Characterization of Bacterial Diversity in Cold-Water Anthothelidae Corals

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Characterization of Bacterial Diversity in Cold-Water Anthothelidae Corals

by

Stephanie Nichole Lawler

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
College of Marine Science
University of South Florida

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DEDICATION

This thesis is dedicated to my parents, Brian and Lorri Lawler, and husband, Jonathon Ellington, for the endless love and support they have shown throughout my graduate career. Thank you for enduring the long hours, success, and failures. It was your loving words that gave me the foundation to believe in myself. As this adventure wraps up, I look forward to where the future will take us.
ACKNOWLEDGMENTS

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ABSTRACT

Cold-water corals, similar to tropical corals, contain a diverse and complex microbial landscape. Comprised of vital microscopic organisms (i.e. bacteria, viruses, archaea), the coral microbiome is a driving factor in the proliferation and survival of the coral host. Bacteria provide essential biological functions within coral holobionts, facilitating increased nutrient utilization and production of antimicrobial compounds. To date, few cold-water octocoral species have been analyzed to explore the diversity and abundance of their microbial associates. For this study, 23 samples of the family Anthothelidae were collected from Norfolk (n = 12) and Baltimore Canyons (n = 11) from the western Atlantic in August 2012 and May 2013. Genetic testing found that these samples comprised two Anthothela species (Anthothela grandiflora and Anthothela sp.) and a new genus. DNA was extracted and sequenced with primers targeting the V4-V5 variable region of the 16S rRNA gene using 454 pyrosequencing with GS FLX Titanium chemistry.

Results demonstrated that the host genus was the primary driver of bacterial composition. The new coral genus, dominated by Alteromonadales and Pirellulales, had much higher species richness and a distinct bacterial community compared to Anthothela samples. Anthothela species had very similar bacterial communities, dominated by Oceanospirillales and Spirochaetes. Core bacterial diversity present across 90% of the Anthothela samples revealed genus-level conservation. This core included unclassified Oceanospirillales, Kiloniellales, Campylobacterales, and Spirochaeta; the functional abilities of which contribute to a nearly
complete nitrogen cycle. Dominant bacterial members of the new coral genus also had functional capabilities in nitrogen cycling. Overall, many of the bacterial associates identified in this study have the potential to contribute to the acquisition and cycling of nutrients within the coral holobiont.
CHAPTER ONE:

Introduction

1.1 Coral Ecosystems

Coral reefs (both tropical and cold-water) are complex and highly productive ecosystems. Housing a myriad of species, these valuable environments are often referred to as “oases” in the oceans (Knowlton et al., 2010; Lumsden et al., 2007). Coral reefs are recognized for their immense biological wealth, which encompasses both socioeconomic and environmental services essential to societies throughout the globe. Each year an estimated $30 billion in net benefits is generated directly from the roles that coral reefs play in tourism, fisheries, and coastal protection (Cesar et al., 2003). Coral reef structures also offer invaluable contributions to the commercial fishing industry, employing millions of fisherman as well as providing livelihoods for many coastal communities (Bryant et al., 1998). Additionally these ecosystems provide habitats for a diverse consortium of organisms ranging from microbes to invertebrate and vertebrate species (Nagelkerken et al., 2000; Reed et al., 2006; Roberts & Hirshfield, 2004), making them one of the leading locations for research and discovery of natural products.

As the main architects of reef habitats, corals (classified under the Class Anthozoa) and their structures are responsible for much of the diversity present (Appeltans et al., 2012). Coral are divided into three subclasses; stony corals (Subclass Hexacorallia, Order Scleractinia), black corals (Subclass Hexacorallia, Order Antipatharia), and soft corals (Subclass Octocorallia; Orders Alcyonacea, Helioporidae, and Pennatulacea) (Berntson et al., 2001). While some species
are globally distributed, corals are generally associated with specific niches, primarily driven by temperature requirements (e.g. warm-water (tropical) corals, temperate, and cold-water corals). In total there are approximately 800 tropical coral species (Roberts et al., 2002) and over 3,000 cold-water coral species (Roberts et al., 2006) known, with many still undescribed.

Tropical reefs are primary biodiversity hotspots, often referred to as the “rainforests of the ocean” (Knowlton & Jackson, 2008). These corals require an intricate balance of ambient environmental factors such as water quality, salinity (32–35 ppt), temperature (25–29°C), tidal variation, and intermittent light intensities in order to ensure health and proliferation (Veron, 2011). Known for their hermatypic (reef-building) capabilities, most tropical corals obtain 50–95% of their nutrients through the translocation of carbon compounds from photosynthetic zooxanthellae (symbiotic dinoflagellate belonging to the genus *Symbiodinium*) (Muscatine & Cernichiari, 1969). Tropical corals are typically found in the euphotic zone, from 0–50 m and within a latitudinal range of 30˚N and 30˚S (Hutson, 1985). However, in areas with very clear waters allowing light penetration, some of these same coral species can be found as deep as 130 m (Lesser et al., 2009). Because the photic zone may extend from the ocean’s surface to depths of 150–200 m in some regions, these ‘twilight’ or mesophotic reef communities consist of a variety of photosynthetic corals as well as azooxanthellate (lacking the symbiotic dinoflagellate) coral species (Kahng et al., 2010).

Unlike tropical or mesophotic species, cold-water corals thrive in aphotic environments, present at depths ranging from 50–4,000 m with preferred temperatures between 4°C–12°C (Roberts et al., 2006). While the majority of these species are present at greater than 50 m (Cairns, 1979), some may occur as shallow as SCUBA-diving depths in the higher latitudes (Roberts et al., 2006). For this reason these corals are referred to as cold-water corals (rather than
deep-sea corals) as their distribution is regulated by temperature and not depth. In comparison to tropical corals, cold-water corals are globally distributed, with populations generally inhabiting continental shelves and seamounts (Roberts & Hirshfield, 2004). Various species of cold-water corals have been described off all coasts of the United States (Lumsden et al., 2007) and from the Caribbean (Cairns, 1979) to the Ross Sea in Antarctica (Stanley & Cairns, 1988). Some of the best studied areas are in the North Atlantic Ocean including the coasts of Norway (Hovland et al., 2002), Canada (Risk et al., 2002), west of the UK (Roberts et al., 2005), and the Azores Islands (Hall-Spencer et al., 2007b).

Similar to tropical corals, cold-water species require a hard substrate for settlement. Structural formations may range in size from individual, isolated colonies (stony corals, soft corals) to small patches (black corals, hydrocorals), to large bioherms (stony corals) (Morgan, 2005; Rogers, 1999). Often, these corals will colonize locations with increased water circulation and elevated seafloor topography to enhance exposure to detritus and particulates transported from surface waters (White et al., 2005). Since cold-water coral species lack the symbiotic dinoflagellates (zooxanthellae) associated with tropical and some mesophotic species, they depend on capture feeding and symbiotic relationships with microorganisms for acquisition and processing of nutrients (Duineveld et al., 2004; Roberts et al., 2006).

1.2 Bacterial Communities

Microbial communities can occupy a range of marine habitats including sediments, water columns, and pelagic and benthic invertebrates (e.g. corals, sponges, etc.) (Bourne & Webster, 2013). Within the last decade, tropical corals have gained increased interest as some of the most biodiverse ecosystems within the world’s oceans. The coral holobiont (which consists of the coral animal and its microscopic associates) includes various members of bacteria, archaea,
fungi, viruses, as well as algal associates, such as endolithic algae and zooxanthellae (notably, only present in tropical and some temperate corals) (Rohwer et al., 2002). Prior to the 1980s, bacterial diversity was studied primarily through the use of culture-dependent techniques. In a study by Ducklow and Mitchell (1979), corals mucus was shown to contain higher concentrations of bacteria than ambient seawater. Additional studies have also addressed the functional roles of bacteria in the processing of nutrients (Ritchie & Smith, 1995; Shashar et al., 1994). While bacteria are recognized for their ubiquitous nature throughout the reef systems, it is estimated that between 90–99% of these microscopic organisms present are unattainable through culturing (Glockner et al., 2011). Thus, the breadth of bacterial diversity obtained through culturing severely underestimates the true total bacterial diversity. Nevertheless, culture-based techniques achieved a first glimpse of the unique bacterial consortia associated with coral hosts.

More recently, hundreds of studies have researched the microbial diversity associated with tropical corals through the use of molecular and culture-based techniques. In 2001, the first culture-independent study sequenced 16S rRNA genes from the bacterial community associated with the tropical coral, *Omicella franksi* (Rohwer et al., 2001). This study was the first to address the immense underrepresentation of bacterial populations present in the coral landscape. Within the last decade, studies have continued to evaluate the microbial associates present in various niches of the coral host, including surface mucus, tissue, and calcium carbonate skeletal structures (Bourne & Munn, 2005; Cook et al., 2013; Ducklow & Mitchell, 1979; Koren & Rosenberg, 2006; Lampert et al., 2006; Lee et al., 2012; Littman et al., 2009; Nithyanand & Pandian, 2009; Ritchie, 2006; Rohwer et al., 2002; Sweet et al., 2010; Sweet et al., 2011). While these studies have increased our understanding of the bacterial diversity present, the potential importance of these communities and their influence on the overall health of coral colonies is
still unclear (Cook et al., 2013; Frias-Lopez et al., 2004; Littman et al., 2009; Sunagawa et al., 2009).

To further evaluate the functions provided by these microorganisms, many studies have addressed their metabolic characteristics. One of the primary beneficial roles bacteria play within the coral holobiont is the acquisition and biochemical transformation of organic and inorganic materials into viable nutrients (Lesser et al., 2004; Shashar et al., 1994). This relationship is especially important in cold-water corals which lack the symbiotic dinoflagellates associated with attaining nutrients in tropical coral species. Other common symbiotic or commensal relationships have been observed between the host and bacteria consortia, including the production of antimicrobial compounds that may act as a defensive line protecting the host organism against predation (Nissimov et al., 2009; Ritchie, 2006; Shnit-Orland & Kushmaro, 2009). In contrast, many bacterial groups have been identified as opportunistic or pathogenic, causing potential harm to the host organism (Bourne & Webster, 2013; Frias-Lopez et al., 2002; Pantos et al., 2003). Additionally, studies have observed fluctuations in bacterial communities based on the transitioning of environmental parameters, such as temperature fluctuation, increased light intensity, and/or an overload of nutrients or pollutants (Ben-Haim et al., 2003a; Guppy & Bythell, 2006; Koren & Rosenberg, 2006; Li et al., 2014; Szmant, 2002). As technology advances, the ability to identify and characterize bacterial composition will redefine our understanding of diverse microorganisms and their influence on the coral holobiont.

Historically, research on the microbial landscape of cold-water corals has been limited in comparison to tropical coral species, due in part to cost and difficulty in sample retrieval (Lumsden et al., 2007). Much like tropical corals, cold-water corals contain a rich and diverse microbial community (Kellogg et al., 2009). While cold-water corals have been identified since
the 1800’s, microbial diversity has only been examined within the last decade. In 2006, two studies characterized the bacterial communities associated with cold-water corals, demonstrating the presence of species-specific bacteria that differed from the surrounding environment. Yakimov et al. (2006) did so by assessing microbiota associated with living and dead samples of scleractinian coral *Lophelia pertusa*. Penn et al. (2006) contributed through the examination of microbiota associated with the octocoral species, *Isididae* sp., and unidentified black corals. Since then, similar relationships have been observed in stony corals *L. pertusa* (Galkiewicz et al., 2011; Kellogg, 2008; Kellogg et al., 2009; Neulinger et al., 2008; Schottner et al., 2009) and *Madrepora oculata* (Hansson et al., 2009). While scleractinian cold-water corals, more specifically *L. pertusa*, have received a lot of attention, comparatively less is understood about cold-water octocoral species (Bruck et al., 2007; Gray et al., 2011; Ransome et al., 2014; Santiago-Vazquez et al., 2007). To date much is still unknown regarding the bacterial composition and community dynamics and functional relationships between of cold-water octocorals and their bacterial associates.

1.3 Overview of Thesis

The research discussed in this study aims to broaden our understanding of the bacterial communities associated with cold-water corals within the family Anthothelidae. To date, few studies have assessed the bacterial assemblages associated with cold-water octocorals (Bruck et al., 2007; Gray et al., 2011; Penn et al., 2006). In an effort to expand on the current knowledge of these unique organisms, this study provided a baseline characterization of the bacterial diversity associated with three Anthothelidae corals; *Anthothela grandiflora*, *Anthothela* sp., and a new unidentified genus. We examined the core microbial associates to identify potential roles conserved by these previously uncharacterized coral hosts. The information gained from this
study provides greater insight into the understanding of these cold-water ecosystems.
CHAPTER TWO:

Coral-Associated Bacterial Diversity is Conserved within the Deep-Sea Genus *Anthothela* spp.

Note to Reader

A modified version of this chapter has been submitted and accepted in the *Frontiers in Microbiology* Journal.

2.1 Introduction

Cold-water coral ecosystems are vital biodiversity hotspots within the deep sea. Thriving in temperatures that range from 4°C–12°C, these corals occur at depths between 50–4,000 m (Roberts et al., 2006). Cold-water corals are globally distributed and the majority of colonies inhabit locations with strong currents and elevated topography such as continental slopes and seamounts (Roberts & Hirshfield, 2004; White et al., 2005). These habitats provide maximum access to particulate and planktonic food sources necessary for non-photosynthetic corals (White et al., 2005). Similar to tropical reefs (Bourne & Munn, 2005; Rohwer et al., 2002), cold-water ecosystems provide critical habitat for many organisms, ranging from benthic and planktonic fauna to microbial associates (Roberts et al., 2006). Coral colonies are home to their own diverse and complex microbial landscape (Bourne & Webster, 2013; Galkiewicz et al., 2011; Gray et al., 2011; Hansson et al., 2009; Kellogg et al., 2009; Neulinger et al., 2009; Neulinger et al., 2008; Penn et al., 2006). In an attempt to further understand bacterial function within the coral
microbiome, studies have examined the roles these bacteria may have within the coral host. While some bacteria appear to play commensal or pathogenic roles (Bourne & Webster, 2013; Nissimov et al., 2009; Shnit-Orland & Kushmaro, 2009), many are not static in function, fluctuating with transitioning environmental conditions (e.g. increased microbial pathogenicity upon exposure to elevated thermal stressors (Ben-Haim et al., 2003a; Bruno et al., 2007)).

Research addressing the microbial communities associated with cold-water corals has been limited due to the expense of sampling, which can be directly linked to the difficulty of sample retrieval at depth. While many of these corals have been identified since the 1800’s, the first microbial study of cold-water corals was not published until 2006. This study assessed microbiota associated with dead and living samples of the scleractinian coral _Lophelia pertusa_ (Yakimov et al., 2006). That same year, Penn et al. (2006) evaluated bacterial communities associated with a black coral and several bamboo corals in the Gulf of Alaska. These two studies were the first to describe the microbial communities associated with stony and soft cold-water coral species as well as demonstrate differentiation between these deep-sea coral-associated communities and those of their surrounding environments (sediment and water column). Since then, studies have characterized the microbial diversity of additional cold-water corals; _L. pertusa_ (Galkiewicz et al., 2011; Hansson et al., 2009; Kellogg, 2008; Kellogg et al., 2009; Neulinger et al., 2008; Schottner et al., 2009), _Madrepora oculata_ (Hansson et al., 2009) and octocorals (_Paragorgia arborea, Plumarella surperba_, and _Cryogorgia koolsae_ (Gray et al., 2011)). Continued research is necessary to broaden our understanding of these complex microorganisms, their relationships with the coral host, and the roles they play in the dynamic deep-sea environment.

In an effort to further our understanding of cold-water octocorals and their microbial
associates, this study evaluated three corals from the family Anthothelidae, initially targeting the species *Anthothela grandiflora*. Endemic to the Atlantic Ocean, *A. grandiflora* was first observed off the coast of Nova Scotia in the mid-1800’s (Whiteaves, 1901), but to date no microbial analysis has been completed. For this study, samples from 23 individual colonies of gorgonian corals visually identified as *Anthothela* were collected from Baltimore and Norfolk canyons off the east coast of the United States in the Mid-Atlantic Bight. The objective of this study was to provide the first characterization of the bacterial diversity associated with the cold-water octocoral genus *Anthothela*.

### 2.2 Materials and Methods

#### 2.2.1 Sample Sites and Collections

In total, 23 individual Anthothelidae colonies were sampled during two research cruises conducted in August 2012 and May 2013. Site locations in the Mid-Atlantic Bight included Baltimore Canyon, which was sampled using the *Kraken II* remotely-operated vehicle (ROV) (University of Connecticut) in 2012 and Norfolk Canyon, sampled using the *Jason II* ROV (Woods Hole Oceanographic Institution) in 2013 (Figure 2.1). Environmental parameters were recorded for each sample site including location (latitude and longitude), depth, temperature, and salinity (Table 2.1). Depths of sample collection ranged from 401–704 m. Bottom types varied from rocky seafloor to rock cliffs and ledges. Common benthic fauna present at sample sites associated with coral colonies included adult galatheid squat lobsters (*Eumunida picta*) and cutthroat eels (*Synaphobranchidae*). Several scleractinian and octocorals were present in the vicinity of Anthothelidae colonies, including *Desmophylum, Paragorgia* and *Primnoa* species.

Because some species of Anthothelidae can grow over other organisms, including dead coral branches and sponges, great care was taken to select sections of polyps from the tips of
branches rather than the main stalk (Lumsden et al., 2007). This technique was employed to avoid accidental contamination of the sampled coral microbiome with that of the supporting organism. Branches were removed using the ROV’s manipulator claw and each sample placed in an individual polyvinyl chloride (PVC) quiver. The quivers were cleaned before deployment using ethanol to remove any interior biofilms, filled with freshwater and sealed with a rubber stopper. This prevented contamination of the containers by the water column prior to sampling, during which the rubber stopper is removed and in situ seawater replaces the freshwater due to density differences. On the ship, coral samples were transferred from the ROV collection quivers to sterile 50 mL tubes containing the preservative RNAlater (Life Technologies, Grand Island, NY). Samples were incubated overnight at 4°C to allow the preservative to penetrate the coral tissues and then stored at -20°C until processing.

2.2.2 Genetic Identification

During ROV collection, coral samples were visually identified as *A. grandiflora*. Of the 23 samples collected for microbiology, sufficient biomass remained in 19 samples to be shared with collaborators conducting genetic analysis (Table 2.1). Mitochondrial genes *mtMutS* (France & Hoover, 2002; Sanchez et al., 2003) and cytochrome oxidase (*cox1*) (McFadden et al., 2011; Smith et al., 2004) were sequenced for genetic assessment of the corals (S. France and R. Clostio, pers. comm.).
Figure 2.1. Map of collection sites
Samples were collected from two submarine canyons, Baltimore and Norfolk, located off the Mid-Atlantic coast of the United States. Symbol shapes defined in the legend distinguish the samples based on genetic identification (e.g. *Anthothela grandiflora*, *Anthothela* sp., and new genus).
2.2.3 Nucleic Acid Extraction

Two polyps (~50 mg) were removed from each coral sample using flame-sterilized forceps and dissecting shears. DNA was extracted using the MOBIO PowerPlant DNA Isolation Kit (MO BIO Laboratories; Carlsbad, CA). Per Sunagawa et al. (2010), modifications to this protocol included the addition of lysozyme and extended incubation periods at room temperature (24°C) and 65°C. Samples were then homogenized using 400 mg each of sterile 0.1 mm and 0.5 mm zirconia/silica beads (BioSpec Products; Bartlesville, OK) in a Mini-BeadBeater-1 (Biospec Products) (Sunagawa et al., 2010). The bacterial and universal primers 63F (5’CAGGCCTAACACATGCAAGTC3’) (IDT; Iowa City, IA) (Marchesi et al., 1998) and 1542R (5’AAGGAGGTGATCCAGCCGCA3’) (IDT) (Pantos et al., 2003) were used to screen the samples to confirm amplification of the target 16S bacterial rRNA genes, rather than the possible amplification of coral 18S ribosomal rRNA genes by polymerase chain reaction (Galkiewicz & Kellogg, 2008). DNA concentrations from the extraction were quantified for each sample using a Quant-iT™ PicoGreen dsDNA Assay Kit (Invitrogen: Eugene, OR) as outlined in the manufacturer’s protocol and sent for sequencing.

2.2.4 16S rRNA Gene Pyrosequencing

Unamplified DNA extracted from the samples was sequenced by 454 pyrosequencing (Selah Genomics; Greenville, SC) using GS FLX Titanium chemistry and V4–V5 targeting primers following Roche 454’s standard protocol for amplicons (Claesson et al., 2010): forward primer (5’ AYTGGGYDTAAAGNG) (IDT) and reverse primer (5’ CGTATCGCCTCCCTCGGCATCAG) (IDT). Sequence data from all samples were deposited in the NCBI Sequence Read Archive (SRA) under Bioproject number PRJNA296835.
Table 2.1. Sample collection and corresponding environmental data. Highlighted samples (n = 16) were used in final analysis.

<table>
<thead>
<tr>
<th>Coral</th>
<th>Year</th>
<th>Canyon</th>
<th>ID #</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Depth (m)</th>
<th>Temp (˚C)</th>
<th>Salinity (psu)</th>
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<td>5.8</td>
<td>34.9</td>
</tr>
<tr>
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<td>NF.18Q6</td>
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<td>-73.849030</td>
<td>524</td>
<td>5.5</td>
<td>35.0</td>
</tr>
<tr>
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<td>581</td>
<td>5.9</td>
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</tr>
<tr>
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<td>Norfolk</td>
<td>RB.687Q3</td>
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<td>6.5</td>
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</tr>
<tr>
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<td>Norfolk</td>
<td>RB.688Q4</td>
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<td>559</td>
<td>5.8</td>
<td>34.9</td>
</tr>
<tr>
<td>sp.</td>
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<td>RB.686Q2</td>
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<td>-74.605852</td>
<td>480</td>
<td>6.6</td>
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</tr>
<tr>
<td>New Genus</td>
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<td>RB.686Q3</td>
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<td>-74.592445</td>
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<td>6.4</td>
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<tr>
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<td>NF.01Q7</td>
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<td>35.0</td>
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<tr>
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<td>Norfolk</td>
<td>RB.687Q4</td>
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<td>-74.580567</td>
<td>704</td>
<td>5.3</td>
<td>35.0</td>
</tr>
</tbody>
</table>

*Samples identified with ND did not have genetic analysis completed
2.2.5 Bioinformatics and Statistical Analysis

Analysis of the sequence data was conducted using the bioinformatics program QIIME 1.5.0 on the Data Intensive Academic Grid (DIAG), a National Science Foundation funded MRI-R2 project #DBI-0959894 and QIIME 1.9.1 on the Amazon Elastic Compute Cloud (Amazon “EC2”) (Caporaso et al., 2010). Our bioinformatics workflow and all resulting processed files are available online as a USGS data release, http://dx.doi.org/10.5066/F7CZ356K (Kellogg & Lawler, 2015).

A total of 1,308,658 raw reads were generated from the 23 individual coral samples. Quality checks were performed using the split_libraries.py with the following parameters: sequence length (minimum sequence length of 200 base pair (bp) and a maximum length of 700 bp), a minimum average quality score of 25, a maximum of one primer mismatch, and a maximum of a six homopolymer run (Kunin et al., 2010). SFF files were split into individual sample libraries based on the designated 10 bp identification barcode assigned to each sample during library preparation. The 889,914 sequences that passed the quality checks were then denoised using denoiser_preprocess.py, denoiser.py, and inflate_denoiser_results.py. This process was employed to reduce the number of erroneous operational taxonomic units (OTUs) and increase the accuracy of the sequence processing (Quince et al., 2011). Samples containing fewer than 10,000 sequences were removed prior to OTU selection to maximize the sequence data available. Furthermore, corals with no confirmed genetic identification were also removed at this stage, leaving a final total of 16 samples (Table 2.1, highlighted). Moving forward, OTUs were selected using an open-reference method (pick_open_reference_otus.py), with a 97% similarity threshold (Rideout et al., 2014). This method clustered sequences from each sample against the Greengenes reference database release 13_8 (DeSantis et al., 2006). Sequences that
were not matched during the reference comparison were reevaluated using the de novo reference method. Sequences were then aligned using usearch (Edgar, 2010), which included the removal of chimeras. Representative OTU sequences (defined as one representative from each OTU) were selected, assigned a taxonomic classification (uclust) (Edgar, 2010), and used to create a phylogenetic tree (Price et al., 2010). Sequences were then filtered to remove absolute singletons (defined as an OTU present only once in the analysis). Sequences classified as chloroplasts and mitochondria were removed from the OTU table as were any sequences classified as Eukarya or Archaea. Samples were then rarified to the number of sequences present in the smallest sample (10,341) before further diversity analysis was completed. Analysis of the core diversity associated with the coral species was completed using compute_core_diversity.py.

Alpha and beta diversity calculations as well as relative abundance summaries were conducted using alpha_diversity.py, beta_diversity.py, and summarize_taxa_through_plots.py. Alpha diversity metrics included Chao index (Chao, 1984), Shannon diversity index (Shannon, 1948), and Simpson diversity index (Simpson, 1949) (Table 2.2). These indices were employed to assess the richness and evenness of the associated microbiota within each individual sample. To assess beta diversity (similarities or differences across samples), three matrices were used based on phylogenetic and taxonomic relationships between sequences. Weighted and unweighted unit fraction (UniFrac) (Lozupone & Knight, 2005) measurements were recorded to evaluate the importance of the presence/absence of specific taxa within the samples (unweighted Unifrac) compared to the abundance of these taxa (weighted Unifrac) (Fukuyama et al., 2012). Bray-Curtis was also assessed to evaluate differences between each sample based on the number of sequences per OTUs. To visualize beta diversity, principal coordinate analysis (PCoA) plots were prepared in R-Studio (R Development Core Team, 2014) using the previously described
metrics. In addition, pairwise analysis of similarities (ANOSIM) was performed to further examine the statistical variation between sample groups (e.g. environmental parameters, location, or species diversity) (Chapman & Underwood, 1999). A similarity percentage (SIMPER) was also used to determine the key contributing families responsible for the observed patterns. This statistical analysis was completed using PRIMER-E Ltd (Clarke & Warwick, 2001) and R-Studio. Figures for this study were produced in R-Studio (R Development Core Team, 2014) using the vegan (Oksanen et al., 2015) and gplots packages (Warnes et al., 2015).

2.3 Results

Genetic testing revealed that of the 23 samples collected, 12 were classified as *Anthothela grandiflora*, five as a new *Anthothela* species, and two as a new unidentified genus within the family Anthothelidae (S. France & R. Clostio, pers. comm.) (Table 2.1). Four of the 23 samples were not analyzed for coral genetics and were removed from our analysis (NF.01Q7, NF.02Q7, NF.16Q6, and RB.687Q4) although their sequence data are included in the SRA data file for completeness. In addition, samples with fewer than 10,000 sequence reads (NF.02Q7, RB.686Q2, RB.687Q3 and RB.688Q2) were removed before primary analysis to increase rarefaction depth. As such, 16 samples were analyzed further, nine from Baltimore Canyon (one *Anthothela* sp. and eight *A. grandiflora* samples) and seven from Norfolk Canyon (one was identified as a new genus, two as *Anthothela* sp. and four as *A. grandiflora*) (Table 2.1, Figure 2.1).

2.3.1 Alpha and Beta Measurements of Bacterial Diversity

Phylogenetic relationships between samples (beta diversity) were compared using three primary diversity matrices (weighted UniFrac, unweighted UniFrac and Bray-Curtis) and visualized using principal coordinates analysis (PCoA). Diversity of samples was first evaluated
based on coral host. Here, samples within the genus *Anthothela* (*A. grandiflora* and *Anthothela* sp.) clustered separately from sample RB.688Q3, accounting for ~57% of the statistical variation (Figure 2.2). Due to deficient sample size (n = 1), the new genus sample could not be included in the analysis of similarities (ANOSIM) to assess the correlation between bacterial diversity and coral species. Samples associated with *Anthothela* sp. and *A. grandiflora* were compared, revealing no significant difference between the two species (ANOSIM: R = 0.03, p = 0.27). Sample site was also assessed, indicating no significant correlation between coral-associated bacterial diversity and the canyon of origin (ANOSIM: R = -0.02, p = 0.45). The influence of depth of sample sites, water temperature, and salinity were also examined by PCoA, but were not correlated with bacterial diversity (results not shown). Similarity percentage analysis (SIMPER) was then used to examine representative bacterial taxa (family level) responsible for the differentiation between *Anthothela* samples and the unidentified genus. Overall, *Anthothela* samples had an average similarity of 63%. Considerable dissimilarity (~72%) was observed between the new genus (sample RB.688Q3) and the *Anthothela* samples. Contributing families included Shewanellaceae (~19%) and Pirellulaceae (~11%) which were only present in sample RB.688Q3. Unclassified Oceanospirillales (16%), unclassified Spirochaetales (12%), Spirochaetaceae (10%) and Colwelliaceae (10%) also defined the differences seen between *Anthothela* samples and RB.688Q3.

To measure the bacterial diversity present within each individual sample, a series of alpha diversity indices were used (Table 2.2). Shannon (Shannon, 1948), Simpson (Simpson, 1949), and Chao 1 (Chao, 1984) diversity indices account for evenness (defined as the abundance of species present) and richness (defined as the number of species or OTUs) as well as the total number of species observed. These measurements (Shannon = 5.54, Simpson = 0.87, and Chao1
revealed greater species richness and evenness in the sample RB.688Q3 compared to the rest of the *Anthothela* samples (Table 2.2). In general, Shannon measurements were fairly consistent across *Anthothela* samples (average Shannon = 2.27) with increased diversity in two samples (RB.688Q4 = 2.68 and NF.18Q6 = 3.09). Similar trends were seen in Chao 1 and Simpson measurements. To visualize the diversity driving these patterns, bacterial communities were characterized at the phylum, order, and family levels for each host genus.

**Figure 2.2. PCoA plot of Weighted Unifrac Distance**
Principal coordinates analysis was used to plot the beta diversity of bacterial communities using the weighted Unifrac Matrix. Red symbols indicate samples collected from Baltimore canyon, while blue symbols indicate samples collected from Norfolk Canyon. Symbol shapes defined in the legend distinguish the samples based on host genetic identification (e.g. *Anthothela grandiflora*, *Anthothela* sp., and new genus).
Table 2.2. Alpha diversity analysis of *Anthothela* samples.

<table>
<thead>
<tr>
<th>Corals</th>
<th>Canyons</th>
<th>Sample ID</th>
<th>No. Reads*</th>
<th>OTUS</th>
<th>ACE Richness</th>
<th>Chao1 Richness</th>
<th>Shannon Index</th>
<th>Simpson Index</th>
<th>Simpson Evenness</th>
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<td>1.53</td>
<td>0.52</td>
<td>0.048</td>
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<tr>
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<td>0.72</td>
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<td>1.59</td>
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<td>0.064</td>
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<td>69.00</td>
<td>1.88</td>
<td>0.65</td>
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<tr>
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<td>457.44</td>
<td>5.54</td>
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</tbody>
</table>

* All samples were rarified to 10,341 sequences before diversity indices were calculated
2.3.2 Bacterial Community Composition of New Coral Genus

Proteobacteria dominated the new genus (RB.688Q3), accounting for ~69% of the relative abundance, with Planctomycetes representing the second most abundant bacterial taxa at ~17% (Figure 2.3A). Additional minor contributors to the bacterial diversity included Bacteroidetes (~2%), Acidobacteria (~1%), Actinobacteria (~1%), Firmicutes (~1%), and Verrucomicrobia (~1%). Phyla representing less than 1% of the relative abundance of the sample were labeled as “Other” (representing ~5% of the sample’s relative abundance); some of these included Chlamydiae, Deferrribacteres, Lentisphaerae, and Nitrospira.

The bulk of the bacterial diversity distinguishable at the order level was found within the new genus RB.688Q3 (Figure 3B). Alteromonadales dominated bacterial diversity at 47% relative abundance, followed by the second most dominate bacterial group, Pirellulales, accounting for 13% of the relative abundances. Additional contributing bacterial populations included α-proteobacteria (Order Rhodobacterales), δ-proteobacteria (Orders Desulfobacterales and Myxococcales), and γ-proteobacteria (Orders Legionellales and Vibrionales). Rhodobacterales and Vibrionales accounted for approximately 4% of the relative abundances with Desulfobacterales, Myxococcales, Marinicellales, Phycisphaerales, and Planctomycetales observed in sample RB.688Q3, but at smaller relative abundance (~2%). Three of these five proteobacterial orders were only present in sample RB.688Q3: Rhodobacterales, Desulfobacterales, and Myxococcales.

Within the order Alteromonadales, Shewanellaceae (~35%) and Colwelliaceae (~11%) were both found at high relative abundance. Additionally, Pirellulaceae (Order Pirellulales) contributed ~13% of the relative abundance of this sample. Other contributing families present in less than 5% of the relative abundance of sample RB.688Q3 included: Verrucomicrobiaceae,
Spirochaetaceae, Planctomycetaceae, Pseudoaltermonadaceae, Marinicellaceae, and Rhodobacteraceae. The bacterial genus *Shewanella* accounted for ~35% relative abundance in RB.688Q3. Approximately 2% of the identifiable genera in RB.688Q3 were named; these included *Phaeobacter* (family Rhodobacteraceae), *Planctomyces* (family Planctomycetaceae), and *Pseudoalteromonas* (family Pseudoalteromonadaceae). With the exception of *Pseudoalteromonas*, each was exclusively identified in sample RB.688Q3.

2.3.3 Bacterial Community Composition of Anthothela

In total, 15 coral samples were classified under the genus *Anthothela*, consisting of both *A. grandiflora* (n = 12) and an unknown *Anthothela* species (n = 3). At the phylum level, roughly half of the 15 samples were dominated by Proteobacteria (~48% average relative abundance). The second most dominant bacterial group, Spirochaetes (Figure 2.3A), accounted for ~42% average relative abundance of the *Anthothela* samples (~43% in *Anthothela* sp. and 41% in *A. grandiflora* samples). Unlike sample RB.688Q3, Proteobacteria and Spirochaetes were the only bacterial groups discernible at the phylum level. Phyla representative of less than 1% of the relative abundance in at least one sample were labeled as “Other” (~10% of the total relative abundance). These included Chloroflexi, Lentisphaerae, and Nitrospira.

At the order level, all bacteria groups were classifiable with the exception of one, unclassified Spirochaetes (Figure 2.3B). Bacterial diversity associated with *Anthothela* genus samples (*A. grandiflora* and *Anthothela* sp.) varied slightly with key communities including Oceanospirillales (34%), unclassified Spirochaetes (24%), and Spirochaetales (17%). Other orders were observed at higher abundance in several of the *Anthothela* samples: Alteromonadales accounted for 26% of *A. grandiflora* sample NF.17Q7; Deltaproteobacteria Sva0853 was present in 6 of the 15 samples, ranging from 1%–16% relative abundance; Kiloniellales was present in
10 samples, ranging from 1%–34% relative abundance. Caulobacterales, with ~36% relative abundance, was present in sample RB.686Q4. Bacterial groups observed in one or two of the Anithothela genus samples at lower relative abundance included: Rickettsiales present in ~2% of three Norfolk Canyon samples (RB.686Q5, RB.687Q5 and RB.688Q5); Legionellales accounting for ~2% of samples RB.688Q5 and NF.18Q7; and Vibrionales present in ~2% of samples RB.687Q5 and NF.18Q6. Three of the bacterial groups present in Anithothela samples were not observed in the new genus RB.688Q3: Caulobacterales, Kiloniellales, and Rickettsiales.

Families and identifiable genera (present at greater than 1% relative abundance in at least one sample) were also assessed for Anithothela samples. Spirochaeta (Order Spirochaetales, Family Spirochaetaceae) were the most abundant bacteria (3%–34% relative abundance) observed in 13 of the 15 samples. The family identified as Endozoicomonaceae accounted for ~2% of two samples RB.687Q5 and RB.688Q1. Because we could not find this family described in any taxonomic literature, sequences identified as Endozoicomonaceae were run through RDP Classifier (Wang et al., 2007) for further assessment. In Classifier, Endozoicomonaceae sequences were categorized as the family Hahellaceae. Lastly, Moritella (Order Alteromonadales, Family Moritellaceae) was observed dominating A. grandiflora sample NF.17Q7, accounting for ~26% of its relative abundance.
Figure 2.3. Relative abundance of bacterial taxa in coral samples
A) Phyla present at $\geq 1\%$ relative abundance in at least one sample. All remaining taxa are summarized under “Other”. B) Orders present at $\geq 1\%$ relative abundance in at least one sample. All remaining taxa are summarized under “Other”. Samples collected from Baltimore Canyon begin with the letters “NF” and those from Norfolk Canyon with “RB”.

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2.3.4 Core Microbiome

To evaluate the potential conserved-core diversity, samples were first assessed at the level of family Anthothelidae, i.e. across all 16 samples. One identifiable genus, *Spirochaeta*, was observed in every sample. Next, we evaluated the core diversity at the *Anthothela* genus level (n = 15). Assessment of *Anthothela* samples revealed no additional unique taxa, with *Spirochaeta* still the only shared taxon. From here, conserved bacteria were assessed at 90% sample coverage revealing four conserved communities; unclassified orders (Oceanospirillales, Kiloniellales, and Campylobacterales) and genus *Spirochaeta* (Figure 2.4). Individual species were examined at 100% sample coverage: *A. grandiflora* (n = 12) and *Anthothela* sp. (n = 3). *A. grandiflora* samples only shared the genus *Spirochaeta*, while *Anthothela* sp. included the genera *Propionibacterium*, *Pseudoalteromonas* as well as unclassified bacteria within *Spirochaetes*, Kiloniellales, Campylobacterales, Oceanospirillales, and Brachyspiraceae.

Figure 2.4. Core Microbiome of *Anthothela* sp. samples
A heatmap displaying the core community of the 15 samples within the *Anthothela* genus. Bacterial taxa represented in this figure were unique to the *Anthothela* spp. core microbiome present in 90% of the samples.
2.4 Discussion

Relatively little is known about cold-water coral microbiomes in comparison to those of tropical corals. Prior to this study no microbial assessment had been completed on cold-water Anthothelidae corals. Because some tropical coral species have shown correlation between their bacterial composition and environmental parameters (e.g. geographic location, depth, ambient water-temperature, and surrounding organisms) (Barott et al., 2011; Littman et al., 2009), similar relationships were anticipated within the host-microbe interactions of the cold-water corals collected during this study. However, bacterial composition of samples was not found to be significantly different based on canyon of origin (Figure 2.1). Beta diversity matrices indicated relationships between the present taxa and their abundance drove the diversity. Alpha diversity measurements also indicated higher bacterial diversity in sample RB.688Q3 (Table 2.2). This as well as the ANOSIM results further supported the PCoA distribution showing the clustering of all Anthothela samples separate from the new genus RB.688Q3.

2.4.1 Proteobacteria

In most of the marine environment, Proteobacteria dominate the bacterial diversity (Amaral-Zettler et al., 2010). This is true for many tropical coral species (Bourne & Munn, 2005; Frias-Lopez et al., 2002; Thurber et al., 2009) as well as cold-water scleractinian and octocorals (Galkiewicz et al., 2011; Hansson et al., 2009; Neulinger et al., 2008; Penn et al., 2006; Van Bleijswijk et al., 2015; Yakimov et al., 2006). In this study similar trends were observed. Proteobacteria, primarily the orders Oceanospirillales, Kiloniellales, and Alteromonadales, accounted for the majority of the diversity in samples. Other minor contributing Proteobacteria included Rhodobacterales, Desulfo bacterales, Myxococcales, Legionellales, and Vibrionales.
2.4.1.1 Proteobacteria in Sample RB.688Q3

Alteromonadales (specifically families Shewanellaceae and Colwelliaceae) were observed as the dominant order within the new genus sample, RB.688Q3. Genera classified under these families are ubiquitous throughout marine environments, described in tropical (Bourne & Munn, 2005; Ritchie, 2006; Shnit-Orland & Kushmaro, 2009; Shnit-Orland et al., 2010; Thompson et al., 2006), temperate (La Rivière et al., 2015), and cold-water coral species (Galkiewicz et al., 2011; Gray et al., 2011; Kellogg, 2008; Kellogg et al., 2009). Their presence in cold-water environments was expected as many members of both Shewanellaceae and Colwelliaceae are psychrophilic (organisms capable of growing in cold, extreme environments) (Bowman, 2014; Satomi, 2014). In tropical ecosystems, Colwelliaceae were observed in association with coral disease (e.g. White Plague) and the deterioration of several microalgal species (Daniels et al., 2015; Fernandes et al., 2012; Roder et al., 2014a; Thompson et al., 2006). Biochemical properties of Colwelliaceae members include energy production through the decomposition of organic matter and nitrate reduction (Bowman, 2014). Species classified under the genus Shewanella (family Shewanellaceae) are generally Gram-negative, facultative anaerobes, recognized for their roles in nitrate and iron reduction (Coursolle & Gralnick, 2012; Hau & Gralnick, 2007; Kim et al., 2012; Satomi, 2014). Prevalent throughout the marine environment, members of this family have been isolated from numerous deep-sea and cold-water environments including the Marianas Trench (Kato et al., 1998) and the Arctic Ocean (Kim et al., 2012). Shewanella isolated from the mucus of tropical coral genus Favia demonstrated both antibacterial properties and antibiotic resistance (Shnit-Orland & Kushmaro, 2009; Shnit-Orland et al., 2010). These beneficial or mutualistic relationships are thought to contribute to the overall health of the host organism, providing protection from pathogenic or opportunistic bacteria.
Members of the order Rhodobacterales, specifically the genus *Rhodobacter* are thought to be among the most abundant, diverse, and metabolically influential bacteria within the marine environment (accounting for ~25% of the total bacteria present in coastal and polar regions) (Wagner-Dobler & Biebl, 2006). Rhodobacterales are found ubiquitously amongst coral reef systems, typically observed in surface waters, reef invertebrates, and their associated biofilms (Galkiewicz & Kellogg, 2008; La Rivière et al., 2015; Roder et al., 2014b; Sharp et al., 2012; Sunagawa et al., 2009). Functionally these bacteria are diverse, capable of contributing to the reduction of trace metals and the production of antibiotic compounds (Brinkhoff et al., 2004). Other notable members also contribute to the global carbon and sulfur cycles through the oxidization of carbon monoxide and production of dimethylsulfide (Wagner-Dobler & Biebl, 2006). To date few Rhodobacterales (particularly members of the family Rhodobacteraceae) have been identified in cold-water coral species; these include *Alcyonium digitatum* (Alsmark et al., 2012) and *L. pertusa* (Kellogg et al., 2009; Neulinger et al., 2008).

Desulfobacterales (class α-proteobacteria) are documented sulfate-reducing bacteria, functionally described as hydrogenotrophs (capable of using H₂ as an energy source within the metabolic pathway) (Kimes et al., 2010; Kuever et al., 2005). These members appear to be common within deep-sea ecosystems, including microbial mats (Burow et al., 2014), sediments (Simister et al., 2015), seeps (Jaekel et al., 2013), and one unidentified cold-water coral (Simister et al., 2015). In contrast, Myxococcales (class α-proteobacteria) are commonly observed in terrestrial environments, with some present in marine ecosystems (though, notably less) (Reichenbach & Dworkin, 1992). Several have been identified in coral species including *Eunicella cavolini* (Bayer et al., 2013a) and *Mussismilia braziliensis* (Garcia et al., 2013). Functionally these bacteria may contribute to the production of nutrients through sulfate
Legionellales and Vibrionales were each identified as a small percentage (~2%) in the new genus. Legionellales, described as facultative or obligate intercellular parasites are recognized for infecting species of both invertebrates and vertebrates (Garrity et al., 2005). While commonly associated with marine environments, members of the Legionellales have rarely been identified in corals (tropical or cold-water) (Meron et al., 2012; Ransome et al., 2014). Unlike Legionellales, Vibrio spp. (Order Vibrionales) are common associates of shallow-water corals (Ben-Haim et al., 2003a; Bourne & Munn, 2005; Kushmaro et al., 2001; Lampert et al., 2006; Ritchie, 2006; Rohwer et al., 2001; Rosenberg et al., 2007) and have also been found in association with the cold-water coral species L. pertusa (Galkiewicz et al., 2011; Neulinger et al., 2008), Eunicella verrucosa (Hall-Spencer et al., 2007a), Paragorgia arborea, Plumarella superba, and Cryogorgia koolsae (Gray et al., 2011). Although these bacteria are primarily acknowledged for their roles as opportunistic or pathogenic bacteria associated with coral disease and bleaching events (Ben-Haim et al., 2003a; Ben-Haim et al., 2003b; Hall-Spencer et al., 2007a; Sussman et al., 2008; Sweet & Bythell, 2012; Toren et al., 1998), they are also recognized common members of the healthy coral microbiome (Ainsworth et al., 2015; Bourne & Munn, 2005; Raina et al., 2009).

2.4.1.2 Proteobacteria in Anthothela Samples

Oceanospirillales was identified as one of the dominant bacterial groups in Anthothela samples, present in eight samples at over 20% relative abundance. Functional characteristics associated with Oceanospirillales members include, but are not limited to carbon fixation, sulfur oxidation, and biofilm production in the presence of trace metals, such as copper (Little et al., 1996; Speck & Donachie, 2012; Swan et al., 2011). These roles define both the acquisition of
nutrients and potential attraction or inhibition of bacterial colonization within the host. In this study, Oceanospirillales were further classified to the family level, with identifiable groups consisting of unclassified Oceanospirillales and Endozoicomonaceae. It is currently unclear if Endozoicomonaceae is an accepted taxon, since those same sequences were identified by RDP Classifier as Hahellaceae with 99% confidence. With that in mind, members of the family Hahellaceae (specifically genus *Endozoicomonas*) are widespread in the marine environment. Numerous studies have assessed *Endozoicomonas* in tropical and temperate corals (Apprill et al., 2013; Bayer et al., 2013a; Bayer et al., 2013b; Cardenas et al., 2012; Carlos et al., 2013; Correa et al., 2013; Jessen et al., 2013; La Riviere et al., 2013; Lee et al., 2012; Morrow et al., 2012; Pike et al., 2013; Ransome et al., 2014; Roder et al., 2015; Sunagawa et al., 2009; Sunagawa et al., 2010; Yang et al., 2010), as well as a sea slug (Kurahashi & Yokota, 2007) and sponge species (Nishijima et al., 2013; Rua et al., 2014). Because *Endozoicomonas* are both common and highly abundant in healthy tropical corals species, relationships between these bacteria and their hosts have been thoroughly examined. Functional characteristics include nitrate reduction, chemotactic activity, and production of antimicrobial compounds (Kurahashi & Yokota, 2007; Rua et al., 2014; Tout et al., 2015). While members of the family Hahellaceae are common in tropical and temperate environments, this does not appear to be the case for deep-sea corals. Few studies have observed bacteria classified under Hahellaceae in deep cold-water (> 100 m) corals (Hansson et al., 2009; Kellogg et al., 2009; Van Bleijswijk et al., 2015). The symbiotic relationships between Hahellaceae and zooxanthellae are thought to be the one of the driving influences of their abundance and presence in tropical corals (Pantos et al., 2015). Because cold-water corals lack algal symbionts, the Hahellaceae bacteria should be minimal, if present at all, in the cold-water coral holobiont. In this study, this seems to be the case with members of the
Hahellaceae (originally classified as Endozoicomonaceae) representing a small minority of the Oceanospirillales present, at ~2% in A. grandiflora samples RB.687Q5 and RB.688Q1.

Similar to Oceanospirillales, members of the order Kiloniellales were present in multiple Anthothela samples at relatively high abundance. Kiloniellales bacteria have been observed in several tropical corals species (Sharp et al., 2012; Soffer et al., 2015) as well as mussels (Cleary et al., 2015), sponges (Cleary et al., 2013), and algae (Wiese et al., 2009). Soffer et al. (2015), identified members of the order Kiloniellales at higher abundances in healthy coral Orbicella annularis than in diseased colonies, suggesting association in a beneficial capacity. Functionally, these chemoheterotrophic bacteria have been found to utilize nitrates within the metabolic process through denitrification (Imhoff & Wiese, 2014; Wiese et al., 2009). To our knowledge, no prior studies have identified Kiloniellales associated with cold-water corals.

Several contributing Proteobacteria were identified in individual Anthothela samples including Alteromonadales, Caulobacterales and Rickettsiales. Genus Moritella (Order Alteromonadales, Family Moritellaceae) was present in A. grandiflora sample NF.17Q7 accounting for ~26% relative abundance. This bacterial group is specific to marine environments and generally classified as halophilic facultative anaerobes (Stanley et al., 2005; Urakawa, 2014). Moritella isolates have been collected from a wide variety of environments ranging from deep-sea sediments (Kato et al., 1998; Nogi et al., 1998; Xu, 2003) to tropical corals (Bourne & Munn, 2005; Bourne, 2005; Rohwer et al., 2001). In cold-water coral species, however, Moritella sequences have only been described in the scleractinian L. pertusa collected from the Gulf of Mexico (Kellogg, 2008). In the individual A. grandiflora sample RB.686Q4, members of the Caulobacterales were found at relatively high abundance (~30%). Recognized for their unique morphology, members of this order contain a stalk-like flagellum utilized for adhesion to
adjacent surfaces, including but not limited to host organisms (Starr & Skerman, 1965). While these bacteria tend to exhibit parasitic tendencies, they have been described as facultative commensals, potentially contributing to the acquisition of nutrients through their roles in carbon cycling (Abraham et al., 1999). These free-living bacterial communities are often found throughout the water column and have been observed in several tropical corals including the gorgonian *Pseudopterogorgia elisabethae* (Correa et al., 2013) and acroporid species (*A. granulosa, A. valida*, and *A. millepora*) (Ainsworth et al., 2015; Littman et al., 2009). While members of the group Caulobacterales have been identified in deep ocean waters (Eloe et al., 2011), to date, no prior studies have observed Caulobacterales in cold-water reef communities.

Members of the order Rickettsiales have previously been described as opportunistic, pathogenic, and/or associated with diseased tropical corals. Contradictory studies have identified members of Rickettsiales as both associates and etiologic pathogens of White Band Disease I (Casas et al., 2004; Peters, 2014; Peters et al., 1983), infecting acroporid species (*A. cervicornis, A. palmata*, and *A. prolifera*) (Casas et al., 2004) as well as *Mussismilia braziliensis* (Garcia et al., 2013). Casas et al. (2004) found a high abundance of Rickettsiales in both healthy and diseased corals. More recently, the possible etiological impact of Rickettsiales in coral disease has been debated. Peters (2014) suggested the influence of phagocytic Rickettsiales-like bacteria in White Band Disease I as the driving force for cell death. Miller et al. (2014) identified Rickettsiales-like bacteria as the infecting agent of acroporid diseases. In contrast, Sweet et al. (2014) addressed the presence of causative bacteria in White Band Disease via antibiotic partitioning, but failed to identify specific pathogens responsible. Several other studies have also attributed the spread of disease to stressed or compromised corals influenced by transitioning environments, ultimately allowing for the proliferation of opportunistic bacteria such as
Rickettsiales (Gignoux-Wolfsohn & Vollmer, 2015; Peters, 2014). While present in this study, Rickettsiales were observed at low relative abundance (~2%) in only three Norfolk Canyon samples (RB.686Q5, RB.687Q5, and RB.688Q5). With the exception of the study by Gray et al. (2011), members of the order Rickettsiales have not been observed in cold-water corals. Because their functional characteristics are generally driven by ambient environmental fluctuations (e.g. increases in temperature and light intensity), we speculate that the opportunistic tendencies exhibited in tropical environments differ from those in cold-water environments. In this case, additional research is necessary to definitively assess the potential pathogenicity and overall functionality of these bacteria within the cold-water ecosystem.

2.4.2 Spirochaetes

The bacterial diversity in Anthothela samples was distinct from that present in the unidentified genus, RB.688Q3. Spirochaetes were observed as one of the primary bacteria, dominating over half of the Anthothela samples. Spirochaetes are recognized as motile free-living, facultative/obligate anaerobes (Leschine et al., 2006). Functional characteristics displayed by members of this group include nitrogen and carbon fixation, as well as chemotactic responses to chemical stimulants (Baker et al., 2015; Greenberg & Canale-Parol, 1977; Kimes et al., 2010; Lilburn et al., 2015). Members of the phylum Spirochaetes are commonly found in association with invertebrates at high abundance, including species of termites (Breznak, 2002), oligochaetes (Blazejak et al., 2005), sponges (Taylor et al., 2005), and tropical corals (Casas et al., 2004; Closek et al., 2014; Kimes et al., 2013; Kimes et al., 2010). Previous studies using clone libraries have observed Spirochaetes in association with some cold-water corals (Gray et al., 2011; Kellogg et al., 2009; Penn et al., 2006), however they have never been identified as a dominant member of the associated bacterial community. In this study, Spirochaetes were recognized as
the prevailing phyla in roughly half of the total Anthothela samples. To our knowledge this is the first study to establish Spirochaetes as a major contributor within the cold-water coral microbiome.

In Anthothela samples (A. grandiflora and Anthothela sp.), genus Spirochaeta (phylum Spirochaetes) continued to dominate the bacterial groups. New representative Spirochaeta sequences from this study were compared to those of environmental and invertebrate studies where Spirochaeta sequences had been observed. Sequences associated with Anthothela samples were most closely related to sequences isolated from deep-sea water (Accession KF758585, E-value of 7e^{-165}) and microbial mats (Accession DQ218325, E-value of 1e^{-161}). While sequences from Anthothela corals were not closely related to those of other coral species, presence of this bacterium across all 16 samples suggests conservation at the family level. Additionally, presence of this bacterium at such a high abundance, as observed in Anthothela samples, suggests a unique microbe-host interaction specific to that coral genus.

2.4.3 Core Microbiome

Because corals are dependent (in part) on their microbe-host interactions, examining the “core conserved” communities may reveal insights into the overall health of the coral host (Krediet et al., 2013; Shade & Handelsman, 2012). Many variables impact the presence of microbes within the coral holobiont ranging from the identity of the host (specific at the host species (Rohwer et al., 2002) or genus (Littman et al., 2009) level), to a niche within the host organism (e.g. tissue vs. mucus) (Ainsworth et al., 2015; Bourne & Munn, 2005; Koren & Rosenberg, 2006; Sweet et al., 2010), as well as fluctuations in the surrounding environment (Ainsworth & Hoegh-Guldberg, 2009; Pantos et al., 2003; Reshef et al., 2006). While bacterial communities may vary based on these parameters, conserved bacteria necessary for coral host
health, defined as the “core” community, are consistently present. Because little is known about
the bacterial functions within the cold-water coral holobiont, it is necessary to identify the core
microbiota of each coral species (defined as those common in more than one of the designated
habitats) (Shade & Handelsman, 2012). In this study we applied a stringent approach to examine
“core” conserved communities, evaluating bacterial groups present at the family (Anthothelidae),
Anthothela genus, and individual species (A. grandiflora and Anthothela sp.) levels.

To begin, samples were evaluated at the family and genus levels at 100% sample
coverage, revealing one core-conserved bacterium present across all samples, a member of the
genus Spirochaeta. As previously described, members of the phylum Spirochaetes are common
throughout coral species, both tropical and cold-water (Casas et al., 2004; Closek et al., 2014;
Gray et al., 2011; Kellogg et al., 2009; Kimes et al., 2013; Penn et al., 2006). In this study, Spirochaeta was found to be one of the dominant bacterial groups accounting for roughly 16% of
the total relative abundance, thus suggesting a significant role within the coral microbiome.
These free-living nonpathogenic anaerobes contain metabolic characteristics that consist of, but
are not limited to, carbon fixation and organic carbon degradation (Baker et al., 2015).

Samples were then assessed at 90% sample coverage, particularly looking for conserved
bacteria specific to Anthothela samples (Figure 2.4). In addition to the genus Spirochaeta, OTUs
classified under the phylum Proteobacteria (Orders; Oceanospirillales, Kiloniellales, and
Campylobacterales) were identified. Previous studies described members of the orders
Oceanospirillales and Kiloniellales as beneficial bacteria, contributing to their host system
through the production of biofilm and antibacterial properties, respectively (Little et al., 1996;
Swan et al., 2011). Oceanospirillales members have also been recognized for their influence in
nutrient dynamics within the coral holobiont through the formation of dissolved inorganic
materials produced during carbon fixation and sulfur oxidation (Swan et al., 2011). In contrast to Oceanospirillales and Kiloniellales, members of the order Campylobacterales are most commonly known for their association with coral disease (Frias-Lopez et al., 2002; Gignoux-Wolfssohn & Vollmer, 2015; Sunagawa et al., 2009; Sweet & Bythell, 2012; Vezzulli et al., 2013), but are also present in healthy corals (Sharp et al., 2012). Functionally these bacteria are recognized for their metabolic influence in nitrogen cycling and communication through bacterial quorum sensing (Golz et al., 2012; Kern & Simon, 2009).

Nitrogen is a critical, but limited resource within the marine environments (Zehr & Kudela, 2011). Previous studies have addressed the importance of nitrogen cycling in photosynthetic systems such as tropical corals, suggesting the influence of fungi, photosynthetic bacteria (e.g. cyanobacteria), and/or dinoflagellate symbionts on the biochemical processes (Lesser et al., 2007; Lesser et al., 2004; Pernice et al., 2012; Shashar et al., 1994; Wegley et al., 2007). While it is evident that nitrogen availability is one of the driving factors in the proliferation and health of tropical coral hosts, little is known about its influence in the cold-water coral holobionts. The core bacterial groups (Spirochaeta, Oceanospirillales, Kiloniellales, and Campylobacterales) observed in the Anthothela genus samples, accounted for a substantial part of the nitrogen cycle (Figure 2.5). Members of the genus Spirochaeta, identified as the most conserved bacterium present were previously recognized for their roles in nitrogen fixation an essential step in the nitrogen cycle (Lilburn et al., 2015). Spirochaeta fulfill one of the most essential steps in the nitrogen cycle. During nitrogen fixation, nitrogen gas (N\textsubscript{2}) is converted to readily available organic compounds that may then be taken in, sustaining the productivity of bacterial and host organisms (Zehr & Kudela, 2011). Several members of the order Campylobacterales have been recognized for their contributions through nitrate ammonification
(Tiedje, 1988). This is the process by which nitrate is converted to ammonium (one of the primary nitrogen sources (Zehr & Kudela, 2011)), thereby recycling nitrogen back into the system (Radecker et al., 2015; Simon, 2002; Tiedje, 1988). Members of the order Oceanospirillales have been observed to contribute through the reduction of nitrate to nitrite, also defined as nitrate reducers (Zehr & Kudela, 2011). Lastly, members of the order Kiloniellales classified as chemoheterotrophic aerobic bacteria, have shown potential in the processing of molecular nitrogen through denitrification (Imhoff & Wiese, 2014). In this process, nitrates are reduced back into N₂ (dinitrogen) to be utilized by nitrogen-fixing bacteria.

Figure 2.5. Core bacterial groups contributing within the Nitrogen Cycle
Each of the bacteria present within the core microbiome of Anthothela samples was previously recognized for their roles within the nitrogen cycle. This diagram illustrates a simplified overview of the bacterial groups with their coinciding functions. This figure was adapted from one presented in Wegley et al. (2007).
Although the core microbiome of the new genus (n = 1) could not be evaluated, bacterial members were assessed for possible functional characteristics associated with the biochemical processing of nitrogen. Similar to *Anthothela* samples, core members associated with the new genus were previously documents for their metabolic properties consistent with nitrogen cycling. Members of the genus *Spirochaeta* have been known to play a role in nitrogen fixation, while bacterial members classified under the families Shewanellaceae and Colwelliaceae (Order Alteromonadales) have been acknowledged for their roles as nitrate reducers in the reduction of nitrates to nitrites (Satomi, 2014). Classified as anaerobic ammonia-oxidizing bacteria, Pirellulales (Phylum Planctomycetes) have been thought to contribute through the removal of metabolic waste within the host microbiome (Mohamed et al., 2010). One of the end products of nitrate ammonification (ammonia) may be taken in by ammonia-oxidizing bacteria such as Pirellulales, resulting in the oxidization of ammonium and formation of nitrites (Zehr & Kudela, 2011). While members of the order Pirellulales and families Shewanellaceae and Colwelliaceae only show potential metabolic properties for a portion of the nitrogen cycle, other bacterial members may be present within RB.688Q3 that complete the remaining metabolic functions.

In an effort to further understand the core diversity, bacterial composition was evaluated in individual *Anthothela* species (*A. grandiflora* and *Anthothela* sp.) at 100% sample coverage. Of *A. grandiflora* samples (n = 12), the genus *Spirochaeta* remained present as the only core microbe. This was not the case in *Anthothela* sp. samples (n = 3), which exhibited higher overall diversity. These samples contained similar conserved communities when compared to those identified at the genus level, with the exception of several bacterial groups (*Propionibacterium*, *Pseudoalteromonas* and unclassified Brachyspiraceae). Of the three members, *Propionibacterium* and *Pseudoalteromonas* were most commonly observed in association with
other coral holobionts. In a recent study, *Propionibacterium* was identified as one of the core bacteria present in association with endosymbiotic cells of tropical corals *Acropora granulose* and *Montipora capitata* (Ainsworth et al., 2015). In addition to tropical and temperate corals (Ainsworth et al., 2015; Bayer, et al., 2013b; De Castro et al., 2010; La Riviere et al., 2013; Lee et al., 2012), *Propionibacterium* species have also been previously described in cold-water corals, which lack the symbiotic dinoflagellates (Neulinger et al., 2008). However, caution is advised due to a recent study by Salter et al. that identified *Propionibacterium* as a common bacterial contaminant found in association with many extraction kits and associated reagents (Salter et al., 2014). Additional research is necessary to confirm the presence of this bacterium in association with cold-water corals. Similar to *Propionibacterium*, members of the genus *Pseudoalteromonas* were observed within many coral species (Nissimov et al., 2009; Radjasa et al., 2005; Rohwer et al., 2001; Shnit-Orland & Kushmaro, 2009; Shnit-Orland et al., 2012). As predominant members of the coral holobiont, *Pseudoalteromonas* species have previously exhibited antibacterial activity against Gram-negative bacteria as well as probiotic properties (Nissimov et al., 2009; Radjasa et al., 2005; Ritchie, 2006; Shnit-Orland & Kushmaro, 2009). Overall, the core diversity present in *Anthothela* sp. samples was similar to those at the *Anthothela* genus level indicating a genus-specific core microbiome.

2.5 Conclusions

Cold-water corals have been a particular topic of interest for the last decade or more. While prior studies have investigated the host-microbe interactions of some cold-water coral species, much is still unknown about these diverse organisms and their bacterial consortia. Our study provides insight into the previously uncharacterized microbiome of cold-water octocorals in the family Anthothelidae. Comparisons between undersea canyons revealed no significant
correlation between bacterial communities and geographic locations. The two species of *Anthothela* shared a very similar bacterial community, in contrast to the new genus in the family (RB.688Q3) which had a highly diverse microbiome distinct from the rest. This suggests genus-specific bacterial associates rather than species-specific or environmentally influenced as seen in previous tropical coral studies.

The bacterial composition of these Anthothelidae corals had some similarity to the microbiota associated with shallow, tropical corals. Several groups present in this study were related to bacteria previously identified as opportunistic or pathogenic bacteria (e.g. Rickettsiales, Vibrionales, and Campylobacterales). These families may be causative agents in coral disease; however, these opportunistic bacteria could be triggered in response to perturbations from the surrounding environment (i.e. increased temperature and/or light intensities). The presence of these bacteria in cold-water corals, which are subject to less dramatic thermal and radiant environmental shifts, may provide new opportunities to determine their underlying roles within healthy coral holobionts.

Additional evaluation of the core microbiome at 90% also revealed a conserved bacterial community associated with the *Anthothela* genus. Many of the core bacteria share potential metabolic functions associated with nutrient provision and properties aiding in the protection of the coral host. Unlike tropical corals, cold-water corals lack a symbiotic dinoflagellate to assist with the acquisition and cycling of nutrients. Overall health and proliferation of cold-water corals are dependent on capture feeding and the presence of microorganisms (Duineveld et al., 2004; Roberts et al., 2006). Members of the core microbiome of *Anthothela* samples were recognized for their potential roles in the uptake and remineralization of organic and inorganic material. More specifically many of the bacterial groups present have been shown to play a role in
nitrogen cycling, including nitrogen fixation (*Spirochaeta*), nitrate ammonification (Campylobacterales), nitrate reduction (Oceanospirillales), and denitrification (Kiloniellales). The new coral genus (RB.688Q3) also contained a microbial community potentially capable of various functions specific to nitrogen cycling. The bacteria present in this sample accounted for three major processes: nitrogen fixation, nitrate reduction, and ammonia oxidation. Unfortunately with a sample size of \( n = 1 \), this evaluation is only a partial representation of the overall bacterial and functional diversity present within the new genus. Further research is necessary to investigate the microbial-host interactions, specifically the functionality of these bacterial associates and their role within the cold-water coral holobionts.

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CHAPTER THREE

Conclusion

The research presented in this thesis assessed bacterial communities associated with three cold-water octocorals classified under the Family Anthothelidae (A. grandiflora, Anthothela sp., and a new unidentified genus). As the first study to assess the microbial landscapes of the three corals, this research expanded the established knowledge of cold-water coral microbial diversity. Bacteria interact within coral hosts in many ways, some of which are still not fully understood. Previous studies have addressed this interaction as well as the presence of, and functional characteristics of, bacteria in tropical corals, defining diverse and essential relationships between host species and their microbiota. While many tropical coral species have been thoroughly explored, little is known about cold-water corals and their microbial associates.

Prior to this study, Anthothela species had been identified in the Atlantic Ocean (Arantes et al., 2009; Lopez-Gonzalez & Briand, 2002; Watling & Auster, 2005; Whiteaves, 1901), but no microbial assessments of these corals had been completed. In an effort to broaden our understanding of these corals, bacterial diversity was characterized through the use of deep-sequencing, giving a first look at the bacterial diversity present in three Anthothelidae. These corals were collected from two different canyons (Norfolk and Baltimore) to test the hypothesis that bacterial diversity was linked to biogeographic location. However, results of this study instead found host-specific associations among the bacteria communities irrespective of the sample origin similar to prior work in tropical corals. Those bacteria associated with Anthothela samples (A. grandiflora and Anthothela sp.) were found to be similar, yet distinct from those
present in the new unidentified coral genus, RB.688Q3. Both alpha and beta diversity supported the separation of samples, revealing host genus as the primary driver of bacterial community diversity. Analysis of the core microbiome also revealed core diversity conserved at the host-genus level. Bacterial groups present in the core microbiome have been previously recognized for their influential roles in the processing of nutrients, specifically nitrogen cycling.

While this study evaluated the bacterial composition through 16s rRNA gene sequencing (which is generally used for characterization of bacterial communities and not functional characteristics), we were able to infer bacterial roles through existing knowledge of the present groups. While this provided a first glimpse into the bacterial community composition, more information is needed. Moving forward, research should focus on bacterial functionality through the use of culture-based and metagenomic techniques. By doing so, studies may be able to address the complex interactions taking place, thus expanding our understanding of these cold-water corals and their symbiosis with complex microbiota.
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ABOUT THE AUTHOR

Stephanie Nichole Lawler was born and raised in Orlando, Florida by her parents Brian and Lorri Lawler. Having a passion toward the marine environment since a young age, Stephanie developed her interest by receiving a Bachelors degree in Environmental Science and Policy at the University of South Florida St. Petersburg in 2012. Here, Stephanie became engaged within the community by organizing beach cleanings, educational sessions on the surrounding watershed, and authoring grants to prevent trash and debris from entering Tampa Bay. During this time Stephanie became SCUBA certified and began volunteering as a diver at two local aquariums (Clearwater Marine Aquarium and The Florida Aquarium). These experiences as well as an internship with Dr. Christina Kellogg at the U.S. Geological Survey, St. Petersburg Coastal and Marine Science Center were the primary reasons motivating Stephanie in her pursuit of a graduate degree.

Stephanie entered the College of Marine Science (CMS) at the University of South Florida in 2013 with co-advisors Drs. Christina Kellogg and Mya Breitbart. While at the CMS, Stephanie received the George Lorton Endowed Fellowship as well as the Aylesworth Scholarship. She participated in various trainings covering a range of topics, including bioinformatics, statistical trainings, and writing workshops. Stephanie was awarded her Masters degree in Marine Science in 2016.