack of amplification of sponge DNA from this study.

In addition, Acinas *et al* (2005) compared PCR-induced artifacts and bias between two 16S rRNA clone libraries from the same sample of bacterioplankton. The 2005 study discovered that these artifacts are categorized by those resulting in unequal PCR amplification or cloning efficiency and those that result in the formation of PCR errors or sequence artifacts. Sequence artifacts arise through the formation of chimerical molecules (Brakenhoff *et al* 1991; Hugenholtz & Huber 2003; Komatsoulis & Waterman 1997; Kopczynski *et al* 1994), heteroduplex molecules (Qui *et al* 2001; Speksnijder *et al* 2001; Thompson *et al* 2002) and *Taq* DNA polymerase errors (Qui *et al* 2001). Also, PCR bias occurs due to differences in template amplification efficiency (Acinas *et al* 2005). These PCR-induced artifacts could have been present when Mo Bio extracts were amplified and cloned.

In Figure 3, there were bands than ran past the 300bp and those could potentially be heteroduplex molecules of smaller fragments. According to Qui *et al* (2001), the presence of heteroduplex molecules lead to the overestimation of the microbial community diversity of *Proteobacteria.* Qui *et al* recommended the detection and elimination of these heteroduplex molecules by PAGE and polyacrylamide gel purification or T7 endonuclease I digestion, respectively, before cloning. These steps were not done in this study therefore detection and elimination of heteroduplex molecules may have altered downstream cloning results. Furthermore, *Taq* DNA polymerase errors occurred more frequently with the lack of PCR reagents, specifically dNTPs. Qui et al observed that when Mg^{2+} concentrations were more than those of dNTP's, *Taq* DNA polymerase fidelity decreased. We had used an NEB High Fidelity PCR Kit whose mastermix had already been prepared with set ratios of Mg^{2+} and dNTPs concentrations. Other studies (Aird *et al* 2011; Lamble *et al* 2013) that have used this master mix for PCR of environmental samples, however, have had successful clone libraries made. This suggests that PCR error in this study could be due to increased PCR cycles and DNA template concentrations and decreased elongation times, which were all positively correlated with PCR artifact increase. The PCR for this study was set to run for 30 cycles and those of Qui *et al* were run at less than 20 cycles. However, the suitable number of cycles should be determined experimentally because the former depends on template amount, amplification efficiency and inhibitory substance presence and degree. (Qui *et al* 2001).

Additionally, the vigorous lysis process of the Mo Bio kit might cause DNA shearing (Simonelli *et al* 2009). This explains the lower DNA yields and A_{260}/A_{280} ratios that were observed with the Mo Bio extracted sponge and water DNA. Sheared DNA samples would have been improperly amplified or not amplified at all thus contributing to PCR errors.

The Microbial community and Restriction Fragment 336

Table 2 showed that *Synechococcus, Actinosynnema mirum, Collimonas*

fungivorans, Rhodococcus opacus and *Candidatus Puniceispirillum marinum,* were all present in *T. ignis*. In this study, *Synechococcus* had the restriction fragments of 65, 98 and 97, which was seen in sponge 4 (clone 1 and 4) and sponge 4 (clone 4*). DNA shearing during sequence cloning may have caused the variation in fragment lengths. Nevertheless, these clones had percent alignments to *Synechococcus sp.* of 100%, 98% and 91% respectively, which confirm the presence of *Synechococcus*. The *Synechococcus* clade belongs to the *Cyanobacteria* phylum and is the most dominant clade in the world's oceans (Hentshel *et al* 2006). *Synechococcus* has also been confirmed in 26 Demospongiae families (Hentshel *et al* 2006). In light of this, the *Cyanobacteria* counts in Figure 1 may have been mostly comprised of *Synechococcus*. Furthermore, Steindler *et al* (2005) demonstrated the existence of a monophyletic *Synechococcus sporangium* clade in 18 marine sponge species from the Caribbean, Zanzibar, Red Sea and Mediterranean, which was phylogenetically distinct from free-living *Synechococcus sp.* Usher *et al* (2001) suggested that the free-living *Synechococcus sp.* were vertically transferred into and selectively enriched by an appropriate marine sponge (Ferris & Palenik, 1998). And so free-living and symbiont *Synechococcus sp.* end up diverging and becoming different because of the latter's sponge-specific interactions. Giles *et al* (2012) suggested that horizontal transmission occurs mainly in HMA sponges, which would mean that *T. ignis* could be using horizontal transmission of bacteria. Further studies would be needed to confirm horizontal transmission in *T. ignis* to be certain.

In this study, *Actinosynnema mirum* was shown to posses a 189 restriction fragment, which was consistently seen in sponge 1 (clone 1 and 2), sponge 3 (clone 2) and water 2 (clone 2) samples. There was an 87% alignment to *Actinosynnema mirum* for all 4 clones. The low percent alignment to *A. mirum* could indicate a new strain of *Actinosynnema* or could be due to the partial 27F sequence recovery due to PCR errors. Nevertheless, *A. mirum* belongs to the *Actinobacteria* phylum and the *Actinomycetaceae* family (Abdelmohsen *et al* 2014). It can also be found terrestrial habitats, seawater, marine snow and marine sediments and have been cultivated from marine sponges (Abdelmohsen *et al* 2014). Actinomycetes have been previously isolated in marine sponges (Zhang *et al* 2006; Xi *et al* 2012) and are known for producing secondary metabolites with pharmacological and medical applications (Abdelmohsen *et al* 2014). Secondary metabolites produced by Actinomycetes include polyketides, alkaloids, peptides, and terpenes (Solanki *et al* 2008; Subramani *et al* 2012). Costantino *et al* (2009) discovered a new brominated and sulfated diterpene alcohol called tedanol that demonstrated a inflammatory activity in the *T. ignis*. Costantino *et al* (2009) suggests *Micrococcus sp.* to be the bacteria producing the tedanol because the latter have been shown to produce diketopiperazines and benzothiazoles in *T. ignis*. However, *Micrococcus sp.* was not found in this study and the *T. ignis* samples analyzed by Costantino et al were from the Bahamas. It is possible that there could be varying microbial communities among *T. ignis* that are situated in different locations. Nonetheless, it is possible that *A. mirum* could be playing a role in the production of this compound.

Collimonas fungivorans was shown to posses a 344-restriction fragment, which was seen in sponge 2 (clone 3). There was an 86% alignment of the fragment to *C. fungivorans*. Low percent alignment could also be indicative of a new strain of

Collimonas or could be due to the partial 27F sequence recovery. *C. fungivorans* is an aerobic β-proteobacteria found in the soil that can grow on living fungal hyphae (Boer *et al* 2004). *Collimonas* that were detected in marine surface water were cable of producing violacein, which is a blue-black indole pigment (Hakvåg *et al* 2009). There is no previous literature that has found *Collimonas* in marine sponges. Nonetheless, its presence in the Reach sponge suggest that runoff of water from land could have brought this bacteria into the water allowing for *T. ignis* to filter it in its aquiferous system.

Rhodococcus opacus possesses an 86-restriction fragment (78% alignment) that is found in sponge 2 (clone 5). According to Holder *et al* (2011), *R. opacus* is an *Actinomycete* that are found in soils. *R. opacus* can synthesize straight-chain odd-carbon fatty acids in high abundance that are stores as energy-rich tryglycerols that allow for the production of biofuel (Holder *et al* 2011). However, Abdelmohsen *et al* (2014) isolated *R. opacus* from marine sponges from the Red Sea and were found to have antifungal properties against *Fusarium* sp. In light of this, *Rhodococcus sp.* found in marine environments could potentially be different from those found in soil since they act as a secondary metabolite producers in sponge-hosts and as a biofuel producer in the soil. Whether or not there is a divergence within this genus, its presence in *T. ignis* shows that it can potentially be producing secondary metabolites for the fire sponge. However, Salter *et al* (2014) found that *Rhodococcus* was a contaminant in DNA isolation kits such as FastDNASpin Kit, Mo Bio UltraClean™ Soil DNA Isolation Kit, Qiagen QIAmp DNA Stool Mini Kit and PSP Spin Stool DNA Plus Kit. Based on this, it is possible that the R. opacus found in this study's sponge samples are contaminants from the Mo Bio kit used for DNA extractions.

Candidatus Puniceispirillum marinum possesses an 85-restriction fragment (82% alignment) that is found in sponge 4 (clone 5). It is an alphaproteobacteria and is the first cultured representative of the SAR116 clade (Oh *et al* 2010). According to Oh et al (2010), this bacteria was found on the ocean surface in the East Sea of Korea and are metabolic generalists that can utilize sunlight CO, C1 compounds and dimethylsulfo-niopropionate. There is no previous literature that has found *Candidatus Puniceispirillum marinum* in marine sponges. It is possible that the 85-restriction fragment belongs to a relative of *Candidatus Puniceispirillum marinum* within the SAR116 clade. Previous studies (Treusch *et al* 2009) found that SAR116 from the Sargasso Sea had a 192-restriction fragment length.

Paenibacillus graminis possesses a 74-restriction fragment (86% alignment) that was from Reach water 2 (clone 1). It is a nitrogen-fixing bacterium that belongs to the genus *Paenibacillus*, which is typically found in soil sediments (Berge *et al* 2002). However, Choi *et al* (2008) was able to isolate another strain of *Paenibacillus* from bottom-ocean sediments in Korea. The *Paenibacillus donghaenisis* was proposed as novel specie that was capable of nitrogen fixation (Choi *et al* 2008). This novel species was a rod-shaped, motile, gram-positive, facultative anaerobe capable of xylan degradation with a G+C content of 53.1 mol% (Choi *et al* 2008). Because *Paenibacillus* are typically in the soil, the 74-restriction fragment might actually be more related to *P. donghaenisis* than. *P. graminis*. However, *P. donghaenisis,* whose sequence has been put in GenBank (No. EF079062), did not come as a search result when the 74-restriction

fragment was blasted. There is no previous literature that has found *P. graminis* in marine sponges. It is possible that the 74-restriction fragment belongs to a relative of *P. donghaenisis* and *P. graminis* within the JH8 strain of *Paenibacillus* that has just not been found yet.

Based on the restriction fragment lengths only one bacteria came close having a 336-restriction fragment length and that was *Collimonas fungivorans*. It had a 344-restriction fragment length after the virtual *HaeIII* digestion. Nevertheless, because of the partial 27F sequence recovery and excess bases in its sequence this study cannot confidently conclude that *C. fungivorans* to possess fragment 336.

Conclusion

T. ignis is an HMA sponge, which is consistent with Jouett's (2012) findings but contradictory to previous literature (Gloeckner *et al* 2014). The discrepancies in sponge collection location and total cell quantification methods may have led to the differences in microbial abundances observed. The DNA extracted from both sponge and water samples yielded higher concentrations and purity using the CTAB method when compared to the Mo Bio UltraClean™ Soil DNA Isolation Kit. However, the CTAB method resulted in genomic DNA that was contaminated with PCR inhibitors and thus could not be amplified by PCR while Mo Bio UltraClean™ Soil DNA Isolation Kit yielded DNA that could be amplified by PCR. The amplification success of Mo Bio Kit is attributed to its inhibitor removal solution, which is absent in the CTAB method. Residual phenol during the CTAB method may have led to the PCR inhibition. Sequence analysis of cloned PCR products generated from the genomic DNA showed inconclusive data when the forward primer sequences did not align well with the sample sequences. The absence of 27F from some of the sequences may be attributed to its being sheared off and vector extension instead of the insert. Also sequence cloning may have been affected by upstream PCR procedures of the Mo Bio and CTAB extracted DNA. This may be attributed to PCR errors through increased PCR cycles and DNA template concentrations and decreased elongation times, which were all positively correlated with PCR artifact increase. Additionally, DNA shearing through the vigorous lysis method of Mo Bio may have contributed to PCR errors.

T. ignis was determined to harbor *Synechococcus sp., Actinosynnema mirum, Collimonas fungivorans, Rhodococcus opacus* and *Candidatus Puniceispirillum marinum* based on the BLAST sequence alignment. Water samples contained *Actinosynnema mirum,* and *Paenibacillus graminis* based on the BLAST sequence alignment. There was no 336-restriction fragment found except for 344-restriction fragment, which belonged to *C. fungivorans*, a soil-dwelling bacterium. Because of the partial 27F sequence recovery and excess bases in its sequence this study cannot confidently conclude that the *C. fungivorans* genome to possesses the fragment 336. However, the fragments 74 and 189 were identified using clone libraries and BLAST sequence alignment as *Paenibacillus graminis* and *Actinosynnema mirum,* respectively.

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