Exploring the cold-water coral holobiont: characterizing the bacterial associates of the octocorals *Paragorgia arborea* and *Primnoa resedaeformis*

by

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Abstract

Corals are among the world's most biodiverse symbiotic ecosystems, and recent work has highlighted the importance of microbes to the health and resilience of reef-building tropical corals. Cold-water corals (CWCs) constitute crucial ecosystems in both shallow and deeper waters, and also form symbioses with microbes. Here, we describe the bacterial associates of two CWCs, *Paragorgia arborea* and *Primnoa resedaeformis*, sampled from the Gulf of Maine. Unique bacterial communities were found in the mucus and the tissues of *P. arborea*, and anatomically distinct bacterial biomarkers were identified. We speculate that some *P. arborea*-associated bacteria may participate in nitrogen cycling, and other contributors such as the dominant taxa *Mycoplasma* may play a role in host health. While bacterial sequences in the mucus of *P. resedaeformis* were low in abundance and diversity, our findings were supported by the recently published *P. resedaeformis* microbiome. The information provided here serves to detail novel findings in CWC microbiome research and promotes future exploration of CWCs.

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List of Abbreviations

ALDEx	ANOVA-like differential expression
ARISA	Automated ribosomal intergenic spacer analysis
CLR	Centered log-ratio
CWC	Cold-water corals
DMSP	Dimethylsulfoniopropionate
DNA	Deoxyribonucleic acid
HTS	High-throughput sequencing
rRNA	Ribosomal ribonucleic acid
SML	Surface mucopolysaccharide layer
OTU	Operational taxonomic unit
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
ROV	Remotely operated vehicle
TDA	Tropodithietic acid

Chapter 1: General introduction

1.1 Cold water corals

Corals are widely distributed across latitudinal and depth gradients, and are often subdivided into tropical (limited to shallow depths at low latitude) and cold-water taxa (extending to greater depths and latitudes) to reflect considerable differences in ecology and biology between those two groups (Roberts *et al.,* 2005). Cold water corals (CWCs) belong to the classes Hexacorallia (Orders: Scleractinia, Zoanthidae, and Antipatharia) and Octocorallia (Orders: Alcyonacea and Pennatulaceae); while most are solitary, some CWCs are colonial, creating complex framework ecosystems that many cold-water organisms depend upon as habitat and refuge (Freiwald *et al*., 2004; Roberts *et al*., 2006; Cairns, 2007; Roberts *et al.,* 2009). CWC habitats can be largely composed of a similar skeletal matrix (either composed of aragonite or calcite) as their warm-water tropical counterparts (Cairns, 2007; Roberts *et al.,* 2009). While CWC reef ecosystems are ecologically critical (Buhl-Mortensen and Mortensen, 2004; Auster, 2005; Buhl-Mortensen and Mortensen, 2005), many CWCs have slow growth rates (Sherwood and Edinger, 2009), which makes these ecosystems particularly sensitive to environmental and anthropogenic disturbances. Anthropogenic perturbations such as oil exploration (Fisher *et al.,* 2014; Cordes *et al.,* 2016) and various fishing practices (Fosså *et al.,* 2002; Edinger *et al.,* 2007b; Miller *et al.,* 2012; Bo *et al.,* 2014) place these corals at risk; even if the stressors are removed, they could take decades to centuries to recover (Sherwood and Edinger, 2009).

CWC ecosystems are highly speciose habitats (Edinger *et al.* 2007a; Roberts and Cairns, 2014) important for organisms across varying life stages (Auster, 2005), including diverse microbial communities (Yakimov *et al.*, 2006; Shöttner *et al.*, 2009). The microbial communities, collectively defined as the "microbiome", associated with CWCs have been described as both beneficial (Middelburg *et al.*, 2015) and detrimental (Rubio-Portillo *et al.*, 2018) to the coral host. The coral host and associated symbionts are collectively termed the coral holobiont and the literature details potential mechanisms involved in the microbe-microbe and microbe-host interactions (Hernandez-Agreda *et al*., 2017). This thesis reviews the available information on CWC microbiomes and provides new information on the bacteria associated with two cold-water gorgonian octocorals, *Paragorgia arborea* and *Primnoa resedaeformis*.

1.2 The coral microbiome

The coral microbiome has been described as one of the world's most diverse symbiotic ecosystems (Rohwer *et al.*, 2002; Mouchka *et al.*, 2010; Blackall *et al.*, 2015; Bourne *et al*., 2016; Hernandez-Agreda *et al.*, 2017; Sweet and Bulling, 2017). Microbiomes, which are composed of eukaryotes, bacteria, archaea, and viruses, have been identified as crucial contributors to the overall health and survivability of multicellular organisms (Knowlton and Rohwer, 2003; Fraune and Bosch, 2010; Hernandez-Agreda *et al.*, 2017) . Within microbiome assemblages, individual taxa may establish commensal or mutualistic relationships with their host, while others may be dysbiotic, parasitic (exploiting the host), or pathogenic (causing disease to the host)

(Hernandez-Agreda *et al.*, 2017). Corals host diverse communities of microbes (Ainsworth *et al.,* 2010; Blackall *et al.,* 2015) that may assist the host in defense against pathogens, nitrogen cycling, and nutrient provisioning (Rosenberg *et al.,* 2007). For example, bacterial-host interactions result in long-term mutualisms with corals and assist them with nutrient uptake (Mouchka *et al.,* 2010; Middelburg *et al.*, 2015). However, coral-microbe associations are sensitive to environmental perturbations and not all associations are beneficial (Egan and Gardiner, 2016); some transient bacteria can be pathogens, leading to coral mortality (Sweet and Bulling, 2017).

In shallow, sunlit environments, tropical corals may form a symbiotic relationship with photosynthetic algae called zooxanthellae, which generate nutrients for the coral host via photosynthesis (Falkowski *et al.,* 1984). CWCs, however, are azooxanthellate (with few exceptions; Wagner *et al*., 2010) and employ different strategies for fitness and survival (Roberts *et al.,* 2005). In most cases, corals found in low light environments acquire nutrients through suspension feeding, i.e. the capture of plankton and suspended organic matter (Roberts *et al.,* 2006). However, suspension feeding is not the only mode of nutrient acquisition used by corals in cold/deep benthic ecosystems – they may also be dependent on associated microorganisms where and/or when food sources are particularly sparse. Bacteria-mediated nutrient cycling is a fundamental strategy used by corals, especially those found outside tropical environments (Naumann *et al.*, 2009; Maier *et al.,* 2011; Middelburg *et al.*, 2015).

The functional roles of coral associated bacteria and other members of the microbiome are largely unknown, although some bacteria in tropical corals potentially benefit the host via antimicrobial defense (Reshef *et al.*, 2006; Ritchie, 2006), sulfur and nitrogen metabolism (Raina *et al.*, 2009; Bednarz *et al.*, 2017), and nutrient recycling (Wild *et al.*, 2004; Naumann *et al.*, 2009). While tropical corals have been explored with some depth (Mouchka *et al.*, 2010; Blackall *et al.*, 2015; Hernandez-Agreda *et al.*, 2017; Sweet and Bulling, 2017), research of the CWC microbiome remains in its infancy (Kellogg *et al.*, 2016; van de Water *et al.*, 2018). Anatomically, both tropical and CWCs have various compartments that function as microhabitats for microorganisms, containing distinct physio-chemical properties (Sweet *et al.*, 2011; Weinbauer *et al.*, 2012). These coral microhabitats have specific features (e.g., the surface mucopolysaccharide layer is in direct contact with the water column) that may select for microbial associates, and can also vary in terms of abiotic factors such as temperature, light, and salinity (Bourne *et al.*, 2016; Sweet and Bulling, 2017). The coral microhabitats most commonly explored in the literature are the coral skeleton, polyp tissues (gastrodermis and/or epidermis), coelenteron, and the surface mucopolysaccharide layer (SML) (Bourne and Munn, 2005; Brown and Bythell, 2005; Koren and Rosenberg, 2006; Sweet *et al.*, 2011; Weinbauer *et al.*, 2012; Krediet *et al.*, 2013; Ainsworth *et al.*, 2015; Pollock *et al.*, 2018).

In corals, microorganisms are predominantly selectively acquired from the surrounding water column and environment (Rohwer *et al.*, 2002; Sweet *et al.*, 2010; Garren and Azam, 2012). Selection based on microbial availability raises the question, do corals from various habitats share similar stable partners? Recently, the concept of a coral

"core" microbiome has been broadly defined as the stable and consistent members of the microbiome community, either across coral colonies within a species (i.e. in different locations) or shared by different coral taxa (Ainsworth *et al.*, 2015; Kellogg *et al.*, 2016; Lawler *et al.*, 2016; Kellogg *et al.*, 2017). Many species of corals across a broad range of habitats (tropical to cold-water) have been documented to house similar microbes (Ainsworth *et al.*, 2015; Kellogg *et al.*, 2016; Lawler *et al.*, 2016; Kellogg *et al.*, 2017; van de Water *et al.*, 2018; Goldsmith *et al.*, 2018). While corals from different taxa can share a core microbiome, this core may not necessarily be shared across microhabitats within a colony (Koren and Rosenberg, 2006; Shöttner *et al.*, 2009; Sweet *et al*., 2011; Weinbauer *et al.*, 2012; van Bleijswijk *et al.*, 2015). The core microbiome is composed of both abundant and *rare* microorganisms, which is crucial when considering the more recently defined coral "pathobiome" (Vayssier-Taussat *et al.*, 2014; Sweet and Bulling, 2017). Rare bacteria have been overlooked for decades due to the difficulty in identifying their relative importance to the host, and only recently have been identified as pervasive members of the coral core microbiome (Ainsworth *et al.*, 2015). However, to understand how any microbial taxon (whether rare or common) interacts with and affects the coral host, extensive research is required using functional omics-based approaches (Franzosa *et al.*, 2015; Gilbert *et al.*, 2016) on both shallow warm-water corals and the much less studied CWCs. In this review, we examine what is currently known about the cold-water coral (both Octocorallia and Hexacorallia) microbiome across coral taxa, microhabitats, and geographical locations.

1.3 Coral microbiomes in cold-water environments: Limitations and current knowledge

Due to limited information in the developing field of CWC microbiomes, exploration of the microbiome is here restricted to the bacterial associates. Bacteria (and even less so, protists and viruses; Weinbauer *et al.*, 2012) have been only moderately explored in CWCs, which illustrates an important gap in our knowledge. The available data nonetheless provide valuable baseline information on the bacterial composition of CWCs (prior to environmental perturbations), for (1) conservation management of CWCs as important habitat-forming ecosystems, and (2) cross comparisons with zooxanthellate warm-water species that are critical to coastal communities. To date, the bacterial associates (colloquially termed the "microbiome" across studies), have been described in several octocoral (Penn *et al.*, 2006; Brück *et al.*, 2007; Gray *et al*., 2011; Bayer *et al.*, 2013; La Rivière *et al.*, 2013; Vezzulli *et al.*, 2013; Holm and Heidelberg, 2016; Kellogg *et al.*, 2016; Lawler *et al.*, 2016; van de Water *et al.*, 2016; van de Water *et al.*, 2017; Goldsmith *et al.*, 2018) and hexacoral CWC species (Yakimov *et al.*, 2006; Neulinger *et al.*, 2008; Hansson *et al.*, 2009; Kellogg *et al.*, 2009; Shöttner *et al.*, 2009; Galkiewicz *et al.*, 2011; Meistertzheim *et al.*, 2016; Kellogg *et al.*, 2017; Röthig *et al.*, 2017) (Table 1.1). Based on these CWC studies, both Hexacorallia and Octocorallia are largely dominated by Proteobacteria, specifically of the classes Gammaproteobacteria and Alphaproteobacteria (Penn *et al.*, 2006; Brück *et al.*, 2007; Neulinger *et al.*, 2008; Hansson *et al.*, 2009; Kellogg *et al.*, 2009; Galkiewicz *et al.*, 2011; Gray *et al.*, 2011; Bayer *et al.*, 2013; Vezzulli *et al.*, 2013; van Bleijswijk *et al.*, 2015; Kellogg *et al.*, 2016;

Lawler *et al*., 2016; Meistertzheim *et al*., 2016; Kellogg *et al.*, 2017; Röthig *et al*., 2017; van de Water *et al.*, 2017; Goldsmith *et al*., 2018). Interestingly, Proteobacteria are also dominant within the microbiomes of warm-water corals (Blackall *et al.*, 2015; Bourne *et al.*, 2016) illustrating a dominance in the coral microbiome, regardless of habitat or host taxonomy. More specifically, ribotypes of the gammaproteobacterial genus *Endozoicomonas* have been observed in the coral microbiome across various taxa and habitats potentiating a significant role in the coral microbiome (Bayer *et al.*, 2013; Vezzulli *et al.*, 2013; Hernandez-Agreda *et al.*, 2017; Neave *et al.*, 2017; van de Water *et al.*, 2017). However, in some CWCs, either Spirochaetes (Holm and Heidelberg, 2016; Lawler *et al*., 2016; van de Water *et al.*, 2016) or Tenericutes (Penn *et al.*, 2006; Kellogg *et al.*, 2009; Gray *et al*., 2011) were the most abundant phylum within the microbiome. In some cases, Actinobacteria (Yakimov *et al*., 2006; Hansson *et al.*, 2009) and Verrucomicrobia (Neulinger *et al*., 2008) were also highly abundant contributors to the CWC microbiome.

Only two CWC studies to date have explored microbiome differences/similarities across microhabitats, focusing on the reef-forming hexacoral *Lophelia pertusa* (Shöttner *et al.*, 2009; van Bleijswijk *et al.*, 2015). However, only one characterized the bacterial community composition across microhabitats (van Bleijswijk *et al.*, 2015); in the other study, Automated Ribosomal Intergenic Spacer Analysis (ARISA) profiles were used

Table 1. 1. Summary of available cold-water coral microbiome studies and their associated methodologies including the most dominant taxa (to lowest taxonomic classification) and their relative contribution if available.

Coral Specimen	Location	Depth Estimate (m)	Micro-habitat[1]	Samples	Method[2]	rRNA Region	Avg. # of Sequences	Avg. # OTU	Dominant Taxa/ Contribution (%)	Reference
Octocorallia										
Bamboo coral	Gulf of Alaska	634-3300	T/S/M	4	CL	NA[3]	136	NA[3]	α-Proteobacteria and Tenericutes	Penn *et al.*, 2006
Lophelia pertusa	Southern Italy	784	T/M	3	CL	NA[3]	37	14.5	*Holophaga-Acidobacterium* (55%)	Yakimov *et al.*, 2006
Iciligorgia schrammi	NW Atlantic Florida coast	45	T/S/M	1	CL	NA[3]	NA[3]	7.1	α-Proteobacteria (18%)	Brück *et al.*, 2007
Leptogorgia minimata	NW Atlantic Florida coast	45	T/S/M	1	CL	NA[3]	NA[3]	7.5	γ-Proteobacteria (33%)	Brück *et al.*, 2007
Swiftia exertia	NW Atlantic Florida coast	45	T/S/M	1	CL	NA[3]	NA[3]	7.5	α-Proteobacteria (26%)	Brück *et al.*, 2007
Cryogorgia koolsae	Aleutian Archipelago	98	T/S/M	2	CL	NA[3]	35	25.5	Tenericutes and α-, γ-Proteobacteria	Gray *et al.*, 2011
Plumarella superba	Aleutian Archipelago	117	T/S/M	2	CL	NA[3]	53	20	Tenericutes and α-, γ-Proteobacteria	Gray *et al.*, 2011
Eunicella cavolini	NW Mediterranean Sea	24-41	T/S/M	9	454	V1-V2	7659	299	*Endozoicomonas* (61%)	Bayer *et al.*, 2013
Paramuricea clavata	NW Mediterranean Sea	20	T	5	CL	V1-V4	140	36.3	Hahellaceae	La Rivière *et al.*, 2013
Paramuricea clavata	Mediterranean Sea	30-90	T/M	5	454	V6	NA[3]	NA	*Endozoicomonas* (90%)	Vezzuli *et al.*, 2013
Eunicella verrucosa	SW England	6-27	T/S/M	37	CL	NA[3]	NA[3]	11.3	γ-Proteobacteria	Ransome *et al.*, 2014
Muricea californica	NE Pacific California Coast	9-16	T/S/M	7	MiSeq	V4-V6	NA[3]	~312	Mycoplasma	Holm and Heidelberg, 2016
Muricea fruticose	NE Pacific California Coast	8-11	T/S/M	9	MiSeq	V4-V6	NA[3]	~470	Mycoplasma	Holm and Heidelberg, 2016
Paramuricea placomus	Baltimore Canyon	379-382	T/M	3	454	V4-V5	5734	126.7	γ-Proteobacteria	Kellogg et al., 2016
Alcyonium grandiflorum	Norfolk Canyon	474	T/M	1	454	V4-V5	13216	423	Proteobacteria	Lawler *et al.*, 2016
Anthothela grandiflora	Baltimore Canyon	500	T/M	8	454	V4-V5	21910	52.3	Proteobacteria and Spirochaetes	Lawler *et al.*, 2016
Anthothela grandiflora	Norfolk Canyon	576	T/M	4	454	V4-V5	87893	63.3	Spirochaetes	Lawler *et al.*, 2016

Anthothela sp.	Baltimore Canyon	524	T/M	1	454	V4-V5	13039	49	Spirochaetes	Lawler *et al.*, 2016
Anthothela sp.	Norfolk Canyon	570	T/M	2	454	V4-V5	143713	141	Proteobacteria	Lawler *et al.*, 2016
Corallium rubrum	NW Mediterranean Sea	30-40	S	23	MiSeq	V1-V2	NA[3]	250	Spirochaetales (68%)	van de Water *et al.*, 2016
Eunicella cavolini	Mediterranean Sea	30-40	S	18	454	V1-V2	NA[3]	57-86	*Endozoicomonas*	van de Water *et al.*, 2017
Eunicella singularis	Mediterranean Sea	30-40	S	20	454	V1-V2	NA[3]	44-133	*Endozoicomonas*	van de Water *et al.*, 2017
Eunicella verrucosa	Mediterranean Sea	30-40	S	3	454	V1-V2	NA[3]	116	*Endozoicomonas*	van de Water *et al.*, 2017
Leptogorgia sarmentosa	Mediterranean Sea	30-40	S	9	454	V1-V2	NA[3]	64-91	*Endozoicomonas*	van de Water *et al.*, 2017
Paramuricea clavata	Mediterranean Sea	30-40	S	4	454	V1-V2	NA[3]	17	*Endozoicomonas*	van de Water *et al.*, 2017
Primnoa resedaeformis	Baltimore Canyon	383-508	T/M	9	454	V4-V5	12494	189.4	Proteobacteria	Goldsmith *et al.*, 2018
Primnoa resedaeformis	Norfolk Canyon	411-576	T/M	5	454	V4-V5	3773	206.8	Proteobacteria	Goldsmith *et al.*, 2018
Primnoa pacifica	Gulf of Alaska	10-16	T/M	6	454	V4-V5	31710	78.5	Rhabdochlamydiaceae	Goldsmith *et al.*, 2018
Hexacorallia										
Black coral	Gulf of Alaska	1100-1950	T/S/M	1	CL	NA[3]	47	NA[3]	α-Proteobacteria	Penn *et al.*, 2006
Lophelia pertusa	Southern Italy	784	T/M	3	CL	NA[3]	37	14.5	*Holophaga-Acidobacterium* (55%)	Yakimov *et al.*, 2006
Lophelia pertusa (Red)	Mid Norway	54-264	T/S/M	12	CL	NA[3]	163	54	Verrucomucrobia (28%) and α-Proteobacteria (28%)	Neulinger *et al.*, 2008
Lophelia pertusa (White)	Mid Norway	54-264	T/S/M	15	CL	NA[3]	177	27	α-Proteobacteria (61%)	Neulinger *et al.*, 2008
Lophelia pertusa	NE Atlantic Ireland Coast	573-781	NA[3]	NA[3]	CL	NA[3]	NA[3]	NA[3]	NA[3]	Hansson *et al.*, 2009
Madrepora oculata	NE Atlantic Ireland Coast	573-781	NA[3]	NA[3]	CL	NA[3]	NA[3]	NA[3]	NA[3]	Hansson *et al.*, 2009
Lophelia pertusa (White)	NE Gulf of Mexico	310-474	T/S/M	7	CL	NA[3]	73	273	Tenericutes (52%) and γ-Proteobacteria (33%)	Kellogg *et al.*, 2009
Lophelia pertusa	Langenuen Fjord	167	M	12	ARISA	NA[3]	NA[3]	7	NA[3]	Shöttner *et al.*, 2009
Lophelia pertusa	Langenuen Fjord	167	S	12	ARISA	NA[3]	NA[3]	14	NA[3]	Shöttner *et al.*, 2009
Lophelia pertusa	NE Gulf of Mexico	315-500	T/S/M	NA[3]	CL	NA[3]	196	13.9	γ-Proteobacteria	Galkiewicz *et al.*, 2011
Lophelia pertusa	NE Atlantic Rockall Bank	528-568	M	2	454	V4	14000	1761	γ-Proteobacteria (49%)	van Bleijswijk *et al.*, 2015
Lophelia pertusa	NE Atlantic	528-635	S	6	454	V4	14000	1878	γ-Proteobacteria	van Bleijswijk *et al.*, 2015

	Rockall Bank									
Lophelia pertusa	NW Mediterranean Sea	520	T	9	454	V1-V3	2518	13.9	γ-Proteobacteria and α-Proteobacteria	Meistertzheim *et al.*, 2016
Madrepora oculata	NW Mediterranean Sea	520	T	8	454	V3	4690	9.5	γ-Proteobacteria (74-99%)	Meistertzheim *et al.*, 2016
Lophelia pertusa	Gulf of Mexico	487-490	T/M	3	454	V4-V5	102026	206.7	Sphingomonadaceae	Kellogg *et al.*, 2017
Lophelia pertusa	Gulf of Mexico	397-413	T/M	3	454	V4-V5	59159	313	Sphingomonadaceae	Kellogg *et al.*, 2017
Lophelia pertusa	Gulf of Mexico	504-543	T/M	3	454	V4-V5	87477	105	Sphingomonadaceae	Kellogg *et al.*, 2017
Lophelia pertusa	NW Atlantic Florida Coast	743-751	T/M	3	454	V4-V5	85870	250.7	Unclassified Oceanospirillales	Kellogg *et al.*, 2017
Dendrophyllia sp.	Red Sea	625-630	T/S/M	4	MiSeq	V5-V6	30625	329	Alteromonadaceae (19%)	Rothig et al., 2017
Eguchipsammia fistula	Red Sea	314-320	T/S/M	4	MiSeq	V5-V6	31274	475	γ-Proteobacteria (31%)	Rothig et al., 2017
Rhizotrochus typus	Red Sea	970-993	T/S/M	4	MiSeq	V5-V6	43228	272	Proteobacteria	Rothig *et al.*, 2017

[1]Coral compartments represented by the microhabitats tissue (T), mucus (M), and skeleton (S).
[2]Sequencing methodologies are clone library (CL), 454 Pyrosequencing (454), and Illumina MiSeq (MiSeq).
[3]NA notation is indicative of information not present in the study or available from the methodology.

to broadly compare community diversity between microhabitats (Shöttner *et al.* 2009). Shöttner *et al.* (2009) found a significant difference in bacterial community structure between *L. pertusa* mucus and skeleton, with mucus housing more bacteria operational taxonomic units (OTUs) than skeleton. Interestingly, *Endozoicomonas* was observed in *L. pertusa* mucus and near-bottom seawater but was not found in sediment or overlying water samples collected at 5 and 10 m above the seafloor, suggesting that the *Endozoicomonas* in surrounding near-bottom seawater may originate from dissolving mucus (van Bleijswijk *et al.*, 2015). The apparent absence of *Endozoicomonas* in sediment and overlying seawater may provide some support that this bacterium is important to the coral (van Bleijswijk *et al.*, 2015). Within *L. pertusa*, *Mycoplasma* was found in low abundance in tissues, but was absent in mucus (van Bleijswijk *et al.*, 2015). Based on the few studies that focused on CWC microhabitat microbiome differences, it appears that there is variation between microhabitats. Future studies should consider separate coral microhabitats in a broader range of CWC to resolve the extent of such microbiome differences across taxa.

Due to a recent focus on the core microbiome in warm-water corals, some of the more recent CWC studies have also explored bacterial taxa that are conserved across samples of particular coral taxa (Bayer *et al.*, 2013; Kellogg *et al.*, 2016; Lawler *et al.*, 2016; van de Water *et al*, 2016; Kellogg *et al.*, 2017; Röthig *et al.*, 2017; van de Water *et al*., 2017; Goldsmith *et al.*, 2018). To define the core microbiome, studies have used different boundaries in determining which bacterial taxon or OTU can be considered part of that coral's core microbiome. For example, cut-off boundaries ranging from 30-100%

have been explored, wherein an OTU must be present in at least some proportion (e.g. 30%) of the coral samples to be considered part of the core microbiome (Hernandez-Agreda *et al.,* 2017; Sweet and Bulling, 2017). This variability in defining what constitutes a core taxon within the microbiome results in a requirement for a standard cut-off to be used to confidently compare data from different coral microbiome studies. Across CWC studies, various *Endozoicomonas* ribotypes (varying OTUs of the same bacterial genera) were found in the core microbiome of *Corallium rubrum* (van de Water *et al.,* 2016), *Eunicella cavolini* (Bayer *et al.,* 2013; van de Water *et al.,* 2017), *Eunicella verrucose* (Ransome *et al.,* 2014), and *Eunicella singularis* (van de Water *et al.,* 2017). Several *Endozoicomonas* ribotypes were observed across many microbiome studies on warm-water corals, suggesting that *Endozoicomonas* is a key player in an overall coral core microbiome (Blackall *et al.,* 2015; Neave *et al.,* 2017; Sweet and Bulling, 2017). Another notable core microbiome genus is *Propionibacterium*, which was reported in *Eunicella cavolini* (Bayer *et al.,* 2013) and *Lophelia pertusa* (Kellogg *et al.,* 2017). A different *Propionibacterium* OTU was shared among the CWCs *Paramuricea placomus* (Kellogg *et al*., 2016), *Anthothela sp.* (Lawler *et al.,* 2016), and two species of *Primnoa* (Goldsmith *et al.,* 2018). Bacteria found within the orders Spirochaetales and Oceanospirillales were observed in the core microbiome of *Corallium rubrum* (van de Water *et al.,* 2016) and *Anthothela sp.* (Lawler *et al.,* 2016). In a study conducted by Brück *et al.* (2007), three co-occurring cold-water corals, *Iciligorgia schrammi*, *Leptogorgia minimata*, and *Swiftia exertia* shared a strain of *Ralstonia pickettii* and multiple species of *Stenotrophomonas sp.* across all coral microbiomes. Lastly, the core microbiomes of two CWCs, *Dendrophyllia sp.* and *Eunicella fistula*, were almost entirely

dominated by Alpha-, Beta-, Delta-, and Epsilonproteobacteria (Röthig *et al.*, 2017). However, while there are variances among microbiomes, some bacterial taxa may be shared among CWCs, as well as between CWC and warm-water corals, despite temperature and depth gradients. Lastly, observations of bacterial similarities across species may be subject to sequencing and kit contamination, and interpretations remain speculative.

Geographical location may have some influence on the observed similarities in CWC microbiomes. For example, *Paramuricea clavata* colonies sampled close to each other had similar dominant bacterial associates (La Rivière *et al*., 2013; Vezzulli *et al.*, 2013; van de Water *et al.*, 2017). However, due to the paucity in CWC microbiome studies, it is difficult to confidently compare multiple coral species across locations. As there has been particular focus on the CWC *Lophelia pertusa*, some tentative patterns in bacterial composition relative to varying sampling locations can be observed. *L. pertusa* samples from Yakimov *et al.* (2006) recovered in southern Italy showed no sequence similarities with *L. pertusa* samples recovered in Norway (Neulinger *et al.*, 2008). Samples of *L. pertusa* recovered in the NE Gulf of Mexico by Kellogg *et al.* (2009) and Galkiewicz *et al.* (2011) also showed no bacterial 16S rRNA gene sequence (cultured isolates and clones) similarities with either of the previous studies (Yakimov *et al.*, 2006; Neulinger *et al.*, 2008). Furthermore, Meistertzheim *et al.* (2016) reported that their findings on *L. pertusa* further supported the evident spatial variations in bacterial community structure reported in all previous studies on *L. pertusa* (Yakimov *et al.*, 2006; Neulinger *et al.*, 2008; Kellogg *et al.*, 2009; Shöttner *et al.*, 2009; Hansson *et al.*, 2009;

Galkiewicz *et al.*, 2011; van Bleijswijk *et al.,* 2015). Importantly, the studies presented here on *L. pertusa* have different extraction, amplification, sequencing, and statistical analysis methodologies, and therefore care must be taken in interpreting trends with relation to geographical locations.

Kellogg *et al.* (2017) reported that *L. pertusa* collected from the SW Atlantic (off the coast of Florida) shared *Propionibacterium* OTU 4447394 (99% identity and 100% coverage) with three reads of *L. pertusa* mucus from the NE Atlantic (van Bleijswijk *et al.*, 2015) and two *Propionibacterium* clones isolated from *L. pertusa* collected in a Norwegian fjord (Neulinger *et al.*, 2008). *Propionibacterium* has been documented in the core microbiome of *L. pertusa* (Kellogg *et al.,* 2017) and in various shallow tropical corals (Ainsworth *et al.,* 2015; Hester *et al.,* 2016). Kellogg *et al.* (2017) also observed that *L. pertusa* shared an OTU of *Mycoplasma* with an octocoral from the Aleutian Islands, *Cryogorgia koolsae* (Gray *et al.,* 2011), and some deep-sea bamboo corals (Penn *et al.,* 2006); however, this same OTU was not reported in any of the previous studies analyzing the microbiome of *L. pertusa.* Lastly, Kellogg *et al.* (2017) mentioned that at a higher taxonomic level, Proteobacteria was the dominant phylum in their study of *L. pertusa* using pyrosequencing of the V4-V5 hypervariable regions of the 16S rRNA gene, which agreed with other studies using clone libraries (Neulinger *et al.,* 2008; Kellogg *et al.*, 2009; Galkiewicz *et al.,* 2011), and pyrosequencing of the V1-V3 (Meistertzheim *et al.*, 2016) and V4 (van Bleijswijk *et al.,* 2015) hypervariable regions, despite differences in extraction technique and geographical location. These comparisons indicate that some OTUs, or closely related groups of OTUs may be specific to one or more coral species

across broad geographical distributions. This apparent specificity suggests that those particular bacteria may be important for the coral host.

Bacteria can potentially have a functional role within the coral host: they may be involved in sulfur and nitrogen metabolism (Raina *et al.*, 2009; Middelburg *et al.*, 2015; Bednarz *et al.*, 2017) and holobiont defense (Reshef *et al.*, 2006; Ritchie, 2006). Sulfur metabolism in corals was initially observed in shallow-water tropical corals (Raina *et al.,* 2009), and recently, chemoautotrophy was reported in the cold-water scleractinian coral *Lophelia pertusa* (Middelburg *et al.,* 2015). Chemolithoautotrophic bacteria were suggested to play a role in moderating the flow of carbon in *L. pertusa*, as significant rates of inorganic carbon fixation were observed in this species. Chemoautotrophs such as sulfur oxidizing bacteria (previously observed in *L. pertusa*; Neulinger *et al.,* 2008) could be contributing to inorganic carbon fixation in this CWC (Middelburg *et al.,* 2015). The same study also reported isotopic evidence for bacterial nitrification, denitrification, nitrogen assimilation and regeneration in *L. pertusa*, indicating that a vast microbial metabolic repertoire can help those corals survive in resource-limited deep-water habitats (Middelburg *et al.,* 2015). More recently, bacterial nitrogen cycles were proposed to be present in the alcyonacean *Anthothela sp.* (Lawler *et al.,* 2016) and *Paramuricea placomus* (Kellogg *et al.,* 2016). In these CWCs, *Bacillus* and Kiloniellales are suggested to be potential denitrifiers and *Bradyrhizobium*, *Magnetospirillum*, and *Spirochaeta* are potential nitrogen fixers (Kellogg *et al.,* 2016; Lawler *et al.,* 2016). In *P. placomus*, Pirellulaceae were proposed to be nitrifiers due to their ammonia oxidizing function in sponges (Kellogg *et al.,* 2016). *Propionibacterium*, *Photobacterium*, and

Oceanospirillales from CWCs were suggested to reduce nitrogen, and lastly Campylobacterales may be involved in ammonification (Kellogg *et al.*, 2016; Lawler *et al.*, 2016).

Finally, bacterial defense mechanisms may be features of the coral holobiont, as bacteria found in the surface mucus layer can constitute a first line of defense for corals (Sweet and Bulling, 2017). Specifically, antimicrobial properties have been explored in tropical corals (Ritchie, 2006), and more recently an antimicrobial compound, tropodithietic acid (TDA) has been isolated from *Pseudovibrio* sp. P12, a coral-associated bacterium that metabolizes dimethylsulfoniopropionate (DMSP) to inhibit the growth of *Vibrio* pathogens (Raina *et al.*, 2016). A *Pseudovibrio* OTU was also abundant in healthy zooxanthellate scleractinian colonies of *Cladocora caespitosa* from the Mediterranean Sea, but present in significantly lower abundance in diseased colonies (Rubio-Portillo *et al.*, 2018). DMSP has been observed to accumulate in CWC such as *L. pertusa* (Burdett *et al.*, 2014) from their diet, and CWC presumably require pathways to metabolize and remove DMSP. It is possible (but yet unproven) that similar DMSP-derived antimicrobials may be present in CWCs, through the action of bacterial associates. Further metagenomic research focusing on the CWC microbiome and pathobiome is required to understand which bacterial groups are present in healthy and diseased corals, and functional metatranscriptomics could provide insights on interactions within the coral holobiont.

1.4 *Paragorgia arborea* and *Primnoa resedaeformis*

Paragorgia arborea and *Primnoa resedaeformis* are two very common co-occurring alcyonacean habitat-forming framework corals found throughout the Northern Atlantic Ocean. Documented from the continental slopes of Maine, northward to the eastern coasts of Canada, the southern continental slopes of Greenland, through the Reykjanes Ridge of Iceland, down the coasts of Norway, through the Celtic Sea and in the Bay of Biscay, *P. arborea* and *P. resedaeformis* occupy a large depth gradient, across a broad range of habitat types (Buhl-Mortensen *et al.*,2015; Brooke *et al.,* 2017). *Paragorgia arborea*, commonly referred to as bubblegum coral, occupies depths of 200 – 1300 m, within a temperature range of 3 - 8˚C, across many habitat types (sediment with emergent hard substrates, isolated rock, consolidated sediment/hard pavement, large boulders, and walls/steep slopes), and has been suggested to be a brooding coral (producing and releasing larvae from parental colony polyps for nearby settlement, resulting in low offspring dispersal) (Tendal, 1992; Murillo *et al.,* 2011; Lacharité and Metaxas, 2013; Buhl-Mortensen *et al.,* 2015; Brooke, *et al.,* 2017). *Primnoa resedaeformis*, often referred to as popcorn coral, occupies a depth range of 150 – 1500 m, within a wider temperature range than *P. arborea* (1-11˚C), across fewer habitat types than *P. arborea* (isolated rock, large boulders, and walls/steep slopes), and has been suggested to broadcast spawn its offspring (buoyant gametes released into water column until fertilization and settlement down current, resulting in large offspring dispersal) (Lacharité and Metaxas, 2013; Buhl-Mortensen *et al.,* 2015; Brooke *et al.,* 2017; Miles, 2018). *P. arborea* and *P. resedaeformis* are both crucial habitat-providers as they are

reportedly very common and their arborescent morphology creates dense thickets (Buhl-Mortensen and Mortensen, 2004; Buhl-Mortensen *et al.*, 2015).

1.5 Thesis objectives

This thesis addresses a clear knowledge gap on cold-water corals and their microbiomes. Chapters 2 and 3 are an exploration of two common and co-occurring (though biologically different) CWCs sampled from submarine canyons in the Gulf of Maine to provide novel information on CWC microbiomes. Chapter 2 focuses on the alcyonacean *Paragorgia arborea* and characterizes the bacterial groups associated with two coral microhabitats: 1) surficial mucus, and 2) polyp tissues and the skeletal matrix. Following the examination of *P. arborea*, a preliminary exploration of the surficial mucus of the gorgonian *Primnoa resedaeformis* is presented in Chapter 3 and our samples are compared with a recent study that has described the bacterial associates of *P. resedaeformis* (Goldsmith *et al.*, 2018) to identify trends and similarities between studies. This thesis provides novel CWC microbiome information to be used in conjunction with future CWC exploration. Understanding the microbial interactions in the coral holobiont and how the microbial assemblage shifts after environmental/anthropogenic perturbations may help inform future coral conservation and management activities.

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Co-Authorship Statement

I am the first author on all manuscripts generated from this thesis. I was primarily responsible for sampling protocols and extractions, statistical analyses, and drafting the manuscripts.

Dr. Suzanne Dufour is the senior co-author on all the manuscripts in this thesis. Dr. Dufour was involved in guidance, conceptual design, and review of all chapters, including the senior author of Chapter 2 for publication.

Joost Verhoeven collected the samples aboard the research cruise, was a large support in sample processing/extraction, bioinformatics, data interpretation, provided guidance in all aspects and stages, reviewed and is a co-author on Chapter 2

Chapter 2: Bacterial communities in tissues and surficial mucus of the cold-water coral *Paragorgia arborea*

2.1 Abstract

Coral bacterial associates can play important functional roles for the holobiont, such as nitrogen cycling, nutrient processing, and supporting immunity. While bacteria found within the microbiome of corals may benefit the host, they can also be linked to pathogenesis. In the deep-sea, cold-water corals, like their warm shallow-water counterparts, host bacterial communities, but have received little attention due to logistical constraints in sampling. In particular, bacteria associated with surficial mucus of cold-water corals have not yet been investigated. Here, tissue and mucus samples of *Paragorgia arborea* were collected from three submarine canyons along the continental slope of the Gulf of Maine. Bacterial DNA was extracted from tissue and mucus samples and sequencing of the V6-V8 hypervariable region of the 16S rRNA gene was performed using Illumina MiSeq. The bacterial communities associated with *P. arborea* compartments (tissue and mucus) and sampling locations (canyon) differed significantly in composition. Proteobacteria, Tenericutes, and Spirochaetes were the dominant phyla across the majority of coral tissue samples, with Gammaproteobacteria and Alphaproteobacteria identified as the largest Proteobacteria contributors across all samples. OTUs belonging to the taxa *Spirochaeta*, *Mycoplasma*, Flavobacteriaceae, Terasakiellaceae, Campylobacterales and Rickettsiales were identified as biomarkers (bacterial taxa significantly more abundant in a specific coral microhabitat) of *P. arborea* tissues, while *Paracoccus* was a biomarker of *P. arborea* mucus. Many of the recovered

biomarker taxa may be involved in nitrogen cycling. Representatives from several bacterial families (Vibrionaceae, Campylobacteraceae, Rhodobacteraceae, Flavobacteraceae, and Burkholderiaceae) previously reported in diseased scleractinians, were present in *P. arborea* as rare bacterial taxa. Characterizing the bacterial associates present in visibly healthy coral colonies provides a benchmark of dominant and rare bacterial groups present in the cold-water coral holobiont. This is the first characterization of bacterial groups associated with *P. arborea*, examining both tissue- and mucus-specific communities.

2.2 Introduction

Corals host a wide range of microbial associates, including bacteria, eukaryotes, archaea, and viruses (Ainsworth *et al.*, 2017). Of these, coral-associated bacteria (herein referred to colloquially as the "microbiome") may play important roles in host nitrogen metabolism (Grover *et al*., 2014; Rädecker *et al*., 2015; Kellogg *et al.*, 2016; Lawler *et al*., 2016), nutrient cycling (Wild *et al*., 2004; Naumann *et al.*, 2009), and antibacterial mechanisms (Kelman *et al*., 1998; Sutherland *et al*. 2004; Ritchie, 2006). The microbiome typically helps maintain coral health and provides a defense system against disease (Krediet *et al.*, 2013). However, it can also harbour low abundances of pathogens that may become dominant when subjected to environmental stressors; such dysbiosis may lead to coral disease and/or death (Mouchka *et al*., 2010; Egan and Gardiner, 2016). The coral microbiome likely consists of a combination of commensals, transients, and long-term, stable partners selected by the host (Ainsworth *et al.*, 2015), and is distributed

among several anatomical compartments: the skeletal tissue, polyp tissue, and the external surface mucopolysaccharide layer (SML) (Bourne and Munn, 2005; Brown and Bythell, 2005; Sweet *et al*., 2011; Krediet *et al*., 2013; Ainsworth, *et al.* 2015; Pollock *et al.*, 2018).

The SML has been reported as a first line of defense to protect the coral host against pathogens from the surrounding water column (Sweet *et al.*, 2011; Bythell and Wild, 2011; Glasl *et al.*, 2016). Additionally, the mucus is important for particulate feeding and can act as an energy carrier/provider and as a particle trap, as it is sloughed-off into the surrounding waters (Coffroth, 1991; Wild *et al*., 2008; Bythell and Wild, 2011). In some shallow-water scleractinian corals, mucus is subject to diurnal or hourly replacement cycles, and its bacterial biodiversity could be changing with these cycles (Ainsworth *et al.*, 2010; Sweet *et al.*, 2011). The contrast between the more stable tissue/skeletal regions and the frequently renewing mucus compartment can explain differences in bacterial compositions between those coral microhabitats (Bourne and Munn, 2005; Sweet *et al.*, 201l; Pollock *et al.*, 2018).

Like their warm, shallow-water counterparts, cold-water corals found in the deep-sea (beyond the photic zone to over 2000 m depth) host diverse microbial communities. However, they are difficult to access due to logistical and financial constraints (Kellogg *et al*., 2016) and therefore little is known about them, and even less about their microbiomes (Holm and Heidelberg, 2016). In particular, the bacterial communities colonizing the SML of corals from deep-sea regions remain unexplored and may differ from those of

shallow-water corals due to expected discrepancies in physical conditions within the mucus layer (e.g. the absence of zooxanthellae-linked diel oxygen fluctuations in the SML of corals from deep habitats) and functions (e.g. a greater importance of particulate feeding in the deep sea). This study aims to characterize the bacterial associates of the cold-water alcyonacean coral, *Paragorgia arborea* (Linnaeus, 1758). *P. arborea* has been suggested to be a brooding azooxanthellate coral (Roberts *et al.*, 2006; Lacharité and Metaxas, 2013) and is widely distributed in the Northwestern and Northeastern Atlantic, from the Gulf of Maine northward along the eastern coasts of Canada and the Davis Strait, to the continental slopes of Greenland, along the Reykjanes Ridge of Iceland, to the shelf of Norway (Cairns and Bayer, 2005; Mortensen and Buhl-Mortensen, 2005; Buhl-Mortensen *et al.,* 2015; Brooke *et al.,* 2017). *P. arborea* occupies a wide range of depth (200-1200 m) where temperatures range between 3-8 ˚C, serving as a foundation species across a broad geographic area (Buhl-Mortensen *et al.,* 2015). The arborescent morphology of *P. arborea* provides niches for prey refuge and creates habitats for facultative and obligate deep-sea symbionts (Buhl-Mortensen and Mortensen, 2004; Lacharité and Metaxas, 2013).

The bacterial communities of tropical, cold water, and deep-sea Alcyonacea and gorgonians are typically dominated by Proteobacteria, specifically Gammaproteobacteria (Penn *et al*., 2006; Bayer *et al*., 2013; Correa *et al*., 2013; La Rivière *et al*., 2013; Vezzulli *et al*., 2013; Ransome *et al*., 2014; La Rivière *et al*., 2015; Kellogg *et al*., 2016; Robertson *et al*., 2016). Additionally, Alphaproteobacteria were identified as notable contributors to the microbial consortia across multiple studies on alcyonaceans and

gorgonians (Gray *et al*., 2011; Bayer *et al*., 2013; Correa *et al*., 2013; La Rivière *et al*., 2013; Vezzulli *et al*., 2013; Ransome *et al*., 2014; La Rivière *et al*., 2015; Holm and Heidelberg, 2016; Kellogg *et al*., 2016; Robertson *et al*., 2016). While Proteobacteria remain largely dominant across alcyonacean microbiomes, in some cases Spirochaetes (Holm and Heidelberg, 2016; Lawler *et al*., 2016; van de Water *et al.*, 2016) and Tenericutes (Gray *et al*., 2011; Holm and Heidelberg, 2016) were the most abundant bacterial associates.

In deep-sea ecosystems, food sources are limited and variable and therefore alternate mechanisms are required by benthic invertebrates for metabolic processes (Mueller *et al.* 2014; Middelburg *et al.,* 2015). Such mechanisms include bacteria-mediated nitrogen cycling, which has been documented in shallow-water corals (Wegley *et al.*, 2007; Rädecker *et al*., 2015), and more recently in the cold-water scleractinian coral *Lophelia pertusa* (Middelburg *et al.,* 2015), and postulated for the octocoral *Paramuricea placomus* (Kellogg *et al.*, 2016) and Anthothelidae corals (Lawler *et al.,* 2016). In these studies, several bacterial species are thought to facilitate nitrogen metabolism, with *Spirochaeta* implicated in nitrogen fixation (Lawler *et al.*, 2016), Pirellulaceae in nitrification (Kellogg *et al.*, 2016), Kiloniellales and *Bacillus* spp. in denitrification (Verbaendert *et al.*, 2011; Kellogg *et al.*, 2016; Lawler *et al.,* 2016), *Propionibacterium* and Oceanospirillales in nitrogen reduction (Kellogg *et al.*, 2016; Lawler *et al.,* 2016), and Campylobacterales in nitrate/nitrite ammonification in a deep-sea octocoral (Kellogg *et al.*, 2016). *P. arborea* has been observed to occupy the same benthic distribution and topography as *L. pertusa* (Buhl-Mortensen *et al.,* 2015) and *P.*

placomus (Buhl-Mortensen and Buhl-Mortensen, 2014), and may show similar host-bacterial interactions.

In the deep-sea, corals play a similar role to their shallow water counterparts, providing prey refuge and rugose habitats to many organisms (Hixon and Beets, 1993; Syms and Jones, 2000; Stone, 2006). However, coral ecosystems are threatened by several anthropogenic stressors and there is increasing concern that cold-water corals located along continental shelves are at risk from disturbances such as bottom trawling and oil exploration (Bavestrello *et al*., 1997; Fosså *et al*., 2002; Husebø *et al.*, 2002; Roberts *et al.*, 2006; Cordes *et al.*, 2016). As only a few cold-water corals have been studied, there is a knowledge gap regarding these organisms' bacterial communities and their functional roles in the host. Previous studies have reported variability in bacterial compositions between octocoral genera (Brück *et al.*, 2007), between congeners (Holm and Heidelberg, 2016; Kellogg *et al.*, 2016), between coral microhabitats (Weinbauer *et al.*, 2012), within species and between sampling locations (Gray *et al.*, 2011). In this study, we characterized the bacterial associates of *P. arborea* across two compartments: (1) skeletal and polyp tissue, and (2) surface mucus, and examined the degree of similarity in bacterial composition across three study locations in the Gulf of Maine (ranging in depth between 411-700 m) to explore the effects of relative sampling location proximity and depth on bacterial composition. Due to biological traits observed in *P. arborea* (i.e. abundant mucus production; Etnoyer *et al.*, 2006, and brooding; Lacharité and Metaxas, 2013), and the previously observed differences in bacterial compositions in tissues and mucus in other coral species, we expect to see between-compartment variation

as well as variability among sampling locations. We compared common/dominant bacterial associates, searched for taxa that could be markers of either tissue or mucus due to significant differences in relative abundance, and identified rare bacterial taxa of particular interest. To our knowledge, this work represents the first description of the bacterial associates recovered from tissue and mucus samples of the alcyonacean coral *P. arborea*.

2.3 Methods

2.3.1 Sample collection and study sites

Paragorgia arborea colony fragments were collected from multiple submarine canyons: Nygren-Heezen Intercanyon (N40°51.96′, W66°32.74′, depth 700 m); Corsair Canyon (N41°21.26′, W66°5.39′, depth 411 m); and Georges Canyon (N41°16.48′, W66°11.59′, depth 423m), on the continental slope of the Gulf of Maine during a research cruise aboard the National Oceanic and Atmospheric Association (NOAA) Ship *Henry B. Bigelow* from June 8th-22nd, 2017. Coral colonies were located and sampled using CSSF-ROPOS (Canadian Scientific Submersible Facility, Remotely Operated Platform for Ocean Sciences), with individual fragments held in separate water-filled chambers until surfacing. Tissue fragments were then dissected from the coral stalk and placed in individual, sterile cryovials. Mucus samples were collected by gently rolling a sterile cotton swab over the exterior of the specimen where mucus was visible. Three tissue replicates, and one mucus swab were collected from individual colonies and frozen (-20^{o}C) until further analysis. At each dive location, a reference water sample was collected

from approximately 1 m from the bottom, within proximity of the coral colonies, using a remotely triggered Niskin bottle attached to the ROV. On board, 50 mL aliquots of each water sample were passed through individual 0.22 µm syringe filters (Millipore Sigma, Canada). The syringe filters were frozen at -20˚C until further analysis.

2.3.2 Nucleic acid extraction

Total DNA was extracted from *P. arborea* mucus and tissue samples, following the protocol described by Sunagawa *et al.* (2010) with modifications. Samples of coral tissue and skeleton (between 0.100 and 0.250 g total weight) were washed in 600 µL phosphate-buffered saline (PBS) three times to remove mucus and loosely associated bacteria. Once washed, the samples were flash frozen in liquid nitrogen and crushed using a sterile mortar and pestle. Crushed samples were transferred to tubes included with the PowerViral Environmental RNA/DNA extraction kit (MO BIO, Carlsbad, CA, USA) containing 0.1 mm glass beads. DNA was extracted from water filters by adding 600 µL PV1 lysis buffer (MO BIO) into the filter, incubating for 2 min at room temperature, and subsequently purging the filter cartridge using a syringe to collect all lysis buffer. The process was repeated for the reverse side of the syringe filter and once more for the original starting side to ensure maximum PV1 based lysis and recovery. Mucus swabs were placed in microfuge tubes and vortexed with PV1 lysis buffer (MO BIO) for 10 min. After initial lysis, DNA was extracted according to the manufacturer's protocol. Prior to high throughput sequencing, the presence of bacterial DNA was confirmed by PCR amplification of 16S rRNA genes using the universal primers ECO8F (Edwards *et al.*,

1989) and 1492R (Stackebrandt and Liesack, 1993), DreamTaq Green PCR Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and thermal cycled: 30 cycles of 95ºC for 5 min, 95ºC for 30 sec, 45ºC for 30 sec, 72ºC for 1 min, and final extension of 72ºC for 2 min. Extracted DNA was checked for quality and quantity using NanoDrop™ 1000 (Thermo Fisher Scientific).

2.3.3 16S rRNA gene sequencing and processing

Coral DNA extracts were outsourced for sequencing at the Centre for Comparative Genomics and Evolutionary Bioinformatics (Dalhousie University, Halifax, Canada). Two times 300-bp paired-end sequencing of the 16S rRNA gene was performed using Illumina MiSeq v3, with all samples amplified using previously published primers targeting the V6-V8 regions (B969F/B1406R): V6 forward: 5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG and V8 reverse: 5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG (Comeau *et al.,* 2011).

Sequence data were processed using the in-house developed SPONS-2 pipeline, as described in Verhoeven and Dufour (2017), with a few modifications. In short, sequences were trimmed to remove both low-quality bases using Trimmomatic version 0.38 (20-base sliding window with a minimum average quality of 15 per base) and short reads (< 100 bases) (Bolger *et al.*, 2014). Reads that passed the initial quality check were merged using PEAR version 0.9 (Zhang *et al.*, 2014). Primers were trimmed from merged reads using CutAdapt (maximum error rate 0.2), filtering out reads that lack forward or reverse

primers (Martin, 2011). Reads with an average Phred score below 20 were removed as a final quality check prior to defining operational taxonomic units (OTU) using SWARM version 2.2 (Mahé *et al.*, 2015). The step wherein Swarm defines OTUs was modified so the maximum difference between amplicons (d) was increased from 1 to 3 (decreasing the potential for overestimating defined OTUs). Defined OTUs were analyzed using the RDP naïve Bayesian classifier (Wang *et al*., 2007) for taxonomic assignment, using the SILVA SSU database (release 132; Quast *et al.*, 2013) with a 51% minimum bootstrap confidence estimate when assigning taxonomy.

2.3.4 Bioinformatics and statistical analyses

Microbiome high-throughput sequence (HTS) datasets are compositional (Gloor *et al.*, 2017), and recent concerns have been raised regarding current microbiome analysis methodologies (i.e. normalizing HTS count data by rarefaction or other subsampling methods) (McMurdie and Holmes, 2014; Fernandes *et al.*, 2014). Here, we used a compositional analysis approach to compare the bacterial communities from coral and seawater samples.

OTU count and taxonomic data were imported in R for analyses (R Development Core Team, 2008). First, filtering was conducted to remove OTUs not classified as bacteria at the Kingdom level. Bacterial alpha diversity in each coral and water sample was then examined through the Hill's series of diversity indices. Sample count data were square-root transformed, and using the vegan package (Oksanen *et al.*, 2016), we

calculated the Hill's diversity series: the raw number of OTUs (H_0), the exponent of Shannon diversity (H_1), and the reciprocal of the Simpson's index (H_2). To test for significant differences between sample diversity index values across geographic locations and between anatomical compartments, t-tests were performed in PAST (Hammer *et al.*, 2001).

Before performing beta diversity analysis to compare bacterial community composition across samples, low abundance OTUs were filtered out (minimum proportional abundance: 0.5%) and zero counts were replaced with non-zero calculated values using the *count zero multiplicative* method from the R packages codaSeq and zCompositions (Martín-Fernández *et al.*, 2015; Palarea- Albaladejo and Martín-Fernández, 2015). The zero-adjusted data were then centered log-ratio (clr) transformed using the codaSeq package (Gloor *et al.*, 2017; Gloor and Reid, 2016). The clr is scale-invariant, meaning that the same ratio is expected from samples with different read counts (Gloor *et al.*, 2017). A Principal Component Analysis (PCA) was performed by plotting a singular value decomposition of clr transformed values to visualize beta diversity. We also performed hierarchical clustering using the "hclust" command, using a Euclidian distance matrix and agglomeration method "Ward. D2". Bacterial community composition bar graphs representing phyla contribution (≥1%) and class contribution (≥3%) per sample were produced to visualize trends in the hierarchical cluster analysis dendrogram.

Quantitative analyses were conducted to examine the meta-data factors contributing towards the observed variance in bacterial community structure using the vegan R package "adonis" command for permutational multivariate analysis of variance (PERMANOVA) using a Euclidian distance matrix. A two-way design was used for the PERMANOVA, where location (canyons) and compartment (mucus and tissue) were both considered as fixed factors, and the interaction between the two factors was tested. Lastly, pairwise quantitative (ANOVA-Like Differential Expression (ALDEx), Fernandes *et al.*, 2013) analysis was performed to identify compartment-specific OTUs, or biomarkers. For this, clr-transformed count data were analyzed using differential relative abundance tests generated by 128 Monte Carlo samples sourced from a Dirichlet distribution. This ALDEx analysis was performed using the ALDEx2 package in R (Fernandes *et al.*, 2013, 2014) to generate a list of OTUs (classified according to lowest available taxonomic rank) that possessed a significant association with either tissue or mucus. Here, we consider an OTU to be positively associated with tissue if the effect size is ≥ 1, and positively associated with mucus if the effect size is <-1.

2.4 Results

2.4.1 Alpha and beta diversity in coral bacterial assemblages

Rarefaction curve analysis showed that all samples were sequenced sufficiently (i.e. to a read depth considered representative of each sample's total microbial diversity), as indicated by the plateauing of the OTU count curves (aside from the Corsair Canyon seawater sample, which was omitted from further analysis; Figure 2.1). Sequence data

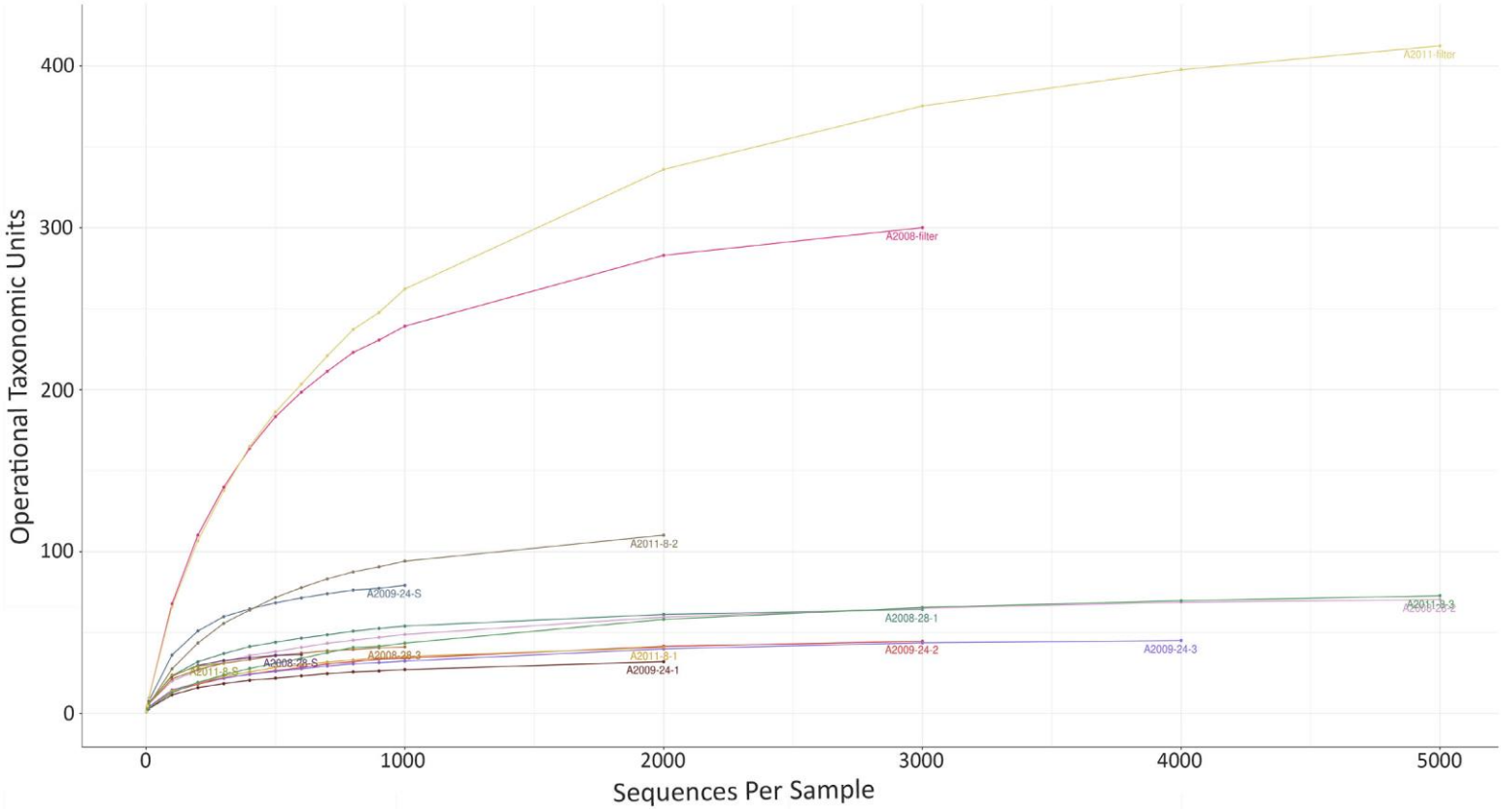

Figure 2. 1. Rarefaction curve showing the number of observed bacterial OTUs as a function of the number of sequences. *Paragorgia arborea* tissue and mucus samples (n = 12) and seawater samples (n = 2). See Collection ID in Table 2.1

were deposited in the NCBI short read archive linked to BioProject accession number PRJNA490387. The mucus swabs were amongst the lowest in sequencing depth, and reference seawater samples were among the highest (Table 2.1). Alpha and beta diversity measures were used to compare diversity trends and similarities between bacterial communities. The reference seawater samples were significantly higher than coral samples in all three Hill's series diversity indices for bacterial communities [H_0, H_1, H_2, two-sample t-test between coral samples (n=12) and seawater (n=2), $p<0.001$ for each metric]. There were no significant differences between coral compartments or among geographic locations for any of the Hill's series diversity indices (H_0, H_1, H_2, two-sample t-test between coral tissue (n=9) and mucus (n=3), $p>0.05$ for each metric).

Due to the significant differences in bacterial alpha diversity between seawater and coral samples, we chose to produce a principal component analysis (PCA) ordination using Euclidian distance to further visualize similarities in bacterial composition across samples (Figure 2.2). The PCA representation of *P. arborea* tissue, mucus, and surrounding seawater bacterial communities showed some separation between coral samples and the reference seawater along the primary PCA axis (PC1= 46.2% of the variation in the dataset) (Figure 2.2). The majority of coral samples clustered together, with some outliers. Differences between coral mucus samples were largely along the secondary PCA axis (PC2 = 16.2% of the variation) (Figure 2.2). PERMANOVA analysis uncovered a significant difference in bacterial composition between compartments (tissue and mucus) in *P. arborea* ($p=0.001$). Furthermore, sample geographic location (canyon)

Table 2. 1. Sample collection, description, number of reads from processed data, and Hill's series diversity summary statistics of bacterial communities determined by analysis of 16S rRNA sequence libraries.

Specimen ID	Collection ID	Species	Submarine Canyon	Description	Depth (m)	Number of Reads	$Hill_0$	$Hill_1$	$Hill_2$
PA-N28-T1	**A2008-28-1**	*P. arborea*	Nygren-Heezen	Tissue Replicate 1	700	3392	69	44.94	29.95
PA-N28-T2	**A2008-28-2**	*P. arborea*	Nygren-Heezen	Tissue Replicate 2	700	6060	77	43.25	26.22
PA-N28-T3	**A2008-28-3**	*P. arborea*	Nygren-Heezen	Tissue Replicate 3	700	1929	48	32.91	23.32
PA-N28-MU	**A2008-28-S**	*P. arborea*	Nygren-Heezen	Mucus	700	696	39	30.72	23.97
PA-C24-T1	**A2009-24-1**	*P. arborea*	Corsair	Tissue Replicate 1	411	2227	37	19.17	9.5
PA-C24-T2	**A2009-24-2**	*P. arborea*	Corsair	Tissue Replicate 2	411	3274	49	26.02	14.16
PA-C24-T3	**A2009-24-3**	*P. arborea*	Corsair	Tissue Replicate 3	411	4038	49	25.88	14.25
PA-C24-MU	**A2009-24-S**	*P. arborea*	Corsair	Mucus	411	1459	87	69.53	54.21
PA-G08-T1	**A2011-8-1**	P. arborea	Georges	Tissue Replicate 1	423	2744	46	25.6	12.72
PA-G08-T2	**A2011-8-2**	*P. arborea*	Georges	Tissue Replicate 2	423	2553	121	85.73	48.14
PA-G08-T3	**A2011-8-3**	*P. arborea*	Georges	Tissue Replicate 3	423	5220	81	39.9	15.86
PA-G08-MU	**A2011-8-S**	*P. arborea*	Georges	Mucus	423	363	35	28.66	22.36
WA-N08	**A2008-filter**	Seawater	Nygren-Heezen	Water	837	3997	322	276.94	230.82
WA-G11	**A2011-filter**	Seawater	Georges	Water	606	6076	446	361.32	276.65

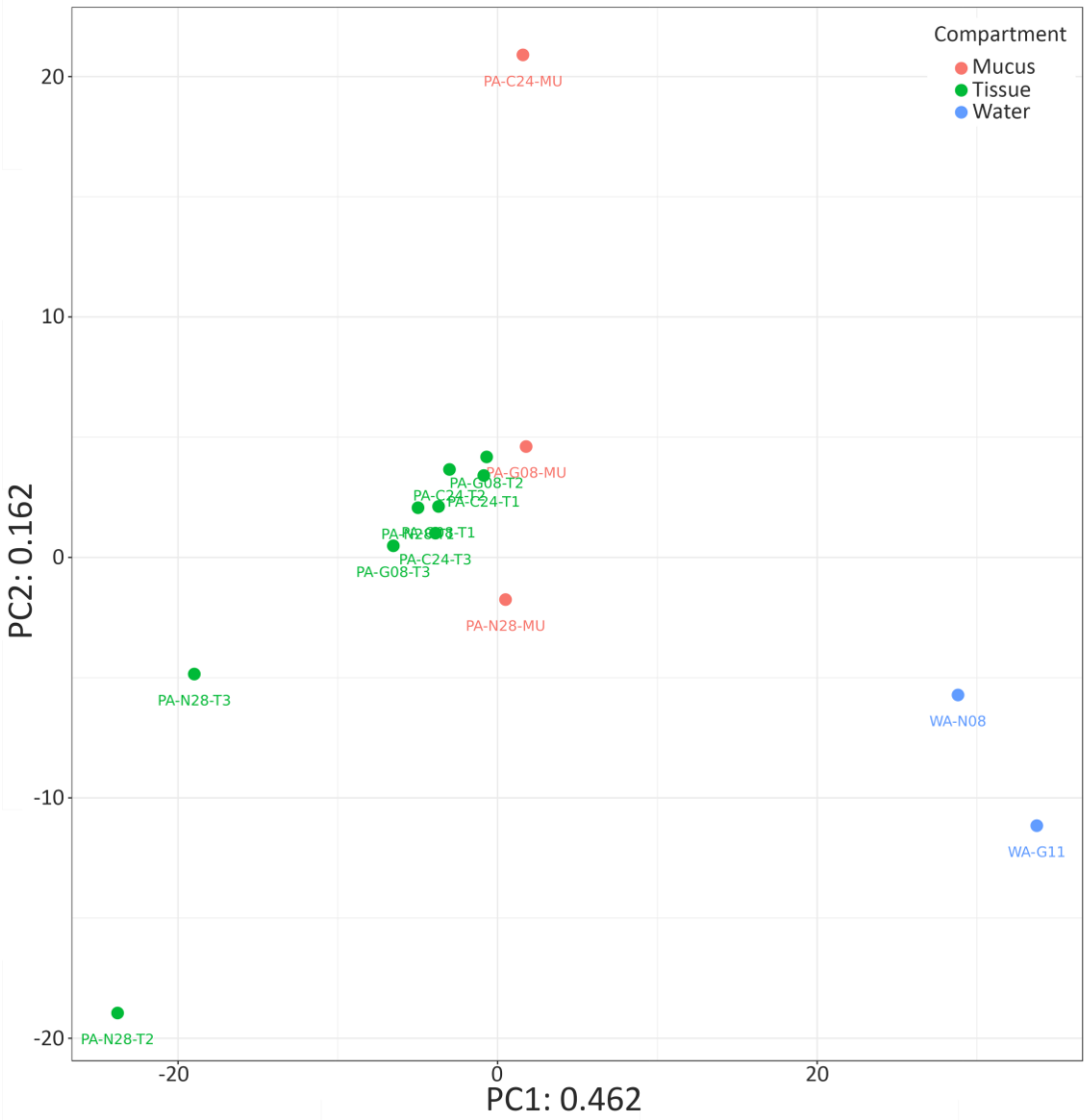

Figure 2. 2. Bacterial community similarity for *Paragorgia arborea* and reference seawater samples. Principal component analysis (PCA) ordination of bacterial communities in *P. arborea* (tissue samples in green and mucus samples in orange) and seawater samples (blue), developed from a singular value decomposition of centered log-ratio (clr)-transformed compositional data. Sample names as in Table 2.1.

was a significant explanatory factor for observed differences within *P. arborea* bacterial composition (p=0.014). The PERMANOVA analysis showed no significant interaction between compartment and canyon. The PERMANOVA results were cross-checked using beta dispersion analysis, wherein significant results were validated by the PERMANOVA's assumption of homogeneity of dispersion.

2.4.2 Bacterial community composition within coral samples

The phyla shown in Figure 2.3A accounted for ~98% of the bacteria found within the samples. Proteobacteria were present in all coral and seawater samples and were dominant in both seawater samples, in two tissue samples from Nygren-Heezen Intercanyon (PA-N28-T1/T3), and in two mucus samples (PA-G08-MU & PA-N08-MU) (Figure. 3.3A). The third mucus sample (PA-C24-MU) had similar proportions of Verrucomicrobia (~37%), Proteobacteria (~27%), and Bacteroidetes (~26%). Tenericutes dominated (~68%) all coral tissue samples from Corsair Canyon and Georges Canyon, with Proteobacteria (~15%) and Spirochaetes (~7%) as notable contributors. Furthermore, Tenericutes were observed in every coral sample with the exception of one mucus sample (PA-C24-MU) and were absent in the seawater sample. The Nygren-Heezen Intercanyon coral tissue samples appeared more variable in composition and contrasted with samples from Corsair Canyon and Georges Canyon, which showed similar bacterial community composition across tissue samples. Cyanobacteria were present in coral samples, with the exception of two tissue samples (PA-C24-T2 & PA-N28-T2). Actinobacteria were observed in all samples recovered from Nygren-Heezen Intercanyon (including seawater)

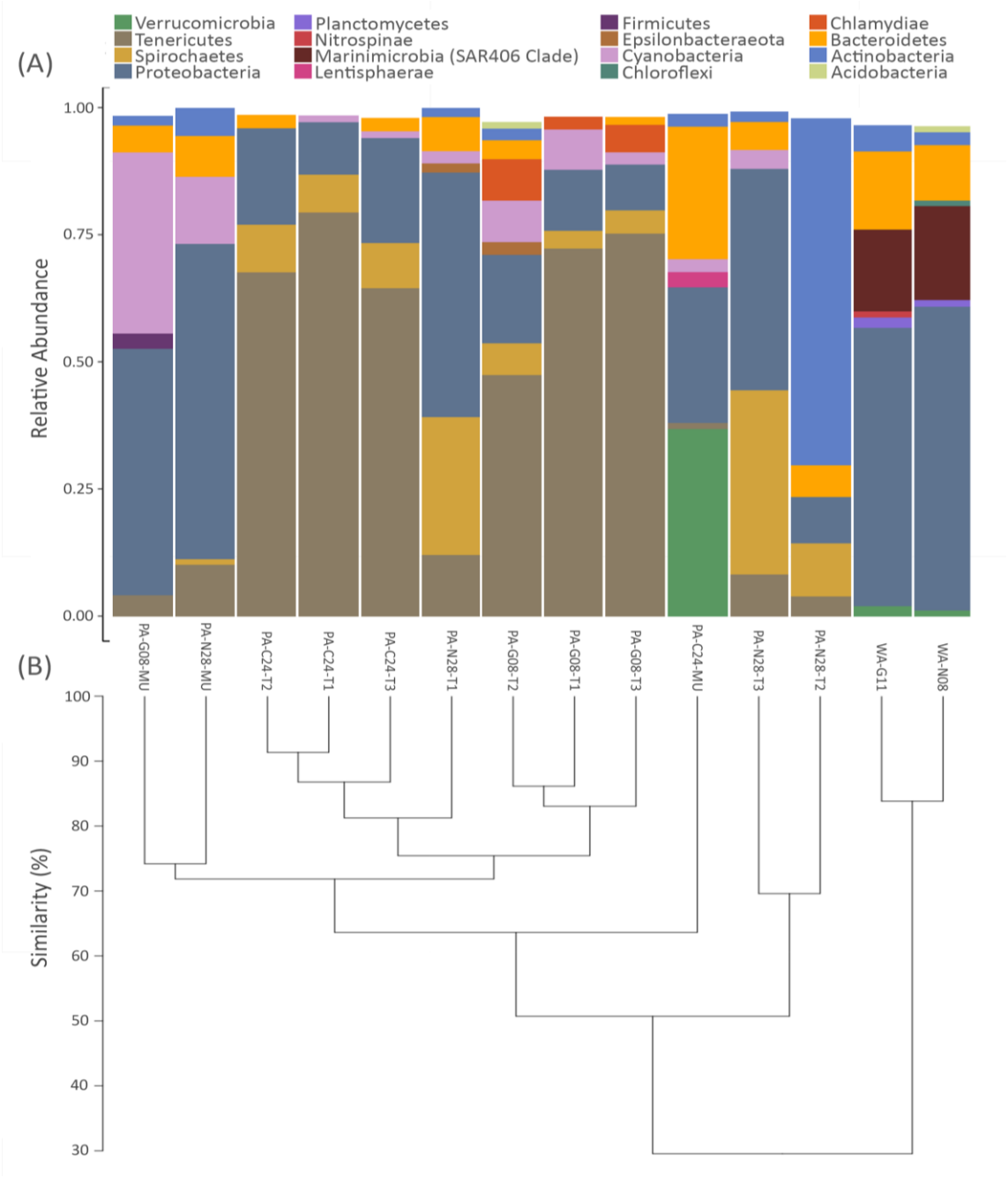

Figure 2. 3. Relative abundance of bacterial phyla and hierarchical dendrogram. (A) X-axis represents samples of *Paragorgia arborea* tissue and mucus, and seawater. Y-axis represents the most dominant bacterial phyla found within samples, with taxa contributing < 1% relative abundance excluded; and (B) Hierarchical clustering dendrogram for *P.*

arborea and reference seawater samples, developed from a Euclidian distance matrix produced from centered log-ratio (clr)-transformed compositional data. Sample names as in Table 2.1.

and were a dominant contributor in one tissue sample from Nygren-Heezen Intercanyon, in all mucus samples, and in the Georges Canyon seawater sample (WA-G11). Lastly, phylum and class Chlamydiae were only present in coral tissue samples from Georges Canyon (~8%), while Firmicutes were only found in coral mucus from Georges Canyon (~3%).

At the class level, bacterial compositions were nearly identical in coral tissue samples from Corsair Canyon, where samples were made up of Mollicutes (~70%), Alphaproteobacteria (~11%), Spirochaetes (~9%), and Gammaproteobacteria (~6%) (Figure 2.4). Coral tissue samples from Georges Canyon were also similar in composition, with dominant contributors being Mollicutes (~65%), Gammaproteobacteria (~9%), Oxyphotobacteria (~6%), Chlamydiae (~5%), and Spirochaetes (~5%). There was less consistency among tissue samples from the Nygren-Heezen Intercanyon, mainly due to the variation in sample PA-N28-T2. Excluding sample PA-N28-T2, dominated by Actinobacteria (68%), the other samples were very similar in composition, made up of Spirochaetes (~32%), Gammaproteobacteria (~28%), Alphaproteobacteria (~18%), Mollicutes (~10%), and Bacteroidia (~6%). Among mucus samples, the one from Corsair Canyon differed from those from Nygren-Heezen Intercanyon and Georges Canyon. Mucus recovered from Corsair Canyon showed a large contribution from

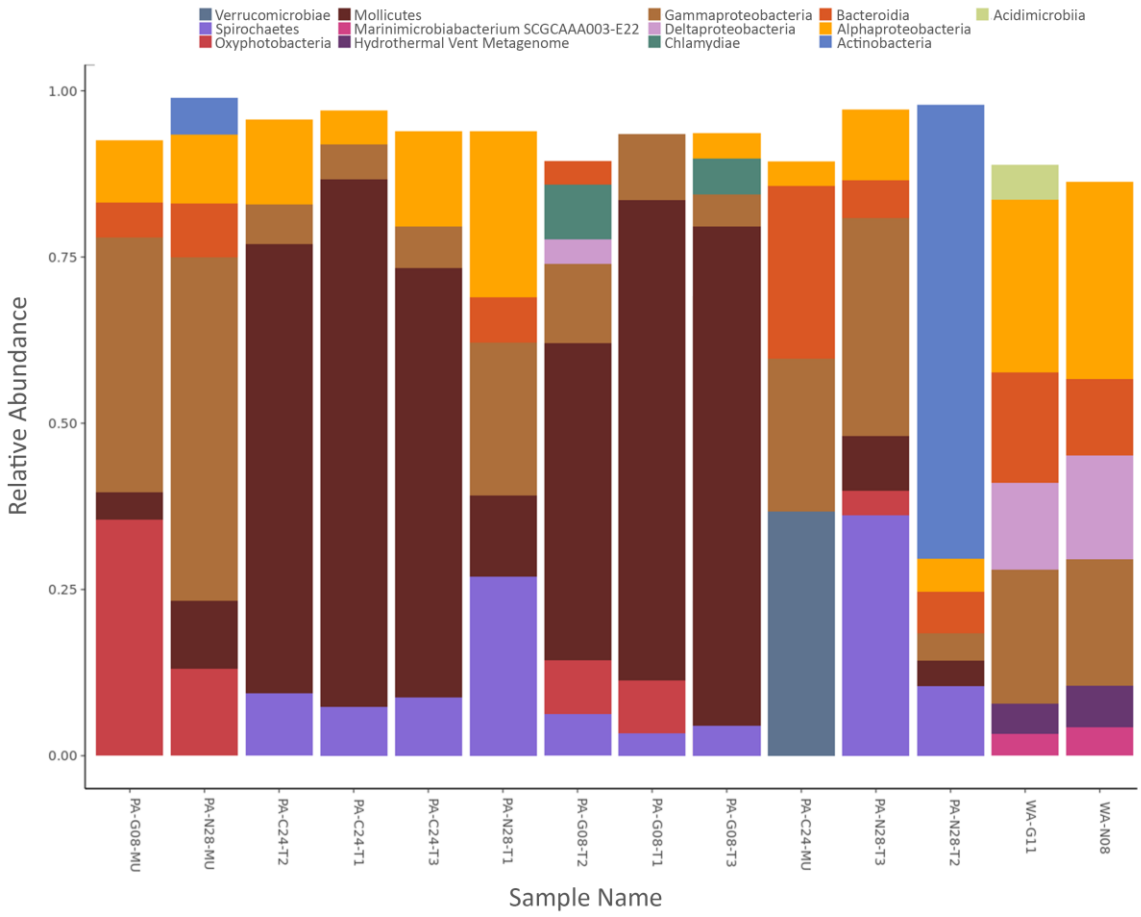

Figure 2. 4. Relative abundance of bacterial classes. X-axis represents samples of *Paragorgia arborea* tissue and mucus, and seawater. Y-axis represents the most dominant bacterial classes found within samples, with taxa contributing < 3% relative abundance excluded.

Verrucomicrobia (~37%), and further composed of Bacteroidia (~26%), Gammaproteobacteria (~23%), and Alphaproteobacteria (~4%). The remaining mucus samples were relatively consistent in composition, containing mostly

Gammaproteobacteria (~45%), Oxyphotobacteria (~24%), Alphaproteobacteria (~10%), Mollicutes (~7%), and Bacteroidia (~7%).

The trends in bacterial composition were observed across all samples in the produced hierarchical dendrogram (Figure 2.3B). Coral and seawater samples were observed to branch apart at 30% similarity, with the two seawater samples showing similar compositions (~85% similarity). Coral tissue samples predominantly formed clusters, with groupings of Corsair Canyon samples (~87% similar) and Georges Canyon samples (~83% similar) most evident. Two mucus samples (PA-G08-MU and PA-N28-MU) contained relatively similar compositions (~74%). Coral tissue samples from all three canyons converged at ~51% similarity.

2.4.3 Bacterial biomarkers within coral anatomical compartments

To observe whether any specific bacterial OTUs (biomarkers) were significantly more abundant in either compartment of *P. arborea* (tissue or mucus), samples were analyzed using an ANOVA-Like Differential Expression analysis using the R package ALDEx2 (Fernandes *et al.*, 2013, 2014). Three OTUS with genus-level assignment were recovered as biomarkers in *P. arborea*, with two recovered in the tissue and one in the surficial mucus (Figure 2.5). Two OTUs recovered as significant within the tissues belonged to the genus *Spirochaeta* of the phylum Spirochaetes and *Mycoplasma* of the phylum Tenericutes (effect sizes = 4.02 and 2.37, respectively) and were observed as relatively large contributors to the associated bacterial assemblage, particularly in

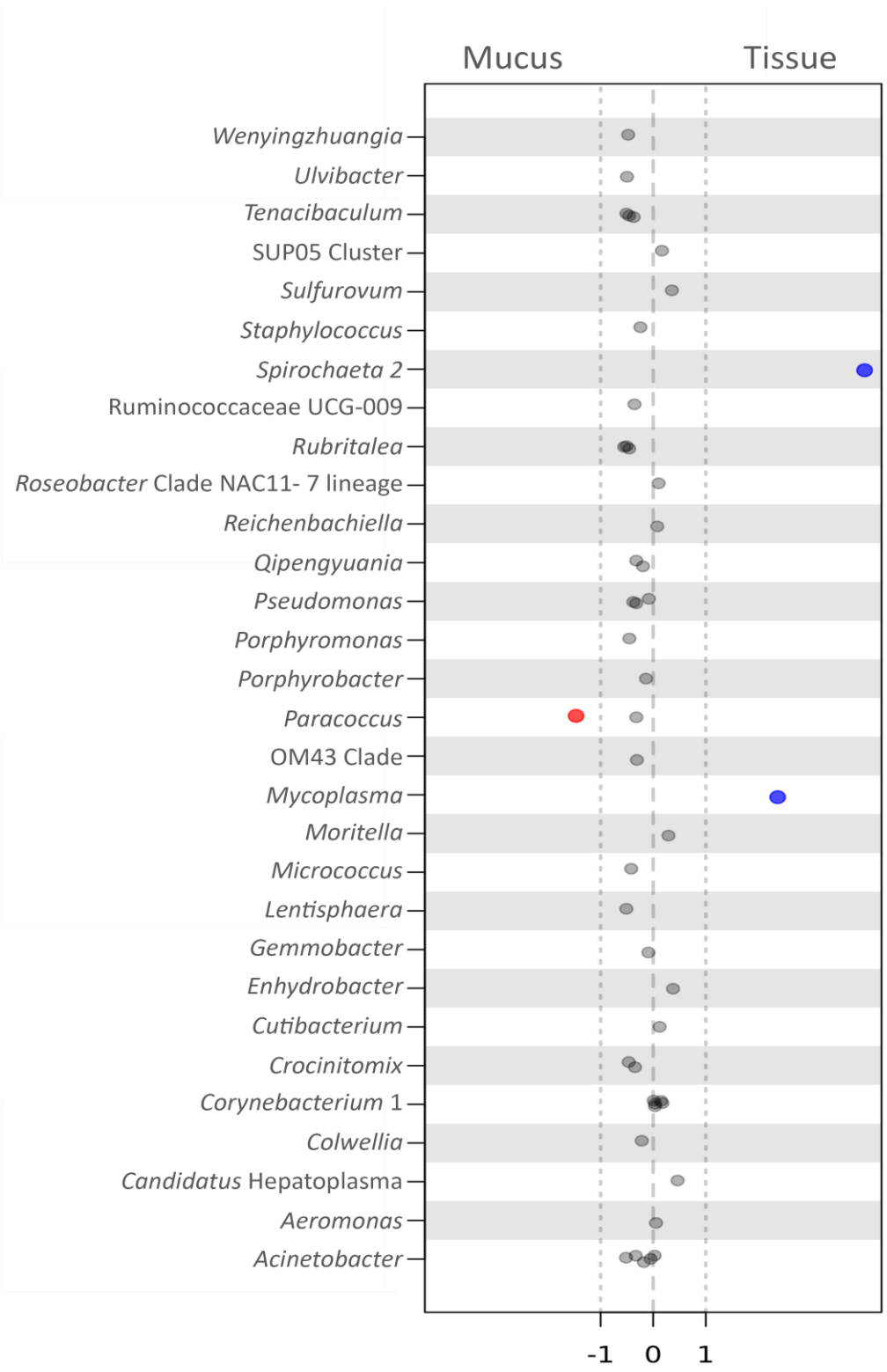
Mucus
Tissue
Wenyingzhuangia
Ulvibacter
Tenacibaculum
SUP05 Cluster
Sulfurovum
Staphylococcus
Spirochaeta 2
Ruminococcaceae UCG-009
Rubritalea
Roseobacter Clade NAC11- 7 lineage
Reichenbachiella
Qipengyuania
Pseudomonas
Porphyromonas
Porphyrobacter
Paracoccus
OM43 Clade
Mycoplasma
Moritella
Micrococcus
Lentisphaera
Gemmobacter
Enhydrobacter
Cutibacterium
Crocinitomix
Corynebacterium 1
Colwellia
Candidatus Hepatoplasma
Aeromonas
Acinetobacter
-1
0
1

Figure 2. 5. Biomarker analysis of bacteria in *Paragorgia arborea* compartments. Effect size scores for bacterial OTUs, classified at the genus level, defined as biomarkers through the use of ANOVA-Like Differential Expression (ALDEx) analysis (R package ALDEx2). Significance is determined by effect size, comparing tissue to mucus: values >1 represent significant, positive associations with tissue (blue), values < -1 show a negative association with tissue and a significant, positive association with mucus (red), and values between -1 and 1 are close to but not significant. Each dot represents an OTU (within the specified genus) tested as a biomarker. The 30 OTUs (having assigned genera) with effect sizes closest to significance are shown here.

samples from Nygren-Heezen Intercanyon (Supplementary Table 2.1). The mucus biomarker was identified as *Paracoccus marcusii* from the phylum Proteobacteria (effect size = -1.47). This biomarker was only recovered in two tissue samples, but was noted in the three mucus samples, comprising ~1.2 – 2.4% of the bacterial assemblage (Supplementary Table 2.1). Four additional OTUs were recovered as significantly associated with tissues, but had no genus level assignment. Those four OTUs belonged to the families Flavobacteriaceae (phylum Bacteroidetes), and Terasakiellaceae (phylum Proteobacteria), and the orders Campylobacterales (phylum Epsilonbacteraeota) and Rickettsiales (phylum Proteobacteria) (effect sizes = 1.72, 1.41, 1.84, and 2.34, respectively). These four OTUs were rarely encountered in mucus and were most abundant in Nygren-Heezen Intercanyon tissue samples (Supplementary Table 2.1 & 2.2).

2.5 Discussion

This is the first described characterization of the bacterial associates for *Paragorgia arborea,* a widely distributed cold-water alcyonacean coral that was collected from three submarine canyons off the Gulf of Maine (Nygren-Heezen Intercanyon, Georges Canyon, and Corsair Canyon). Sequencing of a fragment of the 16S rRNA gene allowed us to observe differences in bacterial composition between coral sample locations, and between compartments (tissue and mucus microhabitats). The numbers of reads obtained from extracts of *P. arborea* tissue samples were much higher than in mucus swabs, suggesting that our mucus sampling methodology could be improved. The low read depth of the water samples was likely due to the small aliquots (50 ml) of seawater at depth available to us on board the research cruise. Coral bacterial associates likely consist of a combination of commensals, long-term, stable partners selected by the host (including potentially intracellular symbionts), bacteria recently ingested and present in the gastro-vascular cavity, and, for mucus samples, either passively adhering bacteria or host-specific taxa (Ainsworth *et al.,* 2015; Pollock *et al.,* 2018). While we are unable to confidently discriminate among those associate categories in this analysis, we draw upon our comparative analyses and on coral microbiomes described in the literature to suggest some bacterial taxa as potentially important members of the bacterial microbiome of those deep-sea, cold-water corals.

2.5.1 Alpha and beta measures of bacterial diversity

As observed in similar studies, the corals examined hosted bacterial communities with significantly lower bacterial biodiversity than those in surrounding seawater (Bayer *et al.*, 2013; Holm and Heidelberg, 2016; van de Water *et al.*, 2016). Within *P. arborea*, there was no significant difference in diversity measures between anatomical compartments (tissue and mucus) and among geographic locations (submarine canyons), suggesting spatial stability in the diversity (as characterized by Hill's indices) of bacterial communities associated with *P. arborea*.

The bacterial communities of seawater were ~70% different in composition (according to hierarchical clustering) from those associated with *P. arborea*; therefore, we consider that the coral microbiome was predominantly comprised of taxa that were uncommon in surrounding seawater and that may show some specificity for this particular host. Other studies on corals provide evidence for bacterial host-specificity: for example, distinct bacterial populations were observed in three octocorals from the coast of Florida, *Leptogorgia minimata, Iciligorgia schrammi,* and *Swiftia exertia* (Brück *et al*., 2007). Similarly, two octocorals from the Eastern Pacific of the genus *Muricea* had distinct microbial assemblages, despite co-occurring habitats and similar morphological structure (Holm and Heidelberg, 2016). Even the alcyonacean congeners *Paramuricea placomus* and *P. clavata* showed negligible bacterial community similarities (Kellogg *et al.*, 2016). However, another study reported a lack of significant variation between microbiomes in two species of Anthothelidae (Lawler *et al.*, 2016). Based on our study, the bacterial

communities associated with *P. arborea* show some species specificity (given the ~50% assemblage similarity among samples from different locations) and are dominated by many of the same taxa reported in other cold-water corals. We note the presence of phototrophic bacteria among the recovered taxa (phylum Cyanobacteria, class Oxyphotobacteria), particularly within mucus samples. These bacteria were likely among phytoplankton and other surficial detritus transported to the deep-sea along the canyons and captured by the corals.

The bacterial assemblages in *P. arborea* samples differed significantly in composition across geographic locations (submarine canyons); substantial variability in bacterial community composition across colonies of a particular coral species has been reported previously (see Hernandez-Agreda *et al*., 2017). Considering only tissue samples, those from Corsair Canyon (depth = 411 m) and Georges Canyon (depth = 423 m) were roughly 75% similar in bacterial community composition (Figure 2.3B); although those sites are within relative proximity to one another, they may differ in environmental and biotic factors, which may play a role in the diversity of available microbial associates and explain observed differences. The bacterial communities in coral tissue samples from the more distant and deeper (700 m) Nygren-Heezen Intercanyon showed greater compositional dissimilarity to the Corsair and Georges Canyon samples. Geographical differences in those bacterial assemblages may be driven mostly by commensals and/or more loosely associated or transient bacteria, but may be influenced by the reproductive behaviour of the host. *P. arborea* has been suggested to be a brooding coral, with fertilization occurring inside or at the surface of the female colony and the

larval offspring settling nearby the parent colony (Lacharité and Metaxas, 2013). Some bacteria may be transmitted vertically in both broadcast and brooding corals (Ceh *et al.*, 2013), and therefore the greater similarity in bacterial community composition between *P. arborea* colonies from nearby canyons could be partly linked to the relatively small larval dispersal distances in this coral species. Further research on vertical bacterial transmission in coral microbial communities is needed to shed light on this matter.

The surface mucus layer (SML) of a coral performs multiple functions and hosts a diverse assemblage of microbes (Sweet *et al.*, 2011; Bythell and Wild, 2011). We observed a significant difference in bacterial composition between anatomical compartments of *P. arborea* and noted compositional differences between mucus samples within a host species. The mucus of shallow-water scleractinian corals is subject to diurnal or hourly replacement cycles, and therefore its bacterial diversity and richness could be constantly changing (Ainsworth *et al.*, 2010; Sweet *et al.*, 2011). In the deep-water scleractinian *Lophelia pertusa*, bacterial communities within the tissues were more stable than those in mucus, which is continuously being replenished following release in the water column (Wild *et al.*, 2008; Weinbauer *et al.*, 2012). It has been previously documented that *P. arborea* produces abundant mucus (Etnoyer *et al.*, 2006). We also observed a large quantity of mucus following *P. arborea* sample collection, and its SML may constitute a perpetually changing microhabitat, as in *Lophelia pertusa* (Wild *et al.*, 2008). Despite the variation in depth and associated physical parameters between shallow and deep-water habitats, we note that the mucus-derived bacterial composition of *P. arborea* shares the high diversity and variability of tropical corals.

2.5.2 Community composition

Based on previous microbiome studies of alcyonaceans, certain bacterial taxa were expected to dominate the bacterial assemblage of *P. arborea*. In most alcyonaceans examined, Proteobacteria (especially Gammaproteobacteria and Alphaproteobacteria) dominate the bacterial assemblage (Penn *et al*., 2006; Gray *et al.*, 2011; Bayer *et al*., 2013; Correa *et al*., 2013; La Rivière *et al*., 2013; Vezzulli *et al*., 2013; Ransome *et al*., 2014; La Rivière *et al*., 2015; Kellogg *et al*., 2016; Lawler *et al.*, 2016; Robertson *et al*., 2016). Our results partly agree with these studies: Gammaproteobacteria and Alphaproteobacteria were present in all *P. arborea* samples, but were not the largest contributor in most samples. In contrast, Tenericutes, or more specifically, Mollicutes were the most dominant across *P. arborea* tissue samples from two canyons (Corsair and Georges Canyon) and were present in all *P. arborea* samples, with the exception of mucus sample PA-C24-MU. Tenericutes were reported to be dominant in a few coral species (Kellogg *et al.*, 2009; Gray *et al.*, 2011; Holm and Heidelberg, 2016). The Tenericutes OTUs observed in *P. arborea* are of the orders Entomoplasmatales and Mycoplasmatales, the latter having been observed in three species of the alcyonacean coral *Muricea* (Ranzer *et al.*, 2007; Holm and Heidelberg, 2016), in two additional alcyonacean species, *Plumarella superba* and *Cryogorgia koolsae* (Gray *et al.*, 2011) and in the scleractinian cold-water coral, *Lophelia pertusa* (Kellogg *et al.*, 2009). Spirochaetes are dominant bacterial contributors in various alcyonaceans (Holm and Heidelberg, 2016; Lawler *et al*., 2016; van de Water *et al.*, 2016). Spirochaetes were

present in all *P. arborea* samples, in some cases as major contributors, and have been described as chemoheterotrophic bacteria that thrive in a wide variety of environments (Ludwig *et al.,* 2010). Spirochaetes dominated some *Anthothela* samples, and were suggested as potential nitrogen fixers (Lawler *et al.,* 2016).

Many of the taxa present at lower abundance may serve crucial roles in coral-bacterial interactions (Ainsworth *et al.*, 2015). Several members of the phylum Firmicutes (orders Bacillales, and Lactobacillales) were observed in extremely low abundance in *P. arborea*. However, Firmicutes were reported in other alcyonacean microbiome studies (Penn *et al.,* 2006; Brück *et al.,* 2007; Kellogg *et al.,* 2009; Correa *et al.,* 2013; Kellogg *et al.,* 2016; Lawler *et al.,* 2016). Closek *et al.* (2014) observed a higher abundance of Firmicutes such as Clostridia in yellow band diseased samples of the scleractinian *Orbicella faveolata*; our samples showed no evidence of disease. Verrucomicrobia were observed in one *P. arborea* mucus sample (PA-C24-MU) and in the seawater samples, and were found in low numbers in other alcyonacean microbiome studies (Gray *et al.,* 2011; Correa *et al.,* 2013; Ransome *et al.,* 2014; Kellogg *et al.,* 2016; Lawler *et al.,* 2016). Verrucomicrobiales from our samples are associated with the family Rubritaleaceae. Bacteria from the Rubritaleaceae are carotenoid pigment producers (Rosenberg, 2014), that give a red colouration to colonies; it is uncertain why these bacteria were highly abundant in PA-C24-MU but absent from all other coral samples. Chlamydiae (taxonomic order Chlamydiales) were only observed in tissue samples from Georges Canyon; the role of Chlamydiales in invertebrates is not known. Campylobacterales were found within all *P. arborea* tissue samples in low abundance,

absent in all mucus samples and were part of the Anthothelidae core microbiome (Lawler *et al.,* 2016). Some members of the order Campylobacterales were suggested to contribute to the nitrogen metabolism cycle for nitrate/nitrite ammonification and denitrification (Tiedje, 1988; Hoffmann *et al.,* 1998; Verbaendert *et al.,* 2011); the Campylobacterales observed here belong to the families Sulfurovaceae, and Thiovulaceae.

Comparing rare taxa found in our study to previously described etiological agents in diseased corals and gorgonians, we found *Vibrio spp.* in one sample of *P. arborea* (PA-G08-T2) in very low relative abundance (data not shown). Various *Vibrio* strains may be etiological agents in diseased *Paramuricea clavata* colonies (Bally and Garrabou, 2007; Vezzulli *et al.*, 2013). Additionally, Daniels *et al.* (2015) found high abundances of mRNA sequences from Vibrionaceae, Campylobacteraceae, Rhodobacteraceae, Flavobacteraceae, and Burkholderiaceae in diseased *O. faveoloata* coral samples. Representatives from the same bacterial families were found in low abundances in our visually healthy coral samples (data not shown). Further research regarding these bacterial groups is required to understand their functional roles in coral microbiomes, as rare bacterial taxa may be as important as dominant taxa and are typically overlooked (Ainsworth *et al.*, 2017).

2.5.3 Bacterial biomarkers within *Paragorgia arborea* anatomical compartments

As previous studies have shown that bacteria found within specific microhabitats may serve particular roles for the host (Bythell and Wild, 2011; Sweet *et al.,* 2011; Glasl

et al., 2016), we explored whether any bacterial OTUs were considered statistically significant biomarkers within *P. arborea* tissues or mucus. Within *P. arborea*, OTUs from the genera *Spirochaeta* and *Mycoplasma*, as well as from the orders Campylobacterales and Rickettsiales and the families Flavobacteriaceae and Terasakiellaceae were biomarkers of tissue, while the genus *Paracoccus* was a biomarker of mucus. While these OTUs have been identified significantly as biomarkers, their abundance can vary markedly across samples and geographic locations and interpretations of key functional roles for these bacteria remain highly speculative.

The genus *Spirochaeta* has been observed to dominate *Anthothela* coral samples and was suggested to be a nitrogen fixer (Lawler *et al.,* 2016). Interestingly, the *Spirochaeta* 2 OTU identified here as a tissue biomarker comprised up to 36% of the identified bacterial assemblage in a tissue sample from Nygren-Heezen Intercanyon. *Mycoplasma* strains have been described as pathogens and/or parasites (Rottem, 2003) and were observed in *Lophelia pertusa* (Kellogg *et al.*, 2009). *Mycoplasma* were abundant in *Muricea* coral samples, with specific strains associated with bleached and unbleached coral samples (Ranzer *et al.*, 2007; Holm and Heidelberg, 2016); although *P. arborea* is azooxanthellate (Roberts *et al.*, 2006), *Mycoplasma* may nonetheless play some role in *P. arborea* health. In previous studies, Campylobacterales were suggested to play a role in denitrification (Verbaendert *et al.*, 2011) and nitrate/nitrite ammonification (Hoffmann *et al.,* 1998). Members of the family Terasakiellaceae and certain *Paracoccus* species were documented to be nitrogen fixers and denitrifiers, respectively, and may be important for alcyonacean nitrogen metabolism (Tiedje, 1988). A microbially-mediated

nitrogen cycle has been uncovered in the cold-water coral *Lophelia pertusa* (Middelburg *et al.,* 2015), and may also be present in alcyonacean corals, such as *P. arborea,* which occupy similar, nutrient-limited environments (Buhl-Mortensen *et al.,* 2015). Notably, we observed the highest proportions of *Spirochaeta*, Campylobacterales, Terasakiellaceae and *Paracoccus*, the biomarkers that might be involved in nitrogen cycling, in Nygren-Heezen Intercanyon, the deepest of our three sampling locations. This could signal a greater importance for bacterial associates involved in nitrogen cycling where food limitation could be more pronounced. Further investigations would be required to characterize any bacterial functions and their impact on the coral host.

2.6 Conclusion

This study provides the first characterization of bacterial associates for *Paragorgia arborea*, detailing the microbiome of this deep-sea cold-water coral during a visibly healthy state. While the bacterial communities of this species did not differ significantly in terms of diversity indices between compartments (mucus and tissue) or sampling location (canyon), there were significant compositional differences found among the bacterial assemblages from different compartments and sampling locations. Bacterial communities appeared more stable across colonies in *P. arborea* tissues than in mucus, and the relative abundance of the more common taxonomic groups tended to fluctuate across samples. In general, the bacterial microbiome of *P. arborea* was dominated by Tenericutes (orders Entomoplasmatales and Mycoplasmatales), with Spirochaetes, Gammaproteobacteria and Alphaproteobacteria also making notable

contributions to the bacterial assemblages. Bacteria from taxa known to contribute to nitrogen recycling and metabolism were identified as tissue and mucus biomarkers in *P. arborea*. Representatives of bacterial families previously found in higher abundance in diseased scleractinians (Vibrionaceae, Campylobacteraceae, Rhodobacteraceae, Flavobacteraceae, and Burkholderiaceae; Daniels *et al.*, 2015) were present (but rare) in our coral samples. The work presented here provides baseline microbiome data for *P. arborea*, a common habitat-forming cold-water coral taxon. Additional research on deep-sea and cold-water coral health and susceptibility to stress is urgently needed for more informed conservation and marine policy planning.

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Chapter 3: Investigating the bacterial associates of the surface mucopolysaccharide layer in the cold-water coral *Primnoa resedaeformis*

3.1 Abstract

Microorganisms such as bacteria found within the microbiome of corals have been suggested to benefit the host through nutrient recycling and host protection, however they can also be linked to pathogenesis. The bacterial communities of corals inhabiting the deep sea have received little attention due mainly to logistical constraints in sampling. Here, tissue and mucus samples of the cold-water coral *Primnoa resedaeformis* were collected from three submarine canyons along the continental slope of the Gulf of Maine. DNA was extracted from tissue and mucus samples and sequencing of the hypervariable V6-V8 region of the 16S rRNA gene was performed on the Illumina MiSeq platform. Read depths from *P. resedaeformis* tissue samples were deemed insufficient to confidently describe the bacterial community structure. The surficial mucus samples did show a plateau in read depth (providing a more reliable representation of the total bacterial diversity), however conclusions drawn from the few samples available remain tentative. The bacterial communities associated with *P. resedaeformis* mucus samples differed slightly in biodiversity across sampling locations (canyons) and showed little variation in composition. Proteobacteria dominated the mucus bacteriome, with Gammaproteobacteria and Alphaproteobacteria identified as the largest Proteobacterial contributors. OTUs belonging to the class Bacilli were also abundant within surficial

mucus. The results presented here are partially supported by a recent study showing that Gammaproteobacteria, Alphaproteobacteria, and Bacilli were abundant in many samples of *P. resedaeformis* (Goldsmith *et al.*, 2018). One genus in particular, *Acinetobacter*, was found in high abundance in our mucus samples, in the core microbiome of the genus *Primnoa* (Goldsmith *et al.*, 2018), in the mucus of *Paragorgia arborea* (Weiler *et al.*, 2018), and mucus of *Lophelia pertusa* (van Bleijswijk *et al.*, 2015). Cold-water coral microbiomes are data deficient and require urgent attention to elucidate the intricate mechanisms of microbe-microbe and microbe-host interactions. Understanding these mechanisms may allow future research to identify potential pathogens that have the ability to decimate entire coral ecosystems.

3.2 Introduction

The coral microbiome helps maintain coral health by providing a defense system against disease (Krediet *et al.*, 2013), and by assisting with nutrient cycling in an otherwise nutrient scarce environment (Wild *et al.*, 2004; Naumann *et al.*, 2009). The coral microbiome (hereafter limited to bacterial associates, and excluding viral, archaeal and eukaryotic taxa) can be described as a combination of transient bacteria and more stable long-term partners selected by the host, and varies depending on the anatomical compartment (Ainsworth *et al.*, 2015; Pollock *et al.*, 2018). The microbiome is distributed among several anatomical compartments: the skeletal matrix, epidermal polyp and gastrovascular tissue, and the external surface mucopolysaccharide layer (SML) (Bourne, *et al.*, 2005; Brown and Bythell, 2005; Sweet *et al.*, 2011; Krediet *et al.*, 2013;

Ainsworth, *et al.* 2015). The SML is suggested to serve a first line of protection for the coral host against pathogenic microorganisms derived from the sediment and surrounding water column (Sweet *et al.*, 2011; Bythell and Wild, 2011; Glasl *et al.*, 2016). As the microbiome can also harbour low abundances of pathogens or be subject to alien microbes that may proliferate when subjected to environmental stressors (e.g. rising sea surface temperatures), this may potentially lead to coral disease and mortality (Mouchka *et al*., 2010; Egan and Gardiner, 2016). However, the SML is also important for heterotrophic particulate feeding acting as a particle trap (Wild *et al*., 2008; Bythell and Wild, 2011). The SML is subject to diurnal or hourly replacement cycles (only observed in shallow-water hard corals), and the bacterial composition may shift and change with mucosal replacement (Ainsworth *et al.*, 2010; Sweet *et al.*, 2011). There have been observable differences in bacterial composition between anatomical compartments (microhabitats), likely due to the contrast between microhabitat types, e.g. stable tissue/skeletal matrix and the variable mucosal layer (Bourne *et al.*, 2005; Sweet *et al.*, 2011; Pollock *et al.*, 2018).

Cold-water corals that are located in the deeper parts of the ocean are studied significantly less than tropical corals due to logistical sampling and financial constraints (Kellogg *et al*., 2016), therefore their microbiome remains data deficient and more attention should be given to these ecologically important habitat providers (Holm and Heidelberg, 2016). The bacterial communities colonizing the SML of corals from deep-sea regions remain largely unexplored (Weiler *et al.*, 2018) and likely differ from those of shallow-water corals due to differences in environmental parameters (e.g., temperature,

salinity, depth, light and nutrient availability) and functional requirements. Notably, corals in the aphotic zone do not host zooxanthellae and are more reliant on particulate food (and potentially nutrient derived from bacterial associates) than most shallow water corals (Roberts *et al.,* 2006). This study aims to characterize the bacterial associates of the cold-water alcyonacean coral *Primnoa resedaeformis* (Gunnerus, 1763). *P. resedaeformis* is an azooxanthellate coral that forms large branching colonies upwards of 2 m in height (Cairns and Bayer, 2005), and has been suggested to broadcast spawn (Lacharité and Metaxas, 2013). *P. resedaeformis* is widely distributed in the Northwestern and Northeastern Atlantic, from the Gulf of Maine northward along the eastern coasts of Canada and the Davis Strait, to the continental slopes of Greenland, along the Reykjanes Ridge of Iceland, to the shelf of Norway (Cairns and Bayer, 2005; Mortensen and Buhl-Mortensen, 2005; Buhl-Mortensen *et al.,* 2015; Brooke *et al.,* 2017). *P. resedaeformis* occupies a depth range of (150-900 m) where temperatures range between 1-11 ˚C, forming "reefs" across a broad geographic area (Buhl-Mortensen *et al.,* 2015). Colonies of *P. resedaeformis* create habitats for various organisms (Buhl-Mortensen and Mortensen, 2004; Lacharité and Metaxas, 2013), similar to shallow-water branching corals.

As mentioned in Chapter 2, the microbiomes of cold-water alcyonaceans (gorgonians) are typically dominated by Proteobacteria, specifically Gammaproteobacteria (Penn *et al*., 2006; Bayer *et al*., 2013; Correa *et al*., 2013; La Rivière *et al*., 2013; Vezzulli *et al*., 2013; Ransome *et al*., 2014; La Rivière *et al*., 2015; Kellogg *et al*., 2016; Robertson *et al*., 2016). In addition, Alphaproteobacteria have been

observed to be important components of microbial communities across multiple studies of alcyonaceans and gorgonians from both shallow and deep environments (Gray *et al*., 2011; Bayer *et al*., 2013; Correa *et al*., 2013; La Rivière *et al*., 2013; Vezzulli *et al*., 2013; Ransome *et al*., 2014; La Rivière *et al*., 2015; Holm and Heidelberg, 2016; Kellogg *et al*., 2016; Robertson *et al*., 2016). In some cases, Spirochaetes (Holm and Heidelberg, 2016; Lawler *et al*., 2016; van de Water *et al.*, 2016) and Tenericutes (Gray *et al*., 2011; Holm and Heidelberg, 2016; Weiler *et al.*, 2018) were the most abundant bacterial associates of cold-water alcyonaceans, while the remainder of the microbial diversity is made up of many less abundant/rare taxa. More recently, concern has been raised with studies overlooking rare taxa, which can be difficult to analyze but could serve important functional roles in the host (Ainsworth *et al.*, 2015).

Coral colonies serve as habitats for many organisms across a large depth gradient (Hixon and Beets, 1993; Syms and Jones, 2000; Husebø *et al*., 2002). However, corals from shallow to deep-water are subject to direct and indirect anthropogenic perturbations, putting many of these corals at risk (Bavestrello *et al.*, 1998; Fosså *et al*., 2002; Roberts *et al.*, 2006; Cordes *et al.*, 2016). As only a few cold-water coral microbiomes have been explored, these organisms are still largely data deficient, especially regarding the associated bacterial communities and their importance to the host. In this study, we aimed to characterize the bacterial associates of *P. resedaeformis* across two compartments: (1) skeleton and polyp tissue combined, and (2) surficial mucus, and examined the degree of similarity in bacterial composition across three study locations in the Gulf of Maine (ranging in depth between 366-668 m) to explore the effects of relative proximity and

depth on bacterial composition. We also examine relationships between bacterial community structure and geographical location in both species. Since this work was undertaken, Goldsmith *et al.* (2018) published a study of the microbiome of *P. resedaeformis* collected from Baltimore canyon and Norfolk canyon in the Atlantic Ocean. We discuss similarities in the bacterial associates of *P. resedaeformis* from the Gulf of Maine to those reported by Goldsmith *et al.* (2018), and to those of a co-occurring coral from another genus, *Paragorgia arborea* (Weiler *et al*., 2018).

3.3 Methods

3.3.1 Sample collection and study sites

Primnoa resedaeformis colony fragments were collected from three submarine canyons on the continental slope of the Gulf of Maine: Nygren-Heezen Intercanyon (N40°51.96′, W66°32.74′, depth 668 m); Corsair Canyon (N41°21.26′, W66°5.39′, depth 366 m); and Georges Canyon (N41°16.48′, W66°11.59′, depth 423m). Sample collection occurred during a research cruise aboard the National Oceanic and Atmospheric Association (NOAA) Ship *Henry B. Bigelow* from June 8th-22nd, 2017. Coral colonies were located and sampled using the remotely operated vehicle (ROV), CSSF-ROPOS (Canadian Scientific Submersible Facility, Remotely Operated Platform for Ocean Sciences), and coral branches separated in individual seawater-filled chambers until surfacing. Aboard the ship, tissue fragments were dissected from the coral branches and placed in sterile cryovials. Coral mucus was extracted from the branches by rolling a sterile cotton swab over the mucosal layer. At each sampling location, three tissue

replicates, and one mucus swab were collected from individual colonies and frozen (-20ºC) until further analysis. At each dive location, a Niskin bottle attached to the ROV was triggered at 1 m from sea floor to collect surrounding seawater within proximity to the coral colonies (these are the same seawater samples from Chapter 2). Processing of the seawater included 50 mL aliquots of each water sample being passed through individual 0.22 µm syringe filters (Millipore Sigma, Canada) and stored at -20˚C until further analysis.

3.3.2 Nucleic acid extraction

Total DNA was extracted from *P. resedaeformis* mucus and tissue samples following the protocol described by Weiler *et al.* (2018). In short, samples of coral tissue and skeleton were washed in phosphate-buffered saline (PBS) three times to remove mucus associated bacteria. Once washed, the samples were flash frozen and crushed using a sterile mortar and pestle. Crushed samples were transferred to the bead tubes included with the PowerViral Environmental RNA/DNA extraction kit and vortexed (MO BIO, Carlsbad, CA, USA). DNA was extracted from water filters by adding lysis buffer (MO BIO) into the filter, subsequently purging the filter cartridge using a syringe to collect all lysis buffer and repeating the process on the back and front sides of the filter. Mucus swabs were placed in microfuge tubes and vortexed with lysis buffer (MO BIO). After initial lysis, DNA was extracted according to the manufacturer's protocol. A detailed description of bacterial DNA detection and extraction is outlined in Weiler *et al*. (2018).

3.3.3 16S rRNA gene sequencing and processing

DNA was sequenced at the Centre for Comparative Genomics and Evolutionary Bioinformatics (Dalhousie University, Halifax, Canada). Two times 300-bp paired-end sequencing of the 16S rRNA gene was performed using Illumina MiSeq v3, and amplified using previously published primers targeting the V6-V8 regions (B969F/B1406R): V6 forward: 5'- CCATCTCATCCCTGCGTGTCTCCGACTCAG and V8 reverse: 5'- CCTATCCCCTGTGTGCCTTGGCAGTCTCAG (Comeau *et al.*, 2011).

Sequence data were processed using the in-house developed SPONS-2 pipeline (Verhoeven and Dufour, 2017) and trimmed to remove both low-quality bases using Trimmomatic version 0.33 (20-base sliding window with a minimum average quality of 15 per base) and short reads (< 100 bases) (Bolger *et al.*, 2014). Once through the initial quality check, reads were merged using PEAR version 0.9 (Zhang *et al.*, 2014). Primers were trimmed using CutAdapt (maximum error rate 0.2) and reads that lack forward and reverse primers were filtered out (Martin, 2011). Operational taxonomic units (OTUs) were defined using SWARM version 2.0 (Mahé *et al.*, 2015) once reads with an average Phred score below 30 were removed. Defined OTUs were compared against the SILVA SSU database (release 132) for taxonomic assignment using VSEARCH version 2.0.3 (Rognes *et al.*, 2016) collecting the lowest common ancestor amongst database hits.

3.3.4 Bioinformatics and statistical analyses

All OTU count and taxonomic data were imported in R for further analyses (R Development Core Team, 2008). Additional filtering in R was conducted to remove OTUs not classified as bacteria at the Kingdom level. Rarefaction curves and alpha diversity analyses for each sample were produced using the PhyloSeq package in R (McMurdie and Holmes, 2014). Alpha diversity analysis was conducted using Shannon's diversity index to calculate species diversity, the inverse of the Simpson's index "Inv. Simpson" to calculate community evenness and the "Chao1" index to estimate community richness. To compare patterns in bacterial community structure across samples, bar graphs were produced at the phylum, class, and family taxonomic levels. Additionally, to examine whether there might be OTUs shared across *P. resedaeformis* colonies at a larger geographic scale, or between co-occurring *P. resedaeformis* and *Paragorgia arborea*, selected OTU sequences from the *P. resedaeformis* mucus database were compared to sequences of similar bacterial genera in *P. resedaeformis* (Goldsmith *et al.*, 2018), and in *Paragorgia arborea* mucus (Weiler *et al.*, 2018) using the sequence comparison feature in BLAST (Altschul *et al.*, 1990).

3.4 Results

Rarefaction curves (Figure 3.1) presented here show that only the *P. resedaeformis* mucus samples and two reference seawater samples reached a plateau (indicating that sequencing depth is likely sufficient to capture a sample's total microbial diversity). The read depth in all *P. resedaeformis* tissue samples was very low (< 20

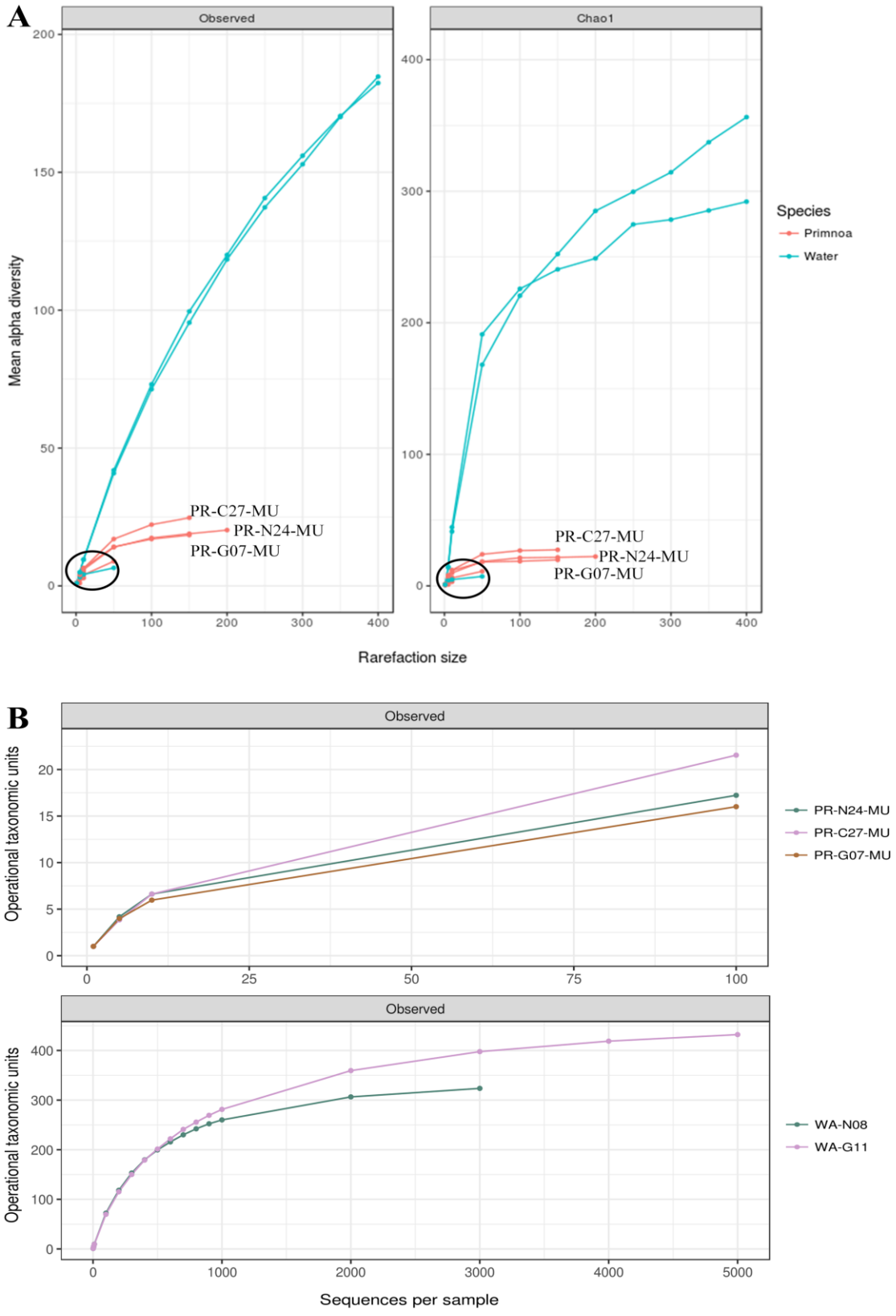
A
Observed
Chao1
Mean alpha diversity
Rarefaction size
Species
Primnoa
Water
PR-C27-MU
PR-N24-MU
PR-G07-MU
B
Observed
Operational taxonomic units
PR-N24-MU
PR-C27-MU
PR-G07-MU
Observed
Operational taxonomic units
WA-N08
WA-G11
Sequences per sample

Figure 3. 1. Rarefaction curves showing the number of observed and Chao1 estimated bacterial OTUs as a function of the number of sequences. (A) *Primnoa resedaeformis* mucus samples (n = 3, red), tissue samples (n = 9, red), and seawater samples (n = 3, blue). Note that tissue samples and Corsair Canyon seawater sample are present and clustered within the black circle; curves for these samples do not approach a plateau. (B) Observed rarefaction curves illustrating seawater approaching a plateau in read depth. Sample names as in Table 3.1.

Table 3. 1. Sample collection, description, and alpha diversity summary statistics of bacterial communities in *Primnoa resedaeformis* mucus and nearby seawater samples determined by analysis of 16S rRNA sequence libraries.

Specimen ID	Submarine Canyon	Description	Depth (m)	Number of Reads	Observed OTUs	Chao1	Inv. Simpson	Shannon
PR-N24-MU	Nygren-Heezen	Mucus	668	116	20	23.33	10.29	2.59
PR-C27-MU	Corsair	Mucus	366	161	28	33.60	7.58	2.61
PR-G07-MU	Georges	Mucus	423	143	18	18.25	7.16	2.37
WA-N08	Nygren-Heezen	Water	837	3712	345	349.13	101.45	5.27
WA-G11	Georges	Water	606	5702	464	466.61	81.54	5.26

sequences per sample, with many OTUs being singletons and doubletons) and failed to reach a plateau. Tissue samples were therefore omitted from further analysis. The mucus sample read depths were also low compared to other studies (18-28 OTUs and 116-161

reads; Table 3.1), but as the read depths reached a plateau (Figure 3.1), tentative observations on community structure are made here. Due to the omitted tissue samples, cross comparisons between the anatomical compartments of *P. resedaeformis* could not be completed and comparisons made between geographical location are speculative due to low sample size (n=1 per sampling location). Additionally, the Corsair Canyon seawater sample (WA-C09) yielded a low number of sequences and was omitted from the study. Results presented here are treated as preliminary observations.

3.4.1 Alpha diversity in coral bacterial assemblages

Alpha diversity measures were used to compare diversity trends and similarities between bacterial communities across mucus and seawater samples. The bacterial communities in seawater samples showed greater read depth, species richness, evenness and Shannon's diversity than those in coral samples. For mucus samples, Corsair Canyon was observed to possess the most OTUs (Chao1 index), suggesting a slightly greater richness in bacterial taxa (Table 3.1). The sample from Nygren-Heezen Intercanyon had the highest Simpson's diversity, and Corsair Canyon the highest Shannon diversity (Table 3.1).

3.4.2 Bacterial community composition within coral samples

The phyla shown in Figure 3.2A accounted for ~98% of the bacteria found within the samples. At the phylum level, Proteobacteria dominated all coral mucus samples (83.6% ± 0.6) and seawater samples (45.2% ± 3.0) (Figure 3.2A). These three samples, while still

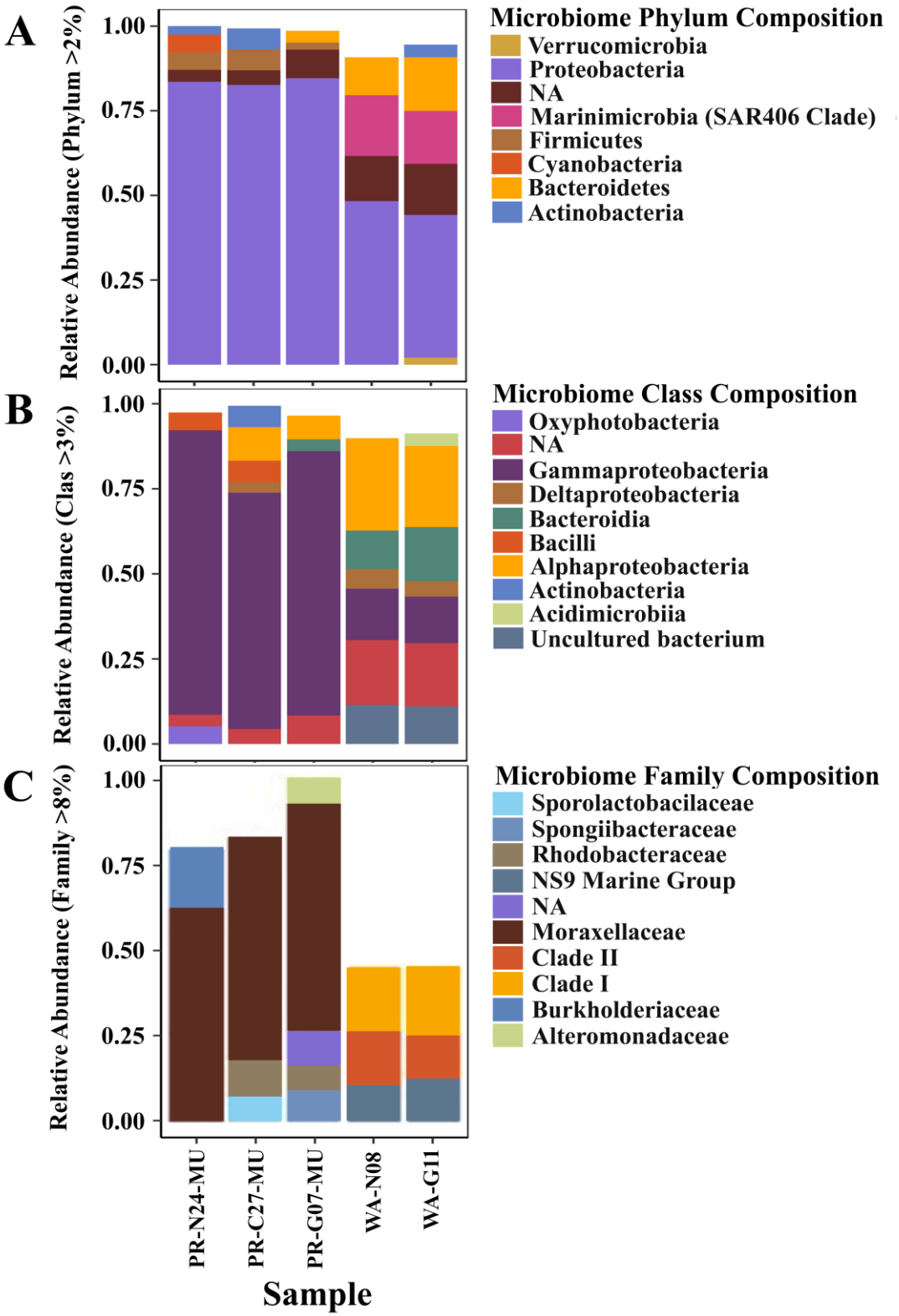
A
Relative Abundance (Phylum >2%)
1.00
0.75
0.50
0.25
0.00
Microbiome Phylum Composition
Verrucomicrobia
Proteobacteria
NA
Marinimicrobia (SAR406 Clade)
Firmicutes
Cyanobacteria
Bacteroidetes
Actinobacteria
B
Relative Abundance (Clas >3%)
1.00
0.75
0.50
0.25
0.00
Microbiome Class Composition
Oxyphotobacteria
NA
Gammaproteobacteria
Deltaproteobacteria
Bacteroidia
Bacilli
Alphaproteobacteria
Actinobacteria
Acidimicrobiia
Uncultured bacterium
C
Relative Abundance (Family >8%)
1.00
0.75
0.50
0.25
0.00
Microbiome Family Composition
Sporolactobacilaceae
Spongiibacteraceae
Rhodobacteraceae
NS9 Marine Group
NA
Moraxellaceae
Clade II
Clade I
Burkholderiaceae
Alteromonadaceae
PR-N24-MU
PR-C27-MU
PR-G07-MU
WA-N08
WA-G11
Sample

Figure 3. 2. Relative abundance of bacterial taxa recovered from *Primnoa resedaeformis* and reference seawater samples. X-axis represents samples; *Primnoa resedaeformis* (PR) and seawater (WA). Y-axis represents the most dominant bacterial (A) phyla, (B) classes, and (C) families found within samples, excluding taxa contributing < 2, 3 or 8% of the relative abundance for phylum, class, and family levels, respectively. Sample names as in Table 3.1.

containing Proteobacteria, also contained Firmicutes (4.5% ± 1.2) and an unclassified bacterial taxon, termed "NA" (5.4% ± 1.5). While Proteobacteria were largely dominant in the seawater samples, the three other phyla forming notable contributions to the microbiome were Bacteroidetes (13.6% ± 2.3), Marinimicrobia (16.7% ± 1.2), and an unclassified bacterial group (14.2% ± 0.8).

At the class level, bacterial compositions were similar across coral mucus samples; however only Gammaproteobacteria were shared in all three samples (Figure 3.2B). Gammaproteobacteria was the dominant bacterial class across *P. resedaeformis* samples (77.0% ± 4.1). The remaining bacterial classes were in low abundance. Seawater samples shared similar bacterial compositions and relative abundances. Notably, the "uncultured bacterium" present across seawater samples belongs to the phylum Marinimicrobia (SAR406 clade). We note the presence of phototrophic bacteria among the recovered taxa (phylum Cyanobacteria, class Oxyphotobacteria). These bacteria were in found in one sample (PR-N24-MU) and were low contributors to the overall abundance and diversity within that sample. At the family level, the only taxon to appear in all three

mucus samples and also the largest contributor overall was Moraxellaceae, contributing 58% ± 1.7 to the relative abundance (Fig. 3.2C). Other large contributors were Burkholderiaceae (15.5%) in PR-N24-MU, Sporolactobacillaceae (6.2%) in PR-C27-MU, Rhodobacteraceae (8.5% ± 2.1) in PR-C27-MU and PR-G07-MU, and Spongiibacteraceae (8.4%) and Alteromonadaceae (7%) in PR-G07-MU. The overall composition of both water samples was highly similar at family level, with Clade I (SAR11 Clade) contributing 17.3% ± 0.2, Clade II (SAR11 Clade) contributing 13.1% ± 1.9, and NS9 marine group (order Flavobacteriales) contributing 10.1% ± 1.5 of the total diversity within those samples.

A total of 48 OTUs were recovered from the *P. resedaeformis* mucus, 10 of those being singletons (OTU appearing once in the mucus dataset) and 5 being doubletons. Only 3 OTUs were shared across all three mucus samples, all belonging to the bacterial phylum Proteobacteria. Among the shared OTUs, the bacterial genus *Acinetobacter* (family Moraxellaceae) and BD1-7 clade (family Spongiibacteraceae) were recovered as the most abundant taxa. OTU sequences were compared with taxonomically similar sequences reported from *P. resedaeformis* tissue samples (Goldsmith *et al.*, 2018) using BLAST to examine whether any OTUs were shared. None of the OTUs recovered from *P. resedaeformis* mucus were similar to those reported in Goldsmith *et al.* (2018). OTUs from *P. resedaeformis* mucus were also compared to those found within the mucus of *Paragorgia arborea* sampled from the same canyons (Weiler *et al.*, 2018). There were 17 OTUs shared between *P. resedaeformis* and *P. arborea* mucus samples (observed in both species with 100% identity and coverage, ~400bp), 14 of which were classified as

Proteobacteria, and the other three were unidentified at the phylum level (Table 3.2). The most abundant shared OTU (OTU 6) was identified as belonging to the phylum Proteobacteria, with no lower level taxonomic assignment provided using the VSEARCH approach; however, when this sequence was compared to a broader nucleotide database using BLAST, it showed 100% similarity and coverage to *Acinetobacter indicus*. Six additional OTUs of *Acinetobacter* sp. were shared between both *P. arborea* and *P. resedaeformis*. Rarer shared OTUs were identified to the bacterial families Alteromonadaceae, Burkholderiaceae, Pseudomonadaceae, Geobacteraceae, and Comamonadaceae.

3.5 Discussion

This work was intended to provide the first characterization of the bacterial associates of *Primnoa resedaeformis*, a common habitat-forming cold-water alcyonacean collected from three submarine canyons off the Gulf of Maine (Nygren-Heezen Intercanyon, Georges Canyon, and Corsair Canyon). However, due to extremely low sequencing read depths for the tissue samples, we were unable to provide a description of the associated bacteria for these samples. The low sequence read yields from *P. resedaeformis* tissues may have been due to the high collagenous content of the skeletal framework in this species, in conjunction with the extraction method we used (Muzik and Wainwright, 1977; Mortensen and Buhl-Mortensen, 2005). The low sequence yields were unlikely due to a low bacterial abundance in *P. resedaeformis*, as Goldsmith *et al.* (2018) successfully sequenced bacterial DNA from *P. resedaeformis* tissue samples, recovering

Table 3. 2. Taxonomic assignment and abundance of OTUs shared between cold-water octocorals, *Primnoa resedaeformis* and *Paragorgia arborea* from three locations in the Gulf of Maine: Nygren-Heezen Intercanyon, Corsair Canyon, and Georges Canyon. All taxonomic assignments are within the phylum Proteobacteria with the exception of "NA", which are undefined organisms from the phylum level. *P. resedaeformis* sample names for each location are found in Table 3.1.

		Taxonomic Assignment				Nygren-Heezen		Corsair		Georges		Total	Total
OTU	Length	Class	Order	Family	Genus	*Primnoa*	*Paragorgia*	*Primnoa*	*Paragorgia*	*Primnoa*	*Paragorgia*	*Primnoa*	*Paragorgia*
6	404	NA	NA	NA	NA	22	163	47	74	38	48	107	285
11	404	γ-Proteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	13	86	28	48	32	15	73	149
82	404	γ-Proteobacteria	Alteromonadales	Alteromonadaceae	*Alishewanella*	0	1	0	7	10	0	10	8
128	401	β-Proteobacteria	Burkholderiales	Burkholderiaceae	NA	8	3	1	0	0	3	9	6
32	404	γ-Proteobacteria	Pseudomonadales	Moraxellaceae	NA	0	10	2	21	6	15	8	46
78	404	γ-Proteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	0	0	3	2	5	1	8	3
94	406	NA	NA	NA	NA	0	0	2	3	6	0	8	3
23	407	NA	NA	NA	NA	4	36	3	1	0	7	7	44
83	404	γ-Proteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	0	3	5	0	2	4	7	7
26	405	γ-Proteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	0	6	3	22	3	17	6	45
101	405	γ-Proteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	0	0	4	2	1	0	5	2
399	406	λ-Proteobacteria	Desulfuromonadales	Geobacteraceae	*Geoalkalibacter*	0	0	4	0	0	3	4	3
59	404	γ-Proteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	0	29	0	7	2	0	2	36
71	381	NA	NA	NA	NA	0	17	2	18	0	0	2	35
64	400	β-Proteobacteria	Burkholderiales	Comamonadaceae	NA	0	4	1	12	0	3	1	19
93	404	γ-Proteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	0	16	1	4	0	0	1	20
733	405	γ-Proteobacteria	Pseudomonadales	Moraxellaceae	*Acinetobacter*	0	2	1	0	0	0	1	2

many operational taxonomic units (OTUs) with large read depths (number of OTUs ranging from 66 to 314, with read depths between 2,557 and 22,448). Both Goldsmith *et al.* (2018) and our study followed similar extraction protocols (despite our study extracting both tissue and mucus separately), however targeted a different variable region of the 16S rRNA gene (V4-V5 compared to our V6-V8), using different primers and sequencing method (Roche's 454). It is difficult to say with confidence what difference in methods may have resulted in the large read count and depth in Goldsmith *et al.* (2018). Here, we present data from one *P. resedaeformis* mucus sample from each sampling location; while read depths for those samples were higher than in tissue samples, they did not quite reach a plateau in rarefaction curves (Figure 3.1, Table 3.1). Here, tentative observations are made of the observed bacterial diversity from the mucus samples, which we compare to the published microbiome of *Primnoa resedaeformis* from Baltimore and Norfolk Canyons (Goldsmith *et al.*, 2018) and of mucus samples in *Paragorgia arborea* (Weiler *et al*., 2018).

The coral bacterial associates identified within *P. resedaeformis* mucus are likely either passively adhering bacteria or host-specific taxa selected by the host (Ainsworth *et al.*, 2015). The bacteria in mucus are suggested to be highly variable (consist of a combination of short-term partners) due to mucosal sloughing from the epidermis (Sweet *et al.*, 2011). While there is a knowledge gap in the literature concerning cold-water coral mucus, *P. resedaeformis* has been documented to produce abundant mucus (Etnoyer *et al.*, 2006) suggesting that mucosal community structure should be highly variable. Here, we do not attempt to classify bacteria as either stable long-term partners or transient taxa,

but instead to describe the bacteria observed in *P. resedaeformis* mucus during a visibly healthy state.

3.5.1 Alpha diversity and community composition within surficial mucus

P. resedaeformis mucus samples hosted bacterial communities with considerably lower (one order of magnitude less) bacterial biodiversity than those in surrounding seawater. A lower diversity in corals compared to seawater has been observed in other cold-water coral studies (Bayer *et al.*, 2013; Holm and Heidelberg, 2016; van de Water *et al.*, 2016; Weiler *et al.*, 2018). Within *P. resedaeformis*, there was little variation in bacterial diversity measures across geographic locations (submarine canyons), despite differences in sampling depths. The low variation in diversity measures across samples (Table 3.1) suggests similarity in bacterial communities within coral colonies, and the lower diversity compared to seawater suggests that bacteria within mucus are not simply an indiscriminate replicate of the surrounding planktonic community, but that there may be some degree of selection taking place within the mucus layer. However, there is the possibility of sequence and kit contamination and therefore the observed differences may be due to methodological issues.

Different coral taxa often host different microbiomes, which may indicate some degree of host specificity. For example, distinct bacterial community structures (abundant/dominating bacterial groups) were observed in three octocorals from the coast of Florida, *Leptogorgia minimata, Iciligorgia schrammi,* and *Swiftia exertia* (Brück *et al*.,

2007). Additionally, two alcyonaceans from the Eastern Pacific of the genus *Muricea* had distinct microbial assemblages, despite co-occurring habitats and similar morphological structure (Holm and Heidelberg, 2016). Similarly, the alcyonacean congeners *Paramuricea placomus* and *P. clavata* showed negligible bacterial community similarities (Kellogg *et al.*, 2016). However, another study reported a lack of significant variation between microbiomes in two species of the genus *Anthothela* (Lawler *et al.*, 2016), in this case suggesting similar microbial assemblages at the host genus level. Here, *P. resedaeformis* may show some specificity towards the bacterial associates appearing in greatest abundance within the mucus layer (*Acinetobacter*), while the microbiota in lower abundance seem more variable and may represent more transient associates, or simply planktonic (environmental) bacteria caught in surficial mucus.

All samples were collected in different submarine canyons and at different depths, but nevertheless were similar in terms of the predominant members of their surficial mucus-associated bacterial communities at the genus level. Geographical similarities in bacterial assemblages within the surficial mucus may be due to the presence of more stable bacteria that the host may actively select for. Microbiome similarities could also be influenced by the reproductive behaviour of the host, as *P. resedaeformis* has been suggested to be a broadcast spawner, with fertilization occurring in the water column, typically near the surface (Lacharité and Metaxas, 2013). Buoyant offspring can disperse over large distances (i.e. between canyons) before settling in a new benthic habitat. Interestingly, bacteria can be transmitted vertically in broadcast-spawning corals (Ceh *et al.*, 2013), and therefore the similarity in bacterial assemblage structure within *P.*

resedaeformis samples could be partly linked to the parental transmission of functionally important bacteria to the offspring. Further research on the role of vertical transmission in coral microbiomes (and whether this might influence the bacteria associated with coral mucus) is needed to shed light on this matter.

The SML of a coral is composed of mucopolysaccharides, which provide a habitat and food source for a diverse assemblage of microorganisms that may provide benefits to the host (Sweet *et al.*, 2011; Bythell and Wild, 2011). The SML is proposed to contain transient, variable bacterial taxa, with the community undergoing some renewal as the mucus sloughs off the coral host and into the surrounding water column. Mucus secretion was abundant upon *P. resedaeformis* collection; Etnoyer *et al.* (2006) also characterized *P. resedaeformis* as an abundant mucus producer. The mucus of shallow-water scleractinian corals is subject to diurnal or hourly replacement cycles, and therefore its bacterial diversity and richness could change constantly (Ainsworth *et al.*, 2010; Sweet *et al.*, 2011). We suggest that the mucus of *P. resedaeformis*, while subject to sloughing and replacement, nevertheless retains some host-level specificity, given the OTU similarities in the three samples studied here.

Previous soft coral microbiome studies have revealed that certain bacterial groups are conserved across coral taxa. In most alcyonaceans examined, Proteobacteria of the class Gammaproteobacteria and Alphaproteobacteria tend to dominate the bacterial assemblage (Penn *et al*., 2006; Gray *et al.*, 2011; Bayer *et al*., 2013; Correa *et al*., 2013; La Rivière *et al*., 2013; Vezzulli *et al*., 2013; Ransome *et al*., 2014; La Rivière *et al*.,

2015; Kellogg *et al*., 2016; Lawler *et al.,* 2016; Robertson *et al*., 2016). The community composition of *P. resedaeformis* agrees with these studies: Gammaproteobacteria were dominant in all coral samples, and Alphaproteobacteria were a large contributor in two samples. In addition, Bacilli were abundant in two of the coral mucus samples presented here and were found within the majority of *P. resedaeformis* samples from Goldsmith *et al.* (2018). While Bacilli are not commonly reported in microbiome studies, they have been found in association with the cold-water coral *Paramuricea placomus* (Kellogg *et al.,* 2016) and other soft corals (Brück *et al.,* 2007; Correa *et al.,* 2013). Kellogg *et al.* (2016) suggest that the *Bacillus* (class Bacilli) recovered from their samples are contributing in the nitrogen cycle of the cold-water coral *P. placomus.* We note the presence of the phototrophic Oxyphotobacteria (Phylum Cyanobacteria), in the Nygren-Heezen Intercanyon sample. This taxon is likely captured in the surficial mucus as detritus/food source from surface waters transported into the submarine canyon.

Some of the bacterial families observed to dominate the *P. resedaeformis* mucus samples from this study were also observed in samples of *P. resedaeformis* recovered in Norfolk and Baltimore Canyon (Goldsmith *et al.,* 2018). Bacterial families Moraxellaceae and Rhodobacteraceae were large contributors for both *P. resedaeformis* mucus samples and the samples recovered by Goldsmith *et al.* (2018). Furthermore, *Acinetobacter* (family Moraxellaceae) was observed as highly abundant in *P. resedaeformis* mucus and was suggested as part of the *Primnoa* genus core microbiome (Goldsmith *et al.*, 2018). Additionally, the families Pseudomonadaceae and Comamonadaceae were in both *P. resedaeformis* mucus samples and those recovered by Goldsmith *et al.* (2018). However,

as comparisons of OTU sequences across studies using BLAST indicated dissimilarity, these bacterial OTUs are likely picked up by the host from the surrounding water column.

The comparison of *P. resedaeformis* and *Paragorgia arborea* mucus samples (Weiler *et al.*, 2018) yielded interesting results. There were 17 OTUs shared between the two species within the mucus, six of which belonged to the genus *Acinetobacter*. The high abundance of *Acinetobacter* may suggest a functional importance for the host. *Acinetobacter* has been observed in the mucus of *Lophelia pertusa*, a cold-water scleractinian coral and suggested to play a role in host defense (van Bleijswijk *et al.*, 2015).

3.6 Conclusion

This study provided preliminary information on the bacteria found within the surficial mucus of the cold-water coral *Primnoa resedaeformis*. Our low sequence yields may be partly linked with the high collagenous content of tissues and perhaps an incomplete or inefficient extraction with the MoBio PowerViral kit, and the mucus sampling method may have been somewhat inefficient in recovering bacteria. The bacterial communities in the SML of this species differed slightly in terms of diversity indices among sampling locations (canyons) and there were no significant compositional differences found among samples. In general, the bacterial microbiome of *P. resedaeformis* was dominated by Proteobacteria (classes Gammaproteobacteria and Alphaproteobacteria), with Bacilli and Actinobacteria also making notable contributions

to the bacterial assemblages. At the genus level, *Acinetobacter* was notable within our *P. resedaeformis* mucus samples, as it was previously described as part of the *Primnoa* genus core microbiome (Goldsmith *et al.,* 2018) and present in the mucus of the cold-water soft coral, *Paragorgia arborea* (Weiler *et al.,* 2018), and in mucus of the cold-water hard coral, *Lophelia pertusa* (van Bleijswijk *et al.*, 2015). *Acinetobacter* was previously suggested to be involved in coral host defensive tactics among other bacterial taxa possessing antibacterial activity in stony corals (Shnit-Orland and Kushmaro, 2009).

Goldsmith *et al.* (2018) described Proteobacterial families Moraxellaceae and Rhodobacteraceae as dominant in their samples, similarly to what we observed in *P. resedaeformis* mucus samples. Bacterial taxa including the families Pseudomonadaceae and Comamonadaceae were observed across studies on *P. resedaeformis*, although there were OTU-level differences across those studies. As there are so few studies on cold-water coral microbiomes, any samples and read data recovered from the deep ocean are of importance to this field. Further research in the field of coral microbiomes (with particular emphasis on cold-water corals) is crucial in understanding the intricate mechanisms regarding microbe-microbe and microbe-host interactions. The interactions occurring within the coral holobiont govern the health of the host and therefore the research recovered from all corals pertaining to their microbiomes during a visibly healthy (and compromised) state will help with future coral microbiome exploration and regional coral reef conservation.

3.7 References

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Chapter 4: Summary and conclusions

4.1 Summary

The research presented in this thesis aimed to provide (1) a review of the underexplored cold-water coral microbiome, (2) a first look at the bacteria associated with the Northwestern Atlantic Ocean cold-water octocoral *Paragorgia arborea* (bubblegum coral), and (3) an investigation of bacteria associated with a co-occurring species, *Primnoa resedaeformis* (popcorn coral). First, we summarized available information in a review (Chapter 1) of the CWC microbiome across a broad geographic distribution, spanning two coral taxa (both Octocorallia and Hexacorallia), and multiple coral microhabitats. This review explored whether corals from varying taxa share bacterial groups, if there are geographical differences in bacterial composition or shared members, and whether there are differences in bacteria across coral microhabitats. Results were compiled from 25 CWC microbiome studies, which examined 26 CWC taxa in some a combination of three different microhabitats (mucus, tissue, and/or skeleton), from a wide depth-range (6-3300 m) across the globe, using four different sequencing methods (clone library, ARISA, 454 Pyrosequencing, Illumina MiSeq).

Regardless of coral subclass (Hexacorallia or Octocorallia), the bacterial phylum Proteobacteria tends to dominate the bacterial component of the cold-water coral microbiome. Within the Proteobacteria, Gammaproteobacteria and Alphaproteobacteria are the most dominant bacterial class within CWC microbiomes (Penn *et al.*, 2006; Brück *et al.*, 2007; Neulinger *et al.*, 2008; Hansson *et al.*, 2009; Kellogg *et al.*, 2009; Galkiewicz *et al.*, 2011; Gray *et al.*, 2011; Bayer *et al.*, 2013a; Vezzulli *et al.*, 2013; van Bleijswijk *et al.*, 2015*;* Kellogg *et al.*, 2016; Lawler *et al*., 2016; Meistertzheim *et al*., 2016; Kellogg *et*

al., 2017; Röthig *et al*., 2017; van de Water *et al.,* 2017; Goldsmith *et al*., 2018). Specifically, the bacterial genus *Endozoicomonas* is reported to be the dominant bacterial taxon within multiple CWC octocoral studies (Bayer *et al.*, 2013a; La Rivière *et al.*, 2013; Vezzulli *et al.*, 2013; van de Water *et al.*, 2017); this genus has been reported to play functional roles for the coral holobiont (Blackall *et al.*, 2015; Sweet and Bulling, 2017). Several studies have suggested that *Endozoicomonas* assists the host with health and probiotic mechanisms (Bayer *et al.*, 2013b; Vezzulli *et al.*, 2013; Ransome *et al.*, 2014; Morrow *et al.*, 2015), protection from bleaching (Pantos *et al.*, 2015), dimethylsulfoniopropionate (DMSP) metabolism (Raina *et al.*, 2009; Bourne *et al.*, 2013; Correa *et al.*, 2013), carbohydrate metabolism and nutrient acquisition (Correa *et al.*, 2013; Morrow *et al.*, 2015). However, the suggested functional roles of *Endozoicomonas* in the animal host still require confirmation (Neave *et al.*, 2016). Additionally, *Propionibacteria* are suggested to play important roles within the coral holobiont (such as reducing nitrate to nitrite; Kellogg *et al.*, 2016) and were documented across several CWC microbiome studies (Bayer *et al.*, 2013a; Kellogg, *et al.*, 2016; Lawler *et al.*, 2016; Kellogg, *et al.*, 2017; Goldsmith *et al.*, 2018). OTUs from the genera *Propionibacterium* and *Mycoplasma* were shared in corals across large geographic distances. A specific *Propionibacterium* OTU was observed in *Lophelia pertusa* from the NE and SW coasts of the Atlantic (van Bleijswijk *et al.*, 2015*;* Kellogg *et al.*, 2017) and a *Mycoplasma* OTU was shared in corals from the SW Atlantic and from Aleutian Islands in the NE Pacific (Gray *et al.*, 2011; Kellogg *et al.*, 2017). Lastly, bacteria were partitioned across microhabitats; for example, *Mycoplasma* was more abundant in tissues and was rare or completely absent in mucus (van Bleijswijk *et al.*, 2015). This review highlighted that the

field of CWC microbiome research is largely data deficient and stressed the need for further work on CWCs, such as that presented in Chapters 2 and 3.

In Chapter 2, we examine the bacterial associates of *Paragorgia arborea* from three submarine canyons along the continental slope of the Gulf of Maine: Nygren-Heezen Intercanyon, Corsair Canyon, and Georges Canyon. The first objective of this study was to characterize the bacteria located within two well-defined coral microhabitats, the surficial mucus surrounding the coral specimen, and the polyp tissue/supportive skeletal framework. The second objective was to examine the degree of similarity in bacterial composition across the three submarine canyons from which coral samples were recovered to test for geographical and bathymetric influences. The results showed a significant difference in bacterial composition between the two microhabitats. The bacterial phyla Proteobacteria, Tenericutes, and Spirochaetes were dominant across *P. arborea* samples, with Tenericutes (orders Entomoplasmatales and Mycoplasmatales) significantly higher in abundance in tissues than in mucus. Several bacterial taxa were significantly more abundant in a particular host microhabitat and were termed bacterial biomarkers. Bacterial OTUs belonging to the taxa *Spirochaeta*, *Mycoplasma*, Flavobacteriaceae, Terasakiellaceae, Campylobacterales and Rickettsiales were biomarkers of *P. arborea* tissues, whereas *Paracoccus* was a biomarker of mucus. These bacterial taxa could be a part of a core *P. arborea* microbiome, with localized microhabitat preference in the host. Additionally, some of the bacterial taxa recovered from *P. arborea* samples have been suggested to play roles in nitrogen cycling (Kellogg *et al.*, 2016; Lawler *et al.*, 2016), which is a mechanism used in nitrogen limited environments by animals such as cold-water corals (Middelburg *et al.*, 2015).

The composition of associated bacteria differed significantly across geographical sampling locations, suggesting bathymetry and other environmental factors (e.g. seawater temperature and nutrient availability) may play a role due to the higher similarity in bacterial composition between Corsair and Georges canyon (depths = 411 m and 423 m, respectively), compared to Nygren-Heezen intercanyon (depth = 700 m). Additionally, significant spatial differences were observed in the bacterial composition of *P. arborea* samples, as both Corsair and Georges canyon samples were higher in similarity and are more proximal to each other, compared to the sample from Nygren-Heezen intercanyon, located further south along the continental slope. The spatial similarities, both within each canyon and between Corsair and Georges Canyon may be associated in part to brooding in *P. arborea*, as bacteria can be vertically transmitted from parent to offspring (Ceh *et al.*, 2013) and larvae are less likely to disperse greater distances from the parental colony compared to non-brooding species. Some bacteria appeared to have been picked up from the surrounding environment: for example, Chlamydiae were abundant in tissue samples from Georges Canyon but were rare elsewhere, suggesting a localized bacterial colonization event. With respect to rare, less abundant bacterial groups, the families Burkholderiaceae, Vibrionaceae, Rhodobacteraceae, Flavobacteraceae, and Campylobacteraceae were present within our coral samples. These rare bacterial families have been previously documented to be present in diseased tropical scleractinian corals (Daniels *et al.,* 2015). The exploration of the bacterial members associated with the octocoral *P. arborea* has yielded important baseline microbiome information for a visually healthy, common habitat-forming species that many organisms rely upon.

In Chapter 3, we examined the bacterial communities associated with the mucus of *Primnoa resedaeformis,* a cold-water octocoral sampled from the same three submarine canyons as the *P. arborea* described in Chapter 3; unfortunately, there were too few OTU sequences recovered from tissue samples to draw meaningful observations. First, the bacterial groups recovered from the mucus of *P. resedaeformis* were described and compared with those reported by Goldsmith *et al.* (2018), to examine within-species similarities and the potential for a regional difference. Next, we discussed similarities in mucus-associated bacteria from the co-occurring species, *P. resedaeformis* and *P. arborea*, both ecologically important to cold-water coral ecosystems. The results suggested low variation in bacterial composition across mucus samples of *P. resedaeformis,* and the presence of bacterial taxa in common with those reported by Goldsmith *et al.* (2018). Specifically, Proteobacteria (Gammaproteobacteria and Alphaproteobacteria) and Bacilli made the largest relative contributions within our samples. The proteobacterial families Rhodobacteraceae and Moraxellaceae were dominant taxa in the *P. resedaeformis* samples recovered by Goldsmith *et al.* (2018) and were also abundant in our mucus samples. Notably, the genus *Acinetobacter* (family Moraxellaceae), which was highly abundant in our mucus samples, was found in the mucus of both the cold-water octocoral *Paragorgia arborea* (Weiler *et al.,* 2018) and the hexacoral *Lophelia pertusa* (van Bleijswijk *et al.*, 2015), and reported in the core microbiome of the genus *Primnoa* (Goldsmith *et al.*, 2018). Additionally, bacteria belonging to the families Comamonadaceae and Pseudomonadaceae were observed in our mucus samples and in the *P. resedaeformis* samples recovered by Goldsmith *et al.* (2018).

The sequencing depths and OTU counts for our *P. resedaeformis* mucus samples were low compared to those obtained from the *P. resedaeformis* samples (combined mucus and tissues) collected and extracted by Goldsmith *et al.* (2018). However, certain similarities between studies are striking and suggest that *P. resedaeformis* may show specificity for taxa such as *Acinetobacter* that might play important roles for the host. Lastly, the OTUs present in the mucosal microhabitat of both *P. resedaeformis* and *P. arborea* could represent bacteria that are coral specialists, as some of these OTUs are in much greater relative abundance with the coral host, compared to that of the surrounding seawater. OTUs belonging to the bacterial genus *Acinetobacter* were found to be highly abundant in *P. resedaeformis* (representing up to 25% of the total microbial composition in mucus samples), compared to seawater (<1% total microbial composition).

4.2 Conclusions and further directions

The bacterial communities of CWCs are highly understudied compared to their warm-water shallow counterparts. Similar to corals within tropical climates, CWCs host diverse microbial assemblages and the bacteria from the microbiome may associate with the host through commensal, mutualistic, dysbiotic, parasitic, or pathogenic interactions. These interactions may be partitioned across host microhabitats due to specific biological requirements, and some of the bacterial members associated with the CWC host may be conserved across geographical distances. The available information on CWC microbiomes are explored in detail within this thesis, along with new characterizations of the bacterial associates in the octocoral *Paragorgia arborea* and in the surficial mucus of *Primnoa resedaeformis*. Across CWC taxa, specific bacterial taxa are found in association with corals regardless of geography and bathymetry. Within CWCs, there

seems to be partitioning according to coral microhabitats. Different bacterial groups are found to colonize mucus, tissues and skeleton microhabitats within CWCs (i.e. Spirochaetes in large abundance within *P. arborea* tissues and in very low abundance in mucus), and similar observations have been found in warm-water environments (Sweet *et al.,* 2011; Pollock *et al.,* 2018). The partitioning of bacterial groups among microhabitats could indicate compartment-specific functions for the host. Lastly, there seems to be some evidence of coral host specificity across CWCs, for example, Tenericutes were highly abundant in *P. arborea* (comprising most of the microbial composition) and absent in *P. resedaeformis*. While the roles of the bacterial groups found in association with CWCs remain largely unknown, several groups have been suggested to benefit the host with nutrient provisioning and nitrogen recycling (Kellogg *et al.,* 2016; Lawler *et al.,* 2016; Weiler *et al.,* 2018), as these organisms inhabit nutrient-limited environments.

While research on the CWC microbiome is still in its infancy, the use of metagenomics is providing the field with descriptive studies characterizing and comparing the prokaryotic members present within various CWCs. To date, the exploration of microbial interactions and their functional importance within the holobiont using metatranscriptomics remains absent from the literature. A review of the available CWC microbiome studies has revealed a huge disparity in the methodologies used to sample, process, sequence, and analyze the CWC microbiome. However similar bacterial members were nevertheless reported across many coral taxa, among geographic locations and within coral microhabitats. While the field of coral microbiome research is still developing due to only recent advancements in molecular approaches (Franzosa *et al.,* 2015; Gilbert *et al.,* 2016), a unified approach in one or more of the aforementioned

variable methodologies would benefit the future of coral microbiome research. Given better standardized methodologies and more advanced molecular approaches (such as metatranscriptomics to explore the individual functional roles of the microbial associates), the coral microbiome can be explored in greater depth to understand the importance of microbes in host health and survival. Many studies have suggested that the future of corals depends on their microbial associates, and the fate of our oceans relies upon the survival of the world's corals, not only within the tropics but across the entirety of our oceans. The present thesis provides the field of coral microbiome research with novel information on the bacteria found in association with CWCs at a visibly healthy state. This work contributes to our understanding of CWC health and susceptibility to stress, and may help improve marine conservation and policy planning.

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Supplementary Materials

Supplementary Table 2. 1. Number of reads for each biomarker OTU, per sample. Sample names are described in Table 1.

				Sample Name													
OTU Identity	Taxonomic Rank	Biomarker	Effect Size	PA-N28-T1	PA-N28-T2	PA-N28-T3	PA-N28-MU	PA-C24-T1	PA-C24-T2	PA-C24-T3	PA-C24-MU	PA-G08-T1	PA-G08-T2	PA-G08-T3	PA-G08-MU	WA-N08	WA-G11
Spirochaeta 2	Genus	Tissue	4.0205858	914	635	696	7	165	307	356	8	94	159	231	3	0	0
Mycoplasma	Genus	Tissue	2.3670641	48	64	71	0	33	73	100	1	17	22	14	0	0	0
Rickettsiales	Order	Tissue	2.3399322	171	214	110	5	67	306	443	3	30	18	157	7	0	0
Campylobacterales	Order	Tissue	1.8426756	61	32	12	0	5	9	21	0	3	9	3	0	0	0
Flavobacteriaceae	Family	Tissue	1.7177874	56	66	64	0	11	69	84	0	0	9	30	0	0	0
Terasakiellaceae	Family	Tissue	1.4104881	599	45	72	0	28	71	93	0	1	0	29	0	0	0
Paracoccus marcusii	Genus	Mucus	-1.46697	1	0	18	17	0	0	0	18	0	0	0	5	3	0
Total reads after processing				3392	6060	1929	696	2227	3274	4038	1459	2744	2553	5220	363	3997	6076

Supplementary Table 2. 2. Proportion of reads for each biomarker OTU. Sample names are described in Table 1.

				Sample Name													
OTU Identity	Taxonomic Rank	Biomarker	Effect Size	PA-N28-T1	PA-N28-T2	PA-N28-T3	PA-N28-MU	PA-C24-T1	PA-C24-T2	PA-C24-T3	PA-C24-MU	PA-G08-T1	PA-G08-T2	PA-G08-T3	PA-G08-MU	WA-N08	WA-G11
Spirochaeta 2	Genus	Tissue	4.0205858	26.95	10.48	36.08	1.01	7.41	9.38	8.82	0.55	3.43	6.23	4.43	0.83	0.00	0.00
Mycoplasma	Genus	Tissue	2.3670641	1.42	1.06	3.68	0.00	1.48	2.23	2.48	0.07	0.62	0.86	0.27	0.00	0.00	0.00
Rickettsiales	Order	Tissue	2.3399322	5.04	3.53	5.70	0.72	3.01	9.35	10.97	0.21	1.09	0.71	3.01	1.93	0.00	0.00
Campylobacterales	Order	Tissue	1.8426756	1.80	0.53	0.62	0.00	0.22	0.27	0.52	0.00	0.11	0.35	0.06	0.00	0.00	0.00
Flavobacteriaceae	Family	Tissue	1.7177874	1.65	1.09	3.32	0.00	0.49	2.11	2.08	0.00	0.00	0.35	0.57	0.00	0.00	0.00
Terasakiellaceae	Family	Tissue	1.4104881	17.66	0.74	3.73	0.00	1.26	2.17	2.30	0.00	0.04	0.00	0.56	0.00	0.00	0.00
Paracoccus marcusii	Genus	Mucus	-1.46697	0.03	0.00	0.93	2.44	0.00	0.00	0.00	1.23	0.00	0.00	0.00	1.38	0.08	0.00