View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by The University of North Carolina at Greensboro

THE ISOLATION, IDENTIFICATION, AND CHARACTERIZATION OF BACTERIA ASSOCIATED WITH THE DEEP-SEA CORAL *LOPHELIA PERTUSA*.

A thesis presented to the faculty of the Graduate School of Western Carolina University in fulfillment of the requirements for the degree of Master of Science in Biology.

by

S. Gold

Director: Seán O'Connell
Associate Professor of Biology
Biology Department

Committee members:
Dr. Christina Kellogg, St. Petersburg Coastal and Marine Science Center
Dr. Jeremy Hyman, WCU Biology Department
Dr. Amanda Storm, WCU Biology Department

April, 2020

ACKNOWLEDGMENTS

I would like to thank Dr. Erik Cordes, chief scientist of the Deep Search cruise, for connecting me with Dr. Chris Kellogg and enabling collection of these samples. I'm so grateful to Dr. Kellogg for providing me with these samples, and for her insights, constructive feedback, and encouragement during the preparation of this thesis. I'm greatly inspired by her work and deeply appreciative of the opportunity to collaborate with her on this project.

I would also like to thank the rest of my thesis committee, Dr. Jeremy Hyman and Dr. Amanda Storm, and especially my advisor, Dr. Sean O'Connell. His guidance in study design, assistance in culture work and molecular tests conducted in the lab, and support in analysis of data and drafting of this thesis were invaluable and critical to the completion of this project. I'd also like to thank Dr. Katherine Matthews for her aid in phylogenetic analysis of isolates, and the Principles of General Microbiology students for their assistance in some of the culture work for this project.

I would also like to express my gratitude for the funding sources for my graduate research, including Western Carolina University's Arts and Sciences Grant as well as WCU's Summer 2019 Graduate Research Assistantship Grant. Without these resources, my research would not have been possible.

Finally, I would like to thank my family, friends, and fellow graduate students for their consistent support and encouragement.

TABLE OF CONTENTS

ACKNOWLEDGMENTS ii

TABLE OF CONTENTS iii

LIST OF TABLES iv

ABSTRACT vii

INTRODUCTION 1

METHODS 9
- Site descriptions and sample collection 9
- Bacterial enrichment and isolation 10
- Bacterial identification by 16S rRNA gene amplification and sequencing 13
- Culture-based characterization of bacterial isolates 14
- PCR analysis of genes for ammonia oxidation and nitrogen fixation 15
- Statistical and phylogenetic analysis 17

RESULTS 18

DISCUSSION 31

CONCLUSION 37

REFERENCES CONSULTED 39

APPENDIX A. MICROBIOLOGICAL MEDIA RECIPES 49
- Medium for Ammonia Oxidizers (Modified from ATCC medium TSD-99) 49
- Medium for Chitin Degraders (Modified from Murthy and Bleakley, 2012) 50
- Medium for Oligotrophic Heterotrophic Marine Organisms (General) 51

APPENDIX B. SUPPLEMENTAL DATA 51

LIST OF TABLES

Table 1. Collection metadata for *Lophelia pertusa* samples. 9
Table 2. Isolate information for subcultures. 12
Table 3. Isolate sequence matches based on 16S rRNA sequence analysis using BLAST.. 19
Table 4. Antibiotic resistance of tested isolates against various antibiotics. 23
Table B1. Binary Key for Heatmap and PCA Analysis. 51

LIST OF FIGURES

Figure 1. Map showing locations of Richardson Reef Complex sample sites.. 10
Figure 2. Heatmap of morphological and physiological features of isolated cultures. 23
Figure 3. Principal components analysis (PCA) of all Gram negative isolates. 25
Figure 4. Principal components analysis (PCA) of isolates cultured from chitin and DSR2B. ... 26
Figure 5. Principal components analysis (PCA) of only *Pseudoalteromonas*. 27
Figure 6. Maximum likelihood tree of *Pseudoalteromonas* isolates. 28
Figure 7. Maximum likelihood tree of *Pseudoalteromonas* isolates with the highest reads. 29
Figure 8. Neighbor-joining tree showing *Pseudoalteromonas* isolates.. 30

LIST OF ABBREVIATIONS

AMO: Ammonia oxidation
AR: Antibiotic resistance
CWC: Cold-water coral
C: Carbon
DSR2B: Dilute, salty Reasoner's 2 broth
ML: Maximum likelihood
N: Nitrogen
NJ: Neighbor joining
PCA: Principal components analysis
SIM: Sulfur, indole, motility

ABSTRACT

Cold-water corals (CWC) form the structural basis of highly diverse and productive ecosystems in the deep, dark ocean, serving as important spawning, nursery, and breeding habitat for many fishes and invertebrates. As such, they play an important role in supporting fisheries that humans rely on, as well as general ocean health, which is of critical importance to Earth as the effects of climate change unfold. CWCs are heterotrophic filter feeders, and their ability to survive in dark, oligotrophic waters may be linked to partnerships with microbial symbionts that participate in nutrient cycling and conservation. While indirect methods–DNA sequencing, whole genome analysis, isotopic analysis–have been used to hypothesize the roles of these symbionts, few studies have grown cultures of associated microbes and directly observed the metabolic processes involved in carbon and nitrogen turnover. In this study, bacteria cultured from the globally distributed, deep-sea coral *Lophelia pertusa* were isolated and characterized according to morphological and physiological characteristics. Sanger sequencing of 16S rRNA from the isolates yielded a diversity of bacterial species in the phylum *Proteobacteria*. In culture, isolates demonstrate the ability to use a variety of organics as carbon, nitrogen, and energy sources, the most notable of which is chitin, a polymer containing both carbon and nitrogen that is abundant in marine systems. Additionally, preliminary evidence suggests the ability of one isolate to fix nitrogen. These findings corroborate evidence of nutrient cycling in CWCs and support the hypothesis that microbial associates of these corals are an important aspect of their ecophysiology and likely help fuel their productivity. Physical and physiological stress induced by changes in the environment resulting from human activities and climate change could influence host-microbe interactions, altering the ability of CWCs to conserve and recycle

limiting resources. Loss of CWC ecosystems would mean loss of critical habitat and a globally relevant carbon sink.

INTRODUCTION

The cold-water coral (CWC) *Lophelia pertusa* is a globally distributed, scleractinian coral that forms the structural basis of highly diverse and productive ecosystems in the deep ocean, serving as important spawning, nursery, and breeding habitat for many fishes and invertebrates (Costello et al. 2005, Mortensen et al. 1995, Cordes et al. 2008). *Lophelia* and other CWCs are ecosystem engineers, generating hotspots of biodiversity and organic material in an otherwise oligotrophic deep sea environment where nutrients are limiting (Soetaert et al. 2016). In the past decade, there has been an increase in focus on these CWCs driven mainly by conservation concerns (Roberts and Hirshfield 2004). CWC gardens are often located in places where large-scale commercial fishing and drilling for oil and gas are occurring, and are subject to oil spills, dredging, and additional environmental stressors including ocean acidification and pollution (Fisher et al. 2014). These corals are of scientific interest for a variety of reasons including bioprospecting--discovery of new commercially useful biological compounds--and in studying the impacts of environmental change (Maxwell 2005, Lu et al. 2015).

Corals (generally) are known for their ability to flourish in nutrient-poor environments due to their ability to take up and recycle nutrients (Radecker et al. 2015). Corals are polytrophic, meaning that they function at multiple trophic levels–as primary producers, primary consumers, and secondary consumers (Muscatine and Porter 1977). This is in part due to their heterotrophic capacity as suspension feeders, and in part due to their close association with microorganisms capable of carrying out biological fixation of carbon and nitrogen and nutrient cycling (Wegley et al. 2007, Radecker et al. 2015, Middleberg et al. 2015). Together, the complex assemblage of coral animal and microbial associates are referred to as the coral holobiont. In tropical corals,

Symbiodinium are largely responsible for supplying their coral host with photosynthate as a carbon and energy source for secondary production (Muscatine and Porter 1977). However, in CWCs like *L. pertusa*, this role is likely played by prokaryotes. The ecology of CWCs is fundamentally different from that of their shallow-water counterparts. CWC can live hundreds to thousands of meters below the sea surface, well below the photic zone (Roberts et al. 2006). At these depths, there is no ambient light, temperatures are between 4-12ºC, and there is increased pressure (Roberts et al. 2006). Under these conditions, *L. pertusa* and other CWCs cannot host photosynthetic partners. Thus, nutrient acquisition and cycling by the *L. pertusa* holobiont is a key area of study.

Studies of the *Lophelia* holobiont have paid particular attention to identifying microbial associates and uncovering how they are involved in nutrient cycling. Biological fixation of both carbon and nitrogen have been demonstrated by the *L. pertusa* holobiont, as well as a complete nitrogen cycle (Middleberg et al. 2015). Biological fixation is restricted to prokaryotes, importantly implicating involvement of bacterial (and potentially archaeal) associates in these processes. Research on *L. pertusa* microbial communities reveals that the coral has a microbiome–an associated bacterial community distinct from its surrounding environment–and that *Lophelia*-specific bacteria may be conserved, even among distant populations such as the Gulf of Mexico and Trondheimsfjord in Norway (Galkiewicz et al. 2011, Kellogg et al. 2009, Kellogg et al. 2017, Neulinger et al. 2008, Schöttner et al. 2009, van Bleijswijk et al. 2015, Yakimov et al. 2005, Meistratzheim et al. 2016). Recent 16S rRNA amplicon analysis of DNA from *Lophelia* samples taken in the Gulf of Mexico and along the Atlantic coastline corroborate this, finding fifteen conserved bacterial operational taxonomic units (OTU) among these corals (Kellogg et al. 2017). Molecular studies using metagenomic data have attempted to identify

bacterial genera and species that may be involved in various nutrient cycles based on functional gene predictions (Neulinger et al. 2008, Kellogg et al. 2009, Kellogg et al. 2017). Taken together, the data suggest that *L. pertusa* has a distinct microbiome consisting of associates involved in carbon and nitrogen cycling.

The ability of *L. pertusa* to survive in dark, nutrient-poor waters appears to be dependent on its ability to conserve limited resources and access new carbon and nitrogen sources, processes that are necessarily mediated by bacterial and archaeal symbionts. The coral organism provides a diversity of habitats for microbes to colonize and access to a variety of food sources. In particular, coral mucus and the coral gastric cavity provide some of the most nutrient-rich habitats, and may very well account for the majority of microbial diversity observed among corals (Fernando et al. 2014, Thompson et al. 2015). Coral mucus is a complex substance made up of carbohydrates, lipids, and proteins (Rix et al. 2016) that the coral secretes and which provides a layer several micrometers thick of cover on the surface of the coral's soft body (Thompson et al. 2015, Brown and Bythell, 2005). The mucus layer is enriched by sugars and coral waste products (such as ammonia and CO_2), and by the nutrient-bearing particulates from the surrounding water column that get trapped in it (Cole and Strathman 1973, Thompson et al. 2015). These compounds provide a source of nutrients for bacteria living on and in the mucus layer.

The coral gastric cavity is another important habitat for microbes where nutrient cycling is likely occurring. Field studies of *L. pertusa* indicate that it feeds on a broad range of food sources including suspended particulate matter, bacteria, phytoplankton, and zooplankton, and lab studies have confirmed these findings (Mueller et al. 2014; van Oevelen et al. 2016). Analysis of food processing by *L. pertusa* reveals incorporation of ^{13}C and ^{15}N from several of

these food sources into bulk tissue, fatty acids, amino acids, and the coral skeleton, and also reveals contribution to coral energy use and respiration (Mueller et al. 2014, van Oevelen et al. 2016). However, bacteria living in the coral gut are also predicted to participate in digestion of ingested particles and may provide the coral with essential nutrients like vitamins and amino acids (Thompson et al. 2015).

Cycling of carbon and nitrogen by the *Lophelia* holobiont serves as a means of conserving limited resources. Functional predictions of bacteria associated with *L. pertusa* suggest that they are capable of using a variety of organics as carbon, nitrogen, and energy sources (Kellogg et al. 2017). Further functional predictions for associated bacteria suggest that they are capable of synthesizing arginine, tyrosine, isoleucine, leucine, lysine, phenylalanine, tryptophan, and valine, which are all amino acids found in coral mucus (Kellogg et al. 2017). This in tandem with findings that carbon and nitrogen isotopes show up in amino acids that the coral animal cannot synthesize (methionine, isoleucine, phenylalanine, valine, threonine (Middleburg et al. 2015) suggest trophic transfer of microbially processed carbon and nitrogen resources from microbial symbionts to the coral host. In addition, observation of ammonium production and assimilation, nitrification, and denitrification in *L. pertusa* in the lab indicate a complete nitrogen cycle mediated by the coral holobiont (Middleburg et al. 2015).

Indirect evidence suggests the role of microbial associates of *L. pertusa* in generating new sources of organic carbon via biological fixation (Middleburg et al. 2015, Kellogg et al. 2017). Phototrophy is not an option in the deep ocean, indicating that chemotrophic prokaryotes associated with the coral are responsible for the input of new organic material into the system, providing the corals with an additional source of energy and carbon. Evidence of microbially fixed carbon being assimilated into coral tissues (Middelburg et al. 2015) confirms the role of

bacterial symbionts in generating organic carbon that is actively used by the coral for secondary production. The energy for carbon fixation by chemolithoautotrophs comes from oxidation of substances such as ammonia or reduced sulfur (Middelburg et al. 2015). This is corroborated by the presence of ammonia-oxidizing bacterial genes and 16S rRNA sequences for species that are metabolically capable of ammonia oxidation (such as *Pseudonocardia*) in *L. pertusa* (Kellogg et al. 2017). Culture-based studies have yet to demonstrate ammonia oxidation and carbon fixation (Galkiewicz et al. 2011), but it is likely that microbial associates are responsible for the carbon fixation that is supplying these corals with organic carbon in the deep sea.

Lophelia-associated bacteria are also likely to be involved in nitrogen fixation. Functional predictions based on amplicon analysis of DNA sequences from *Lophelia* samples in the Gulf of Mexico and Northern Atlantic suggest that nitrogen fixation and cycling are pathways present among bacteria associated with *Lophelia*, yielding high values for nitrogen metabolism (Kellogg et. al, 2017). Studies of *Lophelia* reporting gene sequences for cyanobacteria, *Rhizobiales*, and the genus *Vibrio*–all capable of nitrogen fixation–support the presence of these microbes (Galkiewicz et al. 2011, Kellogg et al. 2017).

In addition to nutrient cycling, the coral microbiome may play a key role in protecting the coral animal from pathogens. The coral probiotic hypothesis proposes that the coral holobiont is capable of developing resistance to disease (Reshef et al. 2006). The coral holobiont is proposed to include specialized microbes that may protect the coral animal from pathogens by producing antibiotics and/or filling particular niches, and that disruption of associated prokaryotic communities could lead to coral disease (Rohwer et al. 2002). Evidence to support this hypothesis includes 1) large and diverse bacterial populations are associated with coral mucus and tissues, 2) changes seen in coral-associated bacterial populations when environmental

conditions change, and 3) the ability of coral to develop resistance to pathogens (Reshef et al. 2006). Results from several studies indicate that coral-associated microbial communities shift in response to stressors such as elevated temperatures, bleaching or disease (Littman et al. 2010, Littman et al. 2011, Kellogg et al. 2013, Frias-Lopez et al. 2002). Research conducted on coral pathogenesis suggests that bacterial symbionts may be responsible for coral resistance to pathogenic bacteria. Mucus-associated microbes from the coral *Acropora palmata* have been shown to demonstrate antimicrobial properties and inhibit the growth of coral pathogens, including a strain of *Serratia marcescens* responsible for white pox disease (Richie, 2006). The coral *Oculina patagonica* has shown the ability to resist *Vibrio shiloi* infection over time, suggesting evolution of a resistance mechanism in associated bacterial symbionts (Rosenberg and Falkowitz, 2004). Additionally, genes for antibiotic resistance were identified in a metagenomic analysis of *P. asteroides* and associated holobiont DNA. Specifically observed were genes for resistance to fluoroquinolones (Wegley et al. 2007). It has also been suggested that corals may have the ability to form associations with new microbial partners in order to resist specific pathogens (Wegley et al. 2007).

An understanding of the prokaryotic symbionts associated with the deep-sea coral *L. pertusa* may provide clues into the role of its microbiome in nutrient cycling and the protection of these organisms. While molecular diversity surveys allow for identification of organisms and provide functional predictions about what role they play, culture-based surveys allow for direct testing to know what the organisms (bacteria and archaea) are capable of and how they respond to changes to their environment. Direct observation and analysis of microbial associates of *L. pertusa* can aid in understanding how these corals are able to conserve and cycle nutrients, which likely fuels their productivity and plays an important role in supporting fisheries that humans rely

on as well as on general ocean health, which is of critical importance to Earth as the effects of climate change unfold.

The main objective of this study was to culture, isolate, identify, and characterize microbial associates of *Lophelia pertusa* samples from the Atlantic Ocean that may be involved in nutrient cycling and antimicrobial properties. A culture-based study has the potential to elucidate active metabolic processes in these bacterial symbionts. In this study, particular attention is paid to carbon and nitrogen metabolism with the goal of identifying ways that associated bacteria may be contributing to general organic turnover and provision of novel sources of carbon and nitrogen for the coral host. It is hypothesized that prokaryotic organisms in association with *L. pertusa* will have metabolic pathways for nitrification, which is a known process used by the coral holobiont, and for the breakdown of chitin, which is likely an important carbon and nitrogen source found in a variety of prey captured by the coral organism–chitin is one of the most abundant sugar polymers in nature and is made and used by many marine organisms including phytoplankton and zooplankton species, fungi, and crustaceans (Svitil et al. 1997). It is also predicted that associated bacteria may be resistant to various antibiotics, suggesting their involvement protecting the coral from pathogens. A general medium was used to encourage the growth of microbes with a wide range of organic metabolisms in addition to enrichment media designed to select for microbes with more specific metabolic pathways for nitrification and chitin degradation. Subsequent analysis of isolates for additional metabolic pathways sought to provide insight into ways that bacterial associates may contribute to nutrient cycling and trophic transfer to their coral host, while assessment of antimicrobial properties of cultures aimed to offer evidence in support of the coral probiotic hypothesis. Isolates were identified using 16S rRNA gene sequencing in order to associate organisms with metabolic

activities and offer greater insight into a *Lophelia*-specific microbiome. Their characterization has the potential to illuminate a key role that microbial associates play in the nutrition and health of these cold-water corals.

METHODS

Site descriptions and sample collection

Coral samples were collected by Dr. Christina Kellogg during the 2019 research cruise of the Deep Search project (April 9–30, 2019). Samples were taken from three individual *L. pertusa* colonies at two Richardson Reef Complex sites (Table 1, Figure 1). Sampling was done using the Jason remotely operated vehicle (ROV) to collect corals at depths of 690 and 756 meters. Environmental parameters (depth, temperature, salinity) were measured (Table 1). Samples RB1903-J2-1128-Biobox3 and RB1903-J2-1128-Biobox6 were collected into PVC boxes such that the corals were not in direct contract with other samples, but the lid does not seal completely so some exposure to water column occurs over the course of the dive. Sample RB1903-J2-1129-Q4 was collected into a single polyvinyl chloride (PVC) quiver with a rubber stopper lid that had been cleaned with ethanol, filled with freshwater and sealed at the surface prior to deployment (Kellogg et al. 2017). At depth, this quiver was individually opened, allowing ambient seawater to replace the freshwater. *Lophelia* branches were collected and placed inside the quiver and sealed at depth. Samples were processed upon reaching the surface using sterile technique.

Table 1. Collection metadata for *L. pertusa* samples.

Sample ID	Date	Collection time (UTC)	Depth (m)	Latitude	Longitude	Temp (°C)	Salinity
RB1903-J2-1128-Biobox3	04/11/19	06:48	756	31°53.093	77°22.225	5.1	35.0

RB1903-J2-1128-Biobox6	04/11/19	05:30	756	31°52.997	77°22.338	5.5	35.0
RB1903-J2-1129-Q4	04/14/19	06:10	690	31°59.093	77°24.646	10	35.3

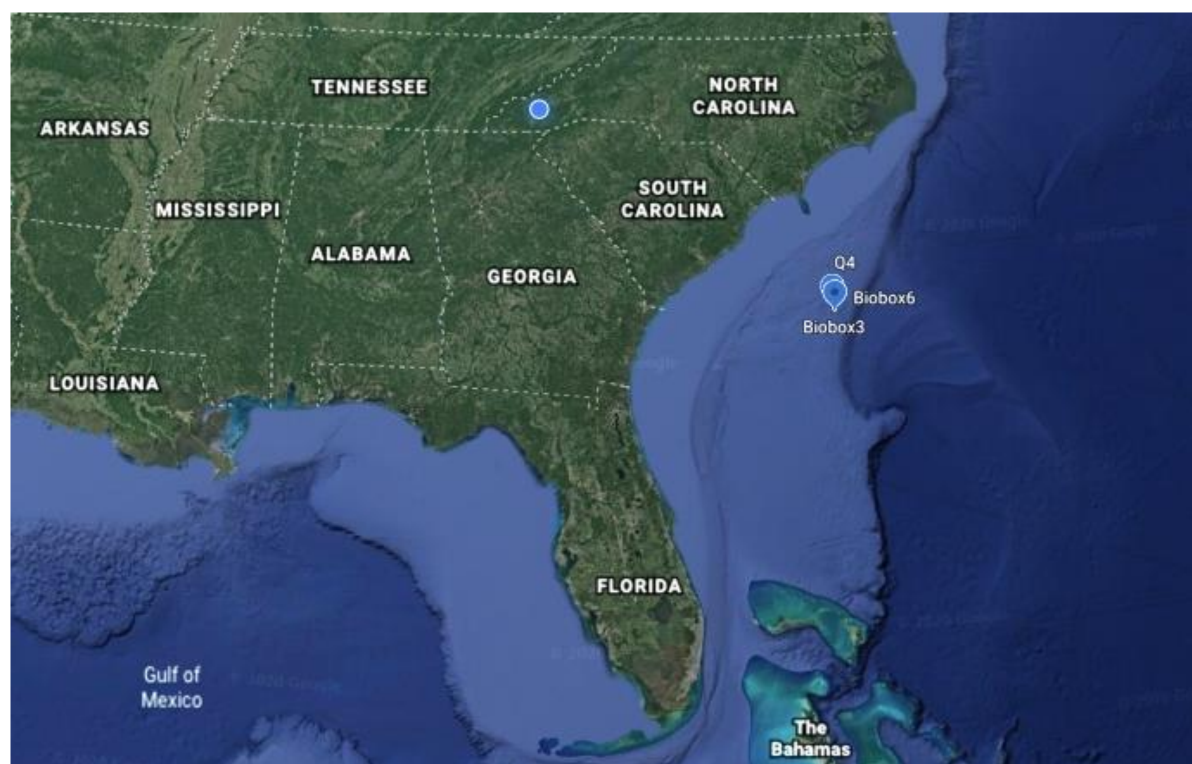

Figure 1. Map showing locations of Richardson Reef Complex sample sites. Sites in this study were collected off the coast of Charleston, SC in the Atlantic Ocean.

Bacterial enrichment and isolation

For each sample, *Lophelia pertusa* pieces with a total of 20 polyps were removed from the collection container (Biobox or Quiver) using sterile forceps, rinsed with 5 ml sterile 1XPBS (phosphate-buffered saline) to remove any loosely associated surface bacteria, and then transferred to a sterile aluminum weigh dish. A flame-sterilized hammer was used to smash open

the calyxes to expose the polyp tissue and sterile forceps were used to separate as much tissue as possible from the skeleton. Ten ml of sterile 1XPBS was added to each dish to create a slurry. The liquid and coral pieces from each sample were transferred to a sterile 15-ml falcon tube and vortexed on high for 5 min to liberate bacteria from mucus, tissue, and skeleton into the supernatant liquid. This slurry was used to inoculate selective microbiological growth media on board the sampling vessel to promote growth of bacteria involved in nitrification, chitin degradation, and general carbon turnover. Twenty plates total for each media type were inoculated. One hundred µl of slurry was spread-plated onto each plate. Spread plating was done inside a plastic box where the interior had been wiped down with 70% ethanol–the purpose being to create a quasi-sterile space with restricted air-flow to reduce contamination since there aren't sterile hoods on the ship. Spreading was done with a glass rod which was dipped into ethanol, flamed, and then touched to a part of the medium that does not contain slurry to quickly cool it, then spreading the liquid in multiple directions until it was evenly distributed. Inoculated plates were stored at 10_{o}C until transfer to WCU.

Three different microbiological enrichment media were used: one medium to target heterotrophs, one medium to select for chitin degraders, and one medium to enrich for ammonia oxidizers. To ensure growth of microorganisms from coral samples, a general heterotrophic medium was used to capture a wide variety of oligotrophic organisms. To simulate the nutrient poor conditions of seawater, the general heterotrophic medium, designated as Dilute, salty R2B (DSR2B) was made with 10% R2B modified with 2.6% NaCl added (see Appendix A). Chitin degrading prokaryotes are chemoorganotrophic and use organic compounds as carbon and energy sources for metabolism. A chitin-enriched media (CH) was used to target bacteria with chitinase activity (Appendix A). Nitrifying *Bacteria* and *Archaea* are chemolithotrophic and

oxidize either ammonia (NH_3) or nitrite (NO_2^-) to form nitrate (NO_3^-), gleaning energy from these reactions to fix CO_2. A third medium (AMO) was used to enrich for these ammonia oxidizing microorganisms (Appendix A). Gellan gum (0.8%) was used as a solidifying agent for all media to prevent desiccation of plates for a prolonged incubation period of several weeks.

Plates were monitored for growth, and three to five colonies from each plate were subcultured and streaked for isolation on designated enrichment media. 109 colonies were subcultured and streaked for isolation. Negative staining with nigrosin was used to assess subcultures for purity. 27 cultures were selected for further evaluation based on sufficient growth and purity. Pure cultures from chitin enrichments were transferred to dilute, salty R2B media plates for further testing to eliminate time and resource-intensive chitin media preparation. All cultures grown and tested on dilute, salty R2B were labeled with an "R" to distinguish them from isolates from the AMO medium, which have only numeric values (Table 2).

Table 2. Isolate information for subcultures showing name used for each culture, the initial culture medium, and source coral sample.

Isolate ID	Enrichment medium	Sample ID/Device
R1	Dilute R2B with salt	RB1903-J2-1128-Biobox6
R12	Dilute R2B with salt	RB1903-J2-1129-Q4
R20	Dilute R2B with salt	RB1903-J2-1129-Q4
R27	Dilute R2B with salt	RB1903-J2-1129-Q4
R35	Dilute R2B with salt	RB1903-J2-1129-Q4
R38	Dilute R2B with salt	RB1903-J2-1129-Q4
R41	Dilute R2B with salt	RB1903-J2-1128-Biobox6
R9	Chitin as sole carbon source	RB1903-J2-1129-Q4

R15	Chitin as sole carbon source	RB1903-J2-1129-Q4
R17	Chitin as sole carbon source	RB1903-J2-1129-Q4
R29	Chitin as sole carbon source	RB1903-J2-1129-Q4
R30	Chitin as sole carbon source	RB1903-J2-1129-Q4
R32	Chitin as sole carbon source	RB1903-J2-1129-Q4
R33	Chitin as sole carbon source	RB1903-J2-1129-Q4
R43	Chitin as sole carbon source	RB1903-J2-1129-Q4
R48	Chitin as sole carbon source	RB1903-J2-1129-Q4
62	Ammonia oxidation	RB1903-J2-1129-Q4
63	Ammonia oxidation	RB1903-J2-1129-Q4
64	Ammonia oxidation	RB1903-J2-1129-Q4
65	Ammonia oxidation	RB1903-J2-1129-Q4
66	Ammonia oxidation	RB1903-J2-1128-Biobox6
67	Ammonia oxidation	RB1903-J2-1129-Q4
68	Ammonia oxidation	RB1903-J2-1129-Q4
69	Ammonia oxidation	RB1903-J2-1129-Q4
70	Ammonia oxidation	RB1903-J2-1129-Q4
71	Ammonia oxidation	RB1903-J2-1129-Q4
72	Ammonia oxidation	RB1903-J2-1129-Q4

Bacterial identification by 16S rRNA gene amplification and sequencing

DNA from bacterial isolates was extracted and amplified by PCR for full length gene sequencing (Sanger). Submitted bacterial colonies underwent a crude NaOH lysis and PCR was performed by GENEWIZ, Inc. (South Plainfield, New Jersey) using universal 16S primer sets to amplify regions V1 through V9 of the 16S rRNA gene. Samples were spot checked on gel to confirm amplification and underwent enzymatic cleanup. After cleanup primer extension

sequencing was performed by GENEWIZ, Inc. using Applied Biosystems BigDye version 3.1. Both forward and reverse strands were sequenced using sequencing primers internal to the amplicon and sequencing outward to ensure the overlap of traces. The reactions were then run on Applied Biosystem's 3730xl DNA Analyzer. 16S rRNA gene sequences for all isolates were analyzed using NCBI BLAST (Altschul et al. 1990) and the Ribosomal Database Project (Wang et al. 2007) to determine the closest relatives of the isolates at the level of genus and species, when possible. Further molecular analysis of isolates using PCR alone described below.

Culture-based characterization of bacterial isolates

All cultures except for AMO isolates were transferred to dilute, salty R2B medium for successive culture work. Agar was used as the solidifying agent for both media as it is easier to work with and desiccation of plates was no longer a concern. Negative and Gram staining techniques were used to determine cell shape, size and Gram reaction. Tests for salinity (0 - 15%), pH (4 - 11), and temperature (4°C - 50°C) ranges were conducted using modified DSR2B media to determine parameters supporting growth. Tests for motility, oxidase and catalase activity, anaerobic growth, nitrate reduction, fermentative metabolism, and other metabolic pathways were conducted on isolates to observe physical and metabolic properties. All culture-based protocols were taken from Cappuccino and Sherman (2014) and supporting materials and kits were purchased from Fisher Scientific. Tests for anaerobic growth were conducted using the BD BBL GasPak system. Tests for catalase and Cytochrome C oxidase activity were performed using hydrogen peroxide and BD DrySlides respectively. Tests for nitrate reduction were conducted using nitrate broth with durham tubes modified with 3% sterile saline solution. SIM tubes modified with 3% salt were used to test sulfur reduction, indole production, and motility.

Semi-solid media tubes modified with 3% salt were also used for separate motility evaluation. Tests for casein hydrolysis, urea hydrolysis, DNase, gelatin hydrolysis, and starch hydrolysis were all conducted using designated enrichment media modified with 3% salt. Fermentation of glucose, lactose, sucrose, and saccharose were all conducted on separate enrichment media modified with 3% salt using a pH color indicator to indicate fermentation of each sugar. Tests for heterocyst formation were conducted on a nitrogen deficient enrichment medium modified with 3% salt.

Isolates were screened for antibiotic resistance and antimicrobial properties. The Kirby-Bauer method (Hudzicki 2009) was used to assess resistance to known antibiotics using dilute salty R2B medium in place of Mueller-Hinton agar. Isolates were tested for resistance to the following antibiotics with standard concentrations: Tetracycline (30 µg), Clindamycin (2 µg), Colistin (10 µg), Chloramphenicol (30 µg), Vancomycin (30 µg), Streptomycin (30 µg), Erythromycin (15 µg), Penicillin G (10 µg), and Ampicillin (10 µg) (Table 4). The cross streak method was used to test *Lophelia*-associated bacterial isolates for antimicrobial properties (Vijayalakshmi and Jawahar, 2011; Balouri et al. 2016). Dilute, salty R2B plates were inoculated with a lawn of *Vibrio* and *Pseudoalteromonas* isolates and then cross-streaked with single streaks of other isolates from coral samples (Vijayalakshmi and Jawahar 2011, Davis et al. 2017, Balouri et al. 2016). Plates were allowed to incubate for a week at room temperature and microbial interactions were examined for signs of inhibition.

PCR analysis of genes for ammonia oxidation and nitrogen fixation

AMO isolates were screened for the presence of archaeal and bacterial 16S rRNA genes using PCR reaction with forward primers 344F and 341F, and reverse primers 915R and 907R

respectively (Casamayor et al. 2000). Products were expected to be 624bp and 585pb respectively. 25uL reaction volume consisting of 12uL Promega nuclease free water, 12.5 uL Promega 2X master mix, and dry cell addition was used for each sample. Genes were amplified using a thermal cycler programmed with an initial denaturation of 5 min at 94°C followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 53.5°C, and elongation for 90 s at 72°C, and a final elongation of 7 min at 72°C. AMO isolates were also screened for a bacterial gene for ammonia oxidation using PCR reaction with forward primers amoA-1F and amoA-2R using the same reaction volumes (Boyle-Yarwood et al. 2008). Expected product size was 283bp. For ammonia oxidation PCR the thermal cycler was programmed with five minutes initial denaturation at 94°C followed by 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and elongation for 90 s at 72°C with final elongation for 7 min at 72°C. Based on potential growth of heterocysts on a nitrogen-deficient enrichment medium, isolates R15 and R27 were screened for the bacterial nitrogenase *nifH* gene using PCR reaction with forward primer IGK and reverse primer DVV (Gaby and Buckley 2012). Expected product size was between 341-394bp. The same reaction volume and components were used for samples. The thermal cycler was programmed with an initial denaturation of 5 min at 94°C followed by 35 cycles of denaturation for 1 min at 94°C , annealing for 1 min at 58°C, and elongation for 1 min at 72°C, with a final elongation for 7 min at 72°C (Gaby and Buckley 2012). All PCR products were visualized using agarose gel electrophoresis at 100V for 20 minutes in a 1 percent agarose gel in 1X TAE buffer. Samples were measured using the Thermo Scientific GeneRuler 100kb. 6X Orange DNA dye from Thermo Scientific was added to the ladder and samples.

Statistical and phylogenetic analysis

Principal components analysis (PCA) and heatmap visualization of morphological and physiological culture characteristics were generated using ClustVis (https://biit.cs.ut.ee/clustvis/) to examine trends in phenotypic variation among isolates. Missing data points were imputed by the ClustVis software. A heatmap was generated for all isolates (including a single, Gram positive isolate) to provide visual analysis of similarities and differences among phenotypic characteristics demonstrated in culture. Separate PCA plots were generated for the 26 Gram negative isolates and the 21 *Pseudoalteromonas* isolates to discern potential relationships among the cultures.

Phylogenetic analysis of *Pseudoalteromonas* isolates was performed using both maximum-likelihood (ML) and neighbor-joining (NJ) methods. The outgroup was identified as *Pseudoalteromonas bactereolytica* (Bowman and McMeekin 2015). The ML analysis was implemented by searching for the best tree using 2 simultaneous threads, followed by a rapid bootstrap with 100 replicates. The GTR+Gamma model was used in raxmlGUI2.0 (Silvestro and Michalak 2011). ML trees were visualized using FigTree1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). NJ trees were constructed in PAUP4.0 (https://paup.phylosolutions.com/) (Swafford 2003). *Pseudoalteromonas* isolates from this Atlantic-based study were contrasted with *Pseudoalteromonas* isolates cultured from *L. pertusa* in the Gulf of Mexico (GOM) (Galkiewicz et al. 2011). Sequences were aligned in Mega (citation) and trimmed to an equal length of 1298bp. Additional analysis of the 5 *Pseudoalteromonas* isolates with the highest amount of reliable sequence data (1464bp) was performed to potentially show greater resolution.

RESULTS

DNA sequencing of the isolates identified organisms from *Proteobacteria* and *Firmicutes* with representatives from the following genera: *Pseudoalteromonas*, *Pseudomonas*, *Vibrio*, *Photobacterium*, *Pantoea*, and *Enterococcus* (Table 3). The majority (80%) of isolates matched to *Pseudoalteromonas* species (Table 3). The DSR2B medium captured the widest diversity of organisms (Table 3) while isolates from AMO plates were restricted to *Pseudoalteromonas* species (Table 3). Phylogenetic analysis of *Pseudoalteromonas* isolates suggests high genetic similarity among isolates cultured from AMO and chitin media, and high genetic similarity generally (Figure 6). Some distinction can be seen between *Pseudoalteromonas* strains from the Atlantic and Gulf of Mexico (Figure 6, Figure 8). Analysis of isolates with the most complete sequence data for 16S rRNA (1463bp) show support for genetic matches of several isolates to type strain *P. tetraodonis* documented in the literature (Figure 7, Figure 8). Both ML and NJ analyses cluster strains from the Gulf of Mexico as more closely related to one another than to Atlantic isolates from this study (Figure 6, Figure 8). All phylogenetic analyses indicate high genetic similarity among isolates within the genus (Figure 6, Figure 7, Figure 8).

Collectively, cultures grew over a wide range of salinities and pH values, and demonstrated metabolism of a variety of carbon and nitrogen sources. The majority of cultures grew between 3-12% NaCl , and between pH 4 and 11 (Figure 2). Three cultures (R1, R27, and R38) grew without salt, and only two cultures (R38 and R15) grew anaerobically (Figure 2); however, several cultures reduced nitrate (R27, R29, R1) and sulfate (R12, R27, R32, R9, R33, R48) (Figure 2). Cultures demonstrated widespread ability to use a variety of organics for growth; however, fermentation of glucose, lactose, sucrose, and saccharose was more limited (Figure 2). Over 50% of isolates showed the ability to degrade chitin (Figure 2). Nitrate

reduction and urea hydrolysis were limited to only a handful of isolates (Figure 2). No evidence of ammonia oxidation was observed in cultures (using Promega kit) or via PCR screening for *amoA* and *amoB* genes. Of the 27 isolates, only two (R15 and R27) showed abnormal growth on an N_2-free enrichment medium. PCR for the *nifH* gene yielded a presumptive positive result for isolate R15. Isolates cultured from chitin and AMO media show similarity in their phenotypic and metabolic characteristics (Figure 3, Figure 4). Colony morphology characteristics showed the most dissimilarity among AMO isolates compared with cultures from other enrichments (Figure 2, Figure 3). Among *Pseudoalteromonas* cultures there were colony and physiological similarities among organisms isolated from the same enrichment medium such as colony size and color, cell size and shape, and ability to metabolize various organic compounds (Figure 2, Figure 5). Evidence of antibiotic resistance to one or more antibiotics was documented among a number of isolates (Figure 2, Table 4). Most isolates and *Pseudoalteromonas* in particular demonstrated resistance to Penicillin, Vancomycin, and Tetracycline (Table 4). *Pantoea* demonstrated additional resistance to Ampicillin, Clindamycin, Erythromycin, and Streptomycin (Table 4). *Vibrio* demonstrated resistance to Colistin (Table 4). *Photobacterium* demonstrated susceptibility to Ampicillin and Tetracycline (Table 4). Results from cross-streaking were inconclusive and failed to provide evidence of antibiotic production among *L. pertusa* associated microbes.

Table 3. Isolate sequence matches based on 16S rRNA sequence analysis using BLAST. Sequence analysis was restricted to samples from coral. Uncultured representatives are also included for isolates due to high genetic similarity.

Closest cultured relative					
Isolates	Species / strain	Sim (%)	Accession no.	Assignment	Culture environment / host coral

R38	*Enterococcus faecalis* strain PRG16	100	MK418939.1	*Firmicutes, Bacilli*	*Porites panamensis*
R27	*Enterobacter cloacae* strain PLA12	94.9	MK418921.1	*Proteobacteria, Gammaproteo-bacteria*	*Pocillopora spp.*
R27	*Pantoea eucalypti* strain 18D	95.1	JF792085.1		*Diploria strigosa*
R17, R20, R30, R32, R33, R35, R43, R48, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72	*Pseudoalteromonas atlantica* strain PQQ20	98.3 - 99.8	KT730056.1		*Oculina patagonica*
R17, R20, R30, R32, R33, R35, R43, R48, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72	*Pseudoalteromonas atlantica strain* SYM2	98.2 - 99.6	KP645203.1		Free-living *Symbiodinium* cultures
R12	*Pseudoalteromonas distincta* strain PQQ84	99.8	KT730063.1		*Oculina patagonica*
R9, R12	*Pseudoalteromonas paragorgicola* strain PQQ1	100	KT730052.1		*Oculina patagonica*
R9	*Pseudoalteromonas tetraodonis* strain PQQ31	100	KT730057.1		*Oculina patagonica*
R12, R17, R20, R30, R32, R33, R35, R43, R48, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72	*Pseudoalteromonas tetraodonis* strain PQQ5	98.3 - 99.8	KT730053.1		*Oculina patagonica*
R1, R41	*Pseudomonas azotoformans* strain 22A	98.5 - 99.6	JF792088.1		*Siderastrea siderea*
R1, R41	*Pseudomonas azotoformans* strain 2S	98.4 - 99.6	JF792068.1		*Siderastrea siderea*
R27	*Serratia proteamaculans* strain F-23	94.9	MK482654.1		*Cinachyra cavernosa* (sponge) associated with coral in Gulf of Mannar

R15	*Vibrio sp.* B-2-1	99.6	KT583432.1		*Alcyonium digitatum*
R15	*Vibrio sp.* CIP 110630	99.6	HG942391.1		*Corallium rubrum*
R15	*Vibrio sp.* C-3-44	99.3	KT583560.1		*Alcyonium digitatum*
R29	*Photobacterium sp.* 34E11	97.4	JF346761.1		*Acropora palmata*
Closest uncultured representative					
Isolates	Organism clone	Sim (%)	Accession no.	Assignment	Culture environment / host coral
R1, R41	Unc. bacterium clone 12F04	98.6 - 99.7	KC668970.1	*Proteobacteria, Gammaproteo-bacteria*	*Stylophora pistillata tissue*
R1, R41	Unc. bacterium clone Apal_K17	98.6 - 99.7	GU118088.1		*Acropora palmata*
R27	Unc. bacterium clone Gven_A12	95.3	GU118494.1		*Gorgonia ventalina*
R27	Unc. bacterium clone Gven_H08	95	GU118359.1		*Gorgonia ventalina*
R29	Unc. bacterium clone RSAE3C31	97.1	JF411535.1		*Platygyra carnosus*
R27	Unc. bacterium clone SGUS1048	95	FJ202675.1		*Montastraea faveolata (aquarium)*
R9, R33, 62, 71	Unc. bacterium clone SPCiL-109	99.5 - 99.9	KC861113.1		*Cinachyra cavernosa (sponge) associated with coral in Gulf of Mannar*
R1, R41	Unc. marine bacterium clone Tc-49	98.7 - 99.8	JF925029.1		*Tubastraea coccinea*
R12, R17, R20, R30, R32, R35, R43, R48, 63, 64, 65, 66, 67,	Unc. *Pseudoalteromonas* clone CI13	98.5 - 99.9	FJ695534.1		*Acropora digitifera mucus*
R9, R12, 65, 67, 68, 69, 70, 72	Unc. *Pseudoalteromonas*	99.8 - 100	FJ695538.1		*Acropora digitifera mucus*

	clone CI17				
R33, 62, 71	Unc. *Pseudoalteromonas* clone CI18	99.5 - 99.7	FJ695539.1		*Acropora digitifera mucus*
R32, R48, 66	Unc. *Pseudoalteromonas* clone CI19	99.7 - 99.9	FJ695540.1		*Acropora digitifera mucus*
R17, R20, R30, R33, R35, R43, 62, 63, 64, 68, 69, 70, 71, 72	Unc. *Pseudoalteromonas* clone CI42	98.5 - 99.9	FJ695534.1		*Acropora digitifera mucus*
R9, R12, R15, R20, R30. R32, R35, R43, R48, 63, 64, 65, 66, 67, 68, 69, 70, 72	Unc. *Pseudoalteromonas* clone CI47	98.6 - 100	FJ695567.1		*Acropora digitifera mucus*

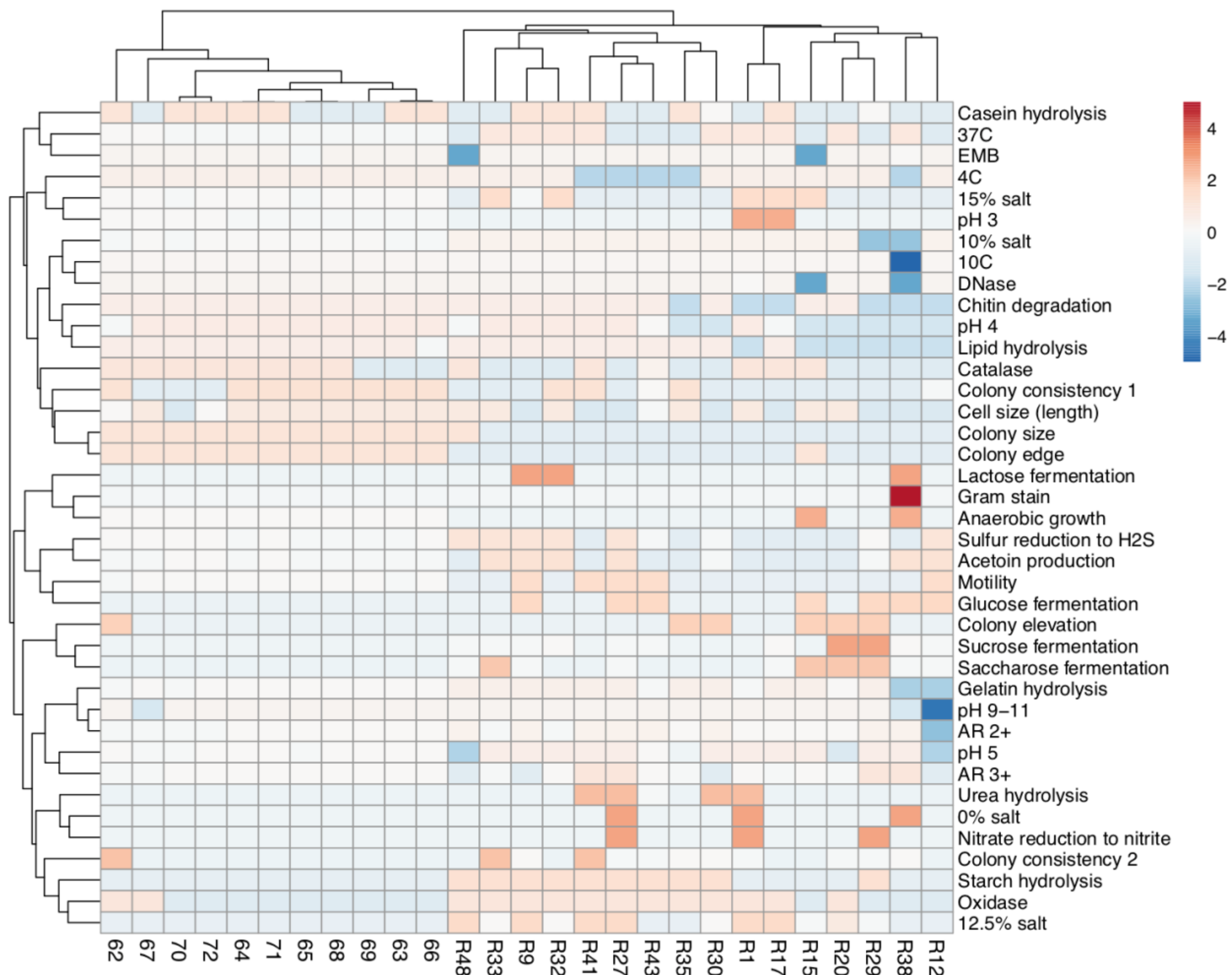

Figure 2. Heatmap of morphological and physiological features of isolated cultures from *L. pertusa*. A binary system was used to designate presence or absence of a trait (Appendix B, Supplemental Table B1). For a given trait, red indicates a positive result, blue indicates a negative result, and white indicates no result. AR stands for antibiotic resistance.

Table 4. Antibiotic resistance of tested isolates against various antibiotics. "R" and "S" indicate resistance and susceptibility respectively based on Kirby-Bauer protocol (Hudzicki 2009). GenBank matches included to indicate closely matching genera of isolates. Empty cells mean not determined.

Isolate ID	Genbank match	Amp	Chl	Clin	Col	Ery	Van	Pen	Str	Tet
R9	*Pseudoalteromonas*						R	R		S
R12	*Pseudoalteromonas*						R	R		R
R15	*Vibrio*				R		R			
R27	*Pantoea*	R		R		R	R	R	R	R
R29	*Photobacterium*	S				R		R	R	S
R30	*Pseudoalteromonas*						R	R		S
R32	*Pseudoalteromonas*							R		R
R33	*Pseudoalteromonas*									
R35	*Pseudoalteromonas*						R	R		R
R38	*Enterococcus*	R						R		
R41	*Pseudomonas*		R				R	R		R
R43	*Pseudoalteromonas*	R						R		

Key: Amp = Ampicilin, Chl = Chloramphenicol, Cln = Clindamycin, Col = Colistin, Ery = Erythromycin, Van = Vancomycin, Pen = Penicillin, Str = Streptomycin, Tet = Tetracylin

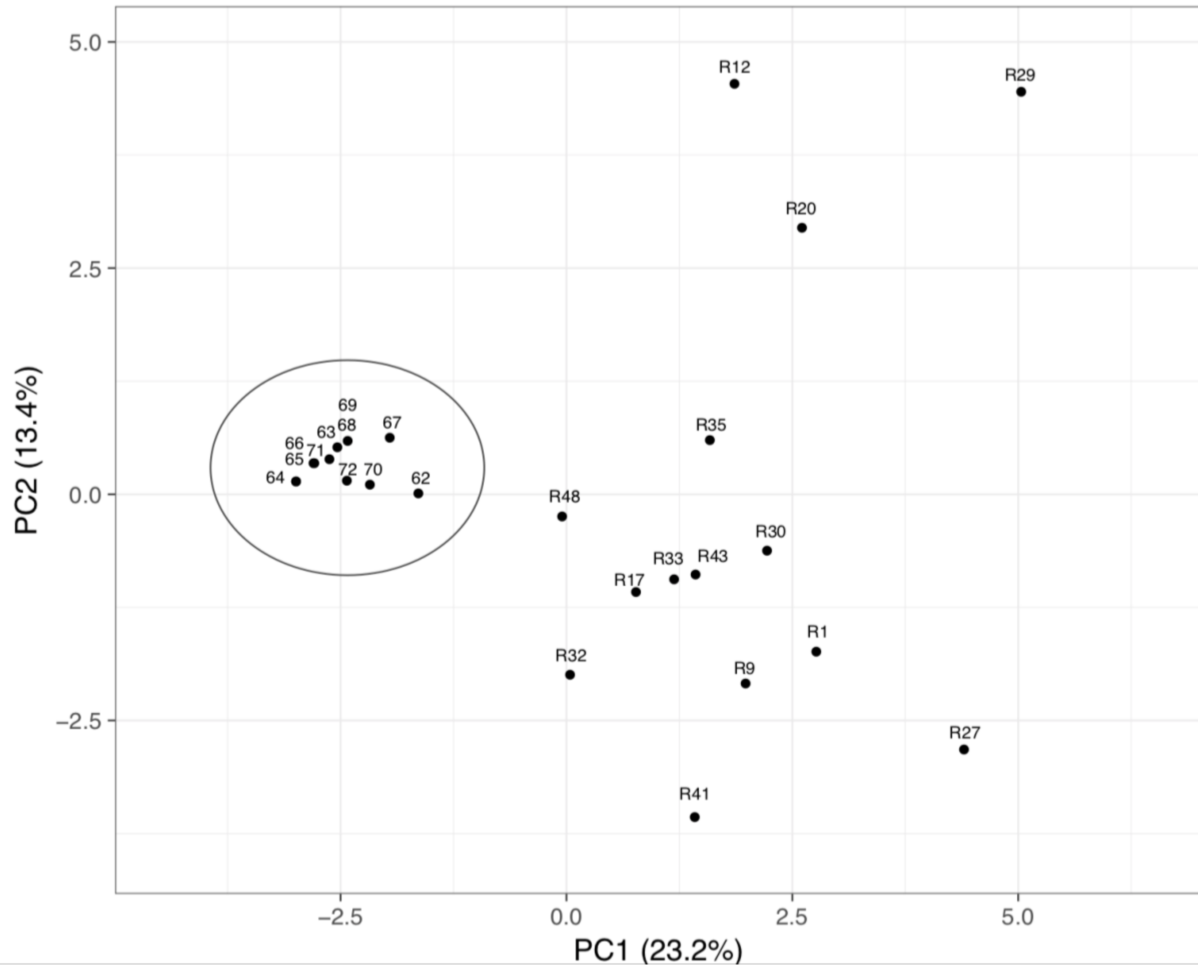

Figure 3. Principal components analysis (PCA) of all Gram negative isolates cultured from *Lophelia* coral, which include all but one of the 26 isolates (R38). Isolate R15 was excluded from the data set as it was an outlier. Circled cluster contains all AMO isolates while the other points represent cultures grown on chitin and DSR2B.

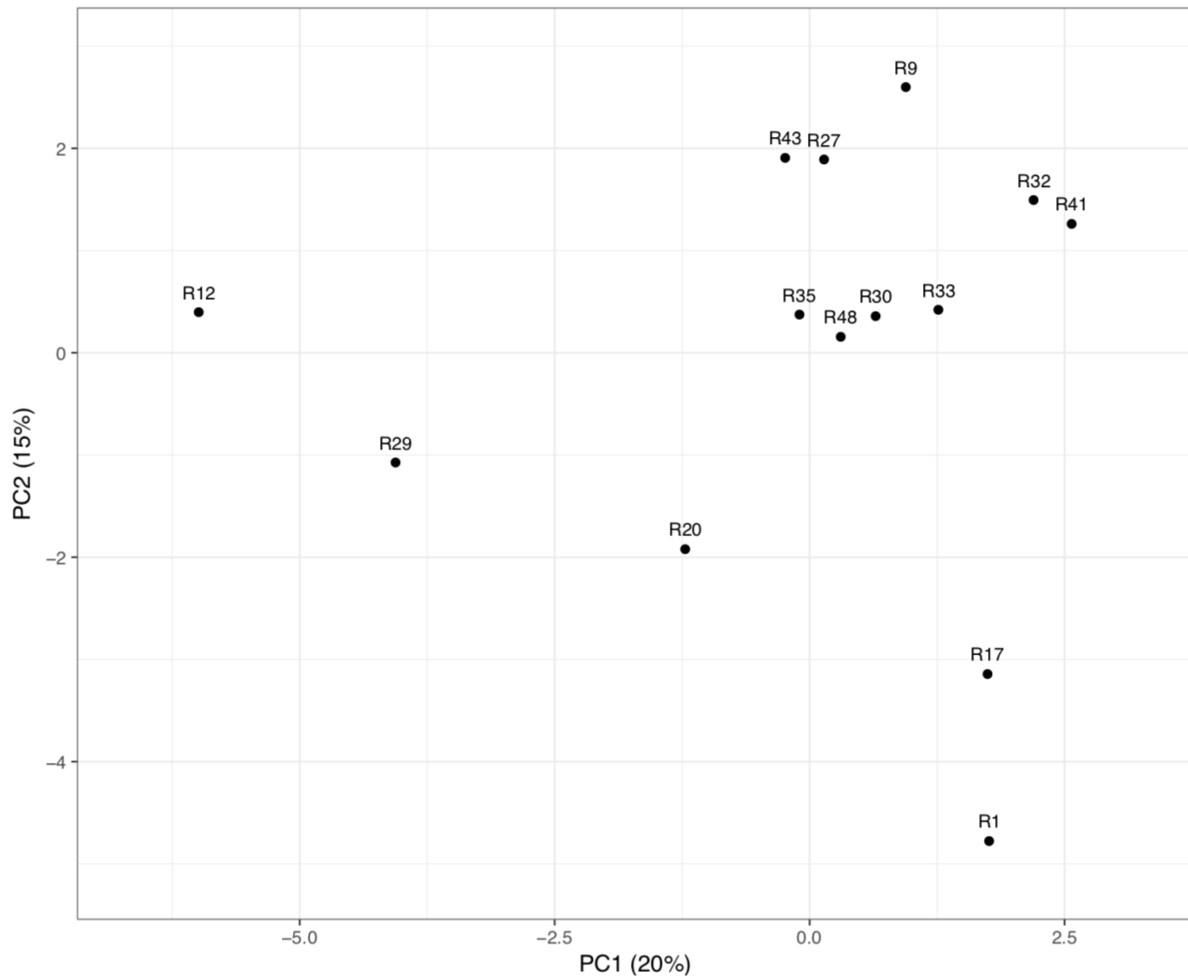

Figure 4. Principal components analysis (PCA) of isolates cultured from chitin and DSR2B enrichment media. R15 was excluded from the data set as it was an outlier. The exclusion of isolates from AMO enrichment media highlights that variation in culture characteristics of these cultures are not highly influenced by media type.

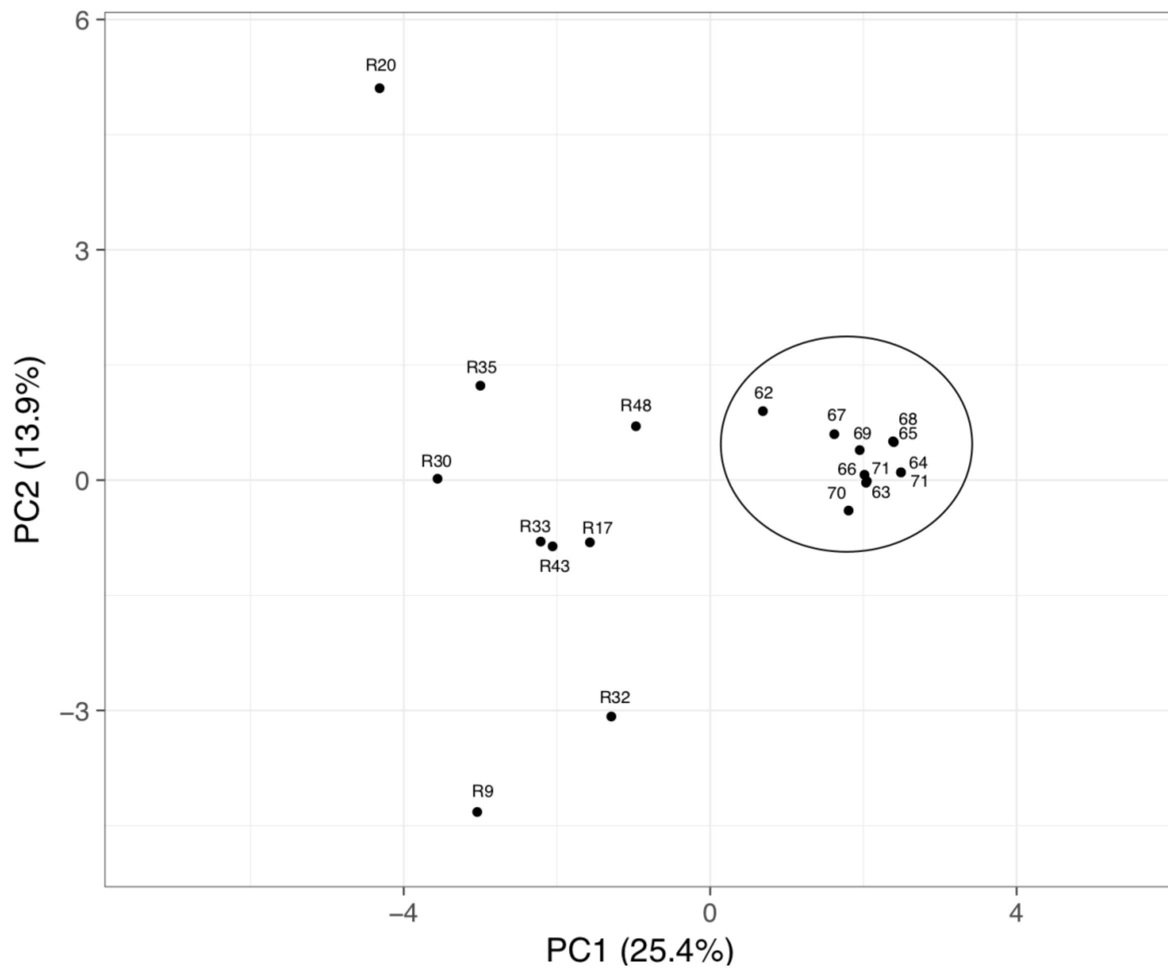

Figure 5. Principal components analysis (PCA) of only isolates identified as *Pseudoalteromonas* based on 16S rRNA sequencing results. R12 was excluded from the data set as it was an outlier. Circled cluster contains all AMO isolates while the other points represent cultures grown on chitin and DSR2B.

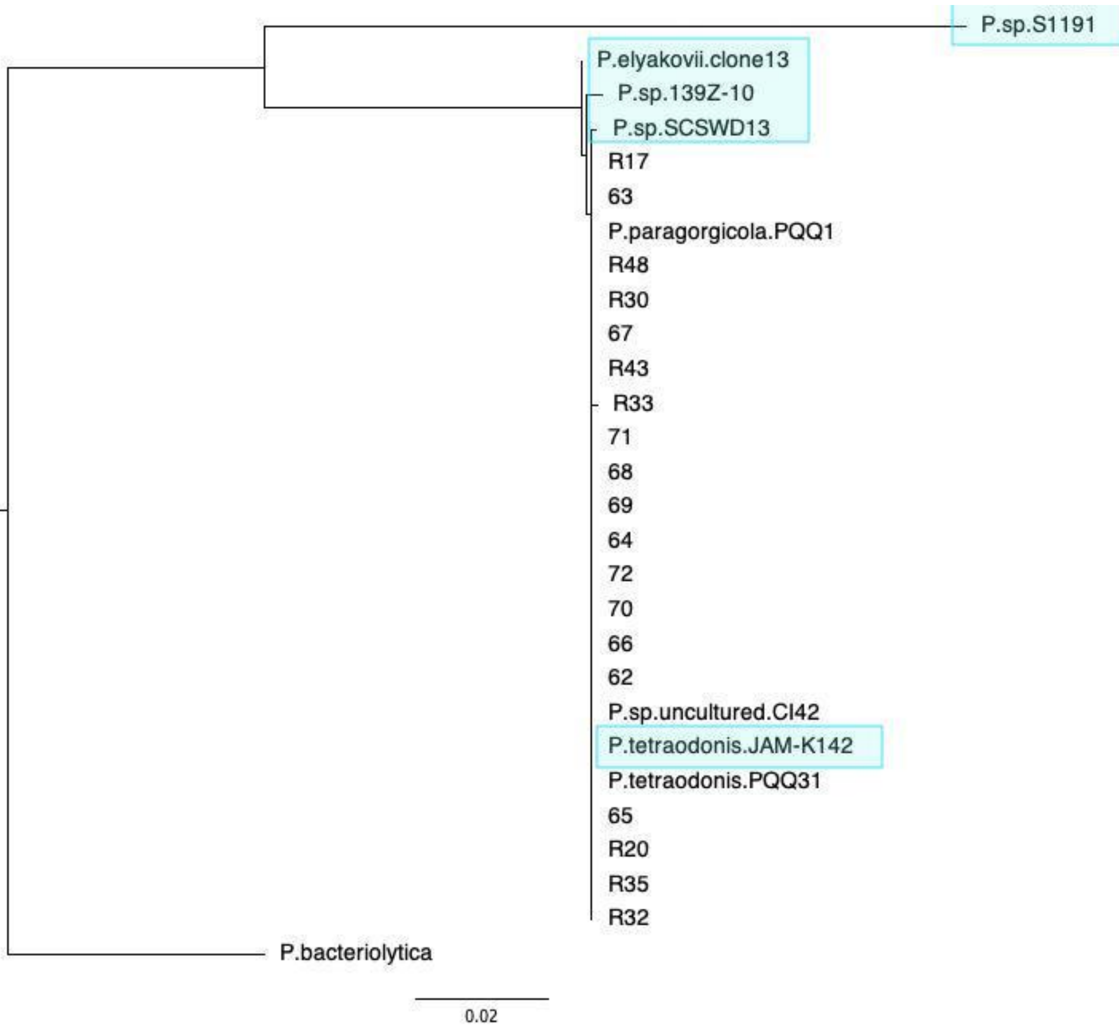

Figure 6. Maximum likelihood tree of *Pseudoalteromonas* isolates cultured from Atlantic *Lophelia* corals along with their closest GenBank matches. Closest GenBank matches for *Pseudoalteromonas* isolates from Gulf of Mexico (GOM) *Lophelia* corals are included and highlighted for comparison (Galkiewicz et al. 2011). Bootstrap values represent confidence intervals. Sequences were aligned and trimmed to an equal length of 1298bp.

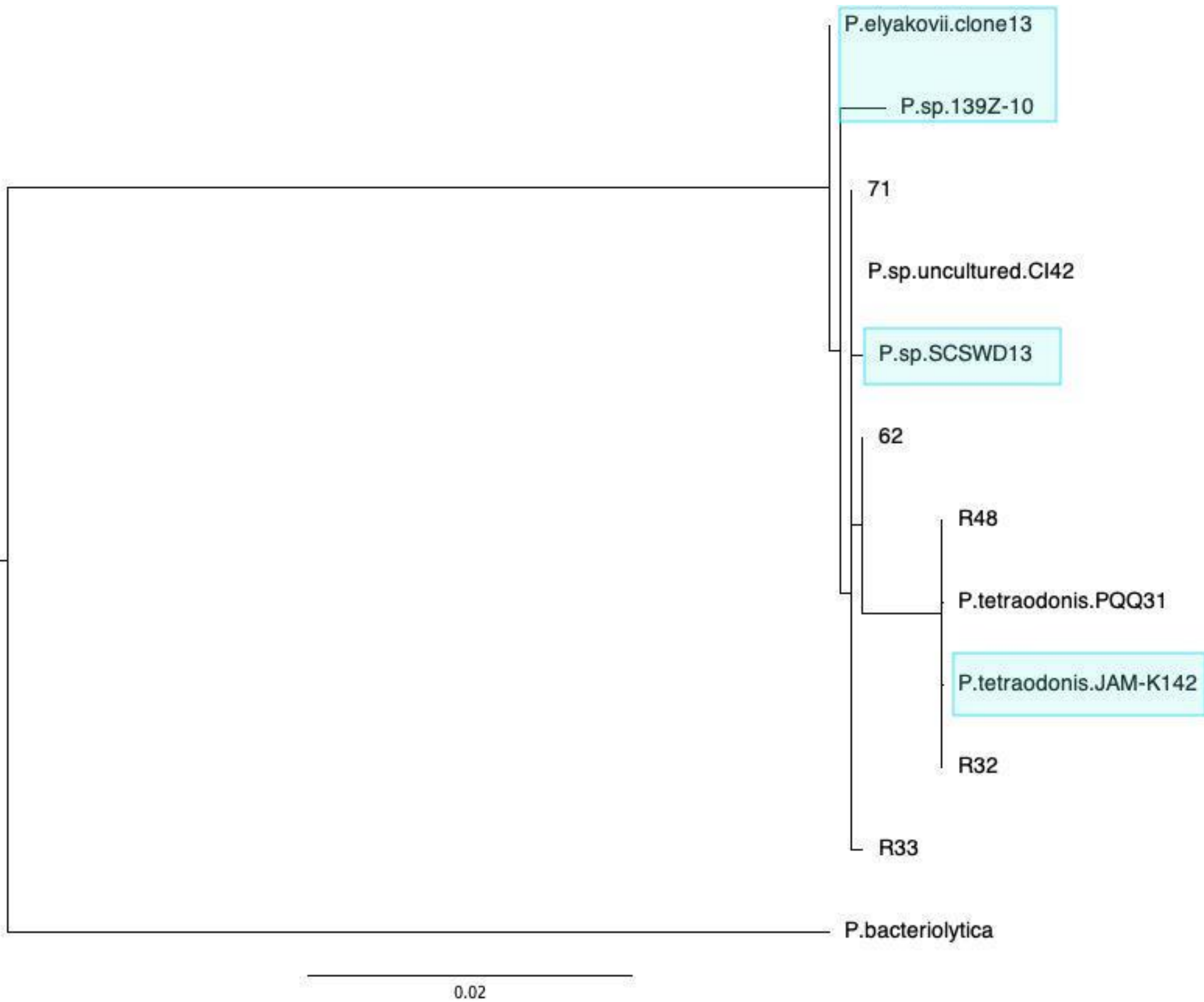

Figure 7. Maximum likelihood tree of *Pseudoalteromonas* isolates with the highest sequence reads (1464bp) cultured from Atlantic *Lophelia* corals along with their closest GenBank matches. Closest GenBank matches for *Pseudoalteromonas* isolates from Gulf of Mexico (GOM) *Lophelia* corals are included and highlighted for comparison (Galkiewicz et al. 2011). Bootstrap values represent confidence intervals.

Figure 8. Neighbor-joining tree showing *Pseudoalteromonas* isolates cultured from Atlantic *Lophelia* corals along with their closest GenBank matches. Closest GenBank matches for *Pseudoalteromonas* isolates from Gulf of Mexico (GOM) *Lophelia* corals are included and highlighted for comparison (Galkiewicz et al. 2011). Sequence lengths are 1298bp.

DISCUSSION

The coral holobiont is a symbiosis of coral animal and microbial associates, and it is thought that both coral and microbes benefit from this relationship. The coral provides a substrate and nutrients (food and energy sources) for microbial colonization, and microbes provide nutrients (food and energy sources) for the coral. This study highlights some of the microbial metabolic pathways that may be relevantly contributing to cycling of carbon and nitrogen by the *L. pertusa* holobiont, which include pathways reserved to prokaryotes that can allow for regeneration of biologically available resources for the eukaryotic coral host. It provides direct observation of general C and N turnover and N cycling by bacteria cultured from *L. pertusa* through a variety of chemoheterotrophic pathways, which has really only been obtained indirectly by previous studies (Kellogg et al. 2009, Kellogg et al. 2017, Galkiewicz et al. 2011, Middelburg et al. 2015). In particular, the results of this study demonstrate bacterial metabolism of organic and inorganic substrates that are likely to be available to the coral holobiont based on available literature on *L. pertusa* ecology and ecophysiology and highlights key pathways in the nitrogen cycle that are performed specifically by microbes. Because of their close association, bacteria living on and inside of these corals are likely to play a role in contributing to the carbon and nitrogen budget of the coral holobiont.

Bacteria isolated in this study demonstrated the ability to degrade a wide variety of organic materials, including various sugars, starch, casein, gelatin, lipids, chitin, urea, and DNA. Many of these substrates are available to associated microbes as part of the *L. pertusa* diet, within the mucus layer, or as metabolic coral waste products. These substrates can serve as carbon, nitrogen, and energy sources for bacteria, and their breakdown is likely to play a role in the cycling and retention of limited C and N resources by the coral holobiont (Wegley et al.

2007, Radecker et al. 2015, Middelburg et al. 2015). Among the various chemoorganotrophic pathways demonstrated, chitinase activity is notable. Degradation of chitin was widespread among isolates in this study, supporting bacterial chitinase activity as a function of the *Lophelia* holobiont. Roughly 80% of isolates showed chitinase ability, including species of *Pantoea*, *Pseudoalteromonas, Pseudomonas, and Vibrio*. Chitinase activity has been documented in some species of *Pseudoalteromonas*, *Pseudomonas*, and *Vibrio* in soil and other freshwater and marine environments (Grimont and Grimont 2015, Bowman and McMeekin 2015, Farmer et al. 2015, Palleroni 2015). Chitin is ubiquitous in the marine environment. Chitinase activity among CWCs associated microbes has been demonstrated indirectly (Neulinger et al. 2008, Yoshioka et al. 2017) and may play a role in assimilation of carbohydrates obtained from phytoplankton and detritus (Bourne, Morrow & Webster 2016) and in defense against fungal pathogens (Kramer & Muthukrishnan 1997).

Cultures from this study also demonstrated important metabolic pathways involved in the cycling and regeneration of nitrogen. Nitrogen cycling is thought to be an important feature of the *L. pertusa* holobiont that influences the ability of the coral to grow in the deep ocean (Wegley et al. 2007, Radecker et al. 2015, Middleburg et al. 2015). Indirect observation of a complete nitrogen cycle by the *L. pertusa* holobiont (Middleburg et al. 2015) is supported by several metabolic pathways demonstrated by bacteria in this study. A single isolate, R15-identified by 16S rRNA gene sequencing as a species of *Vibrio*-yielded a presumptive positive for the *nifH* gene, potentially confirming nitrogen fixation ability in a microbial associate of *L. pertusa*. Nitrogen fixation has been documented among *Vibrio* species cultured from Brazilian coral *Mussismilia hispida* (Chimetto et al. 2008), and inferred in other studies of *L. pertusa* (Galkiewicz et al. 2011, Kellogg et al. 2017). Several cultures were able to reduce nitrate to

nitrite, a key step in the nitrogen cycle that is facilitated solely by prokaryotes. Nitrite can be reduced to ammonia and assimilated by the *L. pertusa* holobiont, or reduced all the way to dinitrogen via denitrification, a process carried out by other microbes. Urea hydrolysis, another important process by which bacteria can cycle nitrogen, converts urea to ammonia, which can then be assimilated. Urea is likely an available resource to bacteria living in close association with the coral as the result of its release into the water column by various marine organisms living in the coral gardens (Crandall and Teece 2012). Several isolates in this study showed urease activity, which supports the presence of this metabolic pathway within the *Lophelia* holobiont.

Evidence of ammonia oxidation and carbon fixation within the *L. pertusa* holobiont (Middleburg et al. 2015) and 16S rRNA sequence data for species capable of this metabolism exist (Kellogg et al. 2017), but in spite of attempts to enrich for nitrifying bacteria and archaea, none were cultured in this study. Their presence is likely due to evidence of ammonia oxidation and carbon fixation within the *L. pertusa* holobiont (Middelburg et al. 2015) and 16S rRNA sequence data for species capable of this metabolism. The AMO enrichment medium used in this study was intended to capture ammonia-oxidizing organisms; however, the only organisms cultured from this medium were bacterial species from the genus *Pseudoalteromonas*, which do not demonstrate nitrifying ability or other chemolithotrophic metabolism (Bowman and McMeekin 2015). It is likely that the trace amount of organics used to create the medium (a ketone derivative of glutaric acid) or possible organic contaminants from the sampling environment allowed for the growth of these organisms. Their proliferation in such a nutrient limited environment might suggest that they are good scavengers.

The diversity of bacteria cultured in this study importantly confirms and expands upon both culture-dependent and culture-independent studies of the *L. pertusa* microbiome. Genera cultured in this study include *Pseudoalteromonas, Pseudomonas, Vibrio,* and *Photobacterium,* which have been documented in association with *L. pertusa* (Kellogg et al. 2017, Galkiewicz et al. 2011, Kellogg et al. 2009, Neulinger et al. 2008), and species of *Pantoea* and *Enterococcus*, which to date have not been documented at the genus level in association with *L. pertusa. Pantoea*, however, has been documented in association with corals affected by white plague disease (Cárdenas et al. 2011), suggesting that its presence in association with *L. pertusa* may not be to the coral's benefit. With the exception of *Enterococcus*, these genera are all representatives of *Gammaproteobacteria*, one of the most metabolically diverse bacterial phyla. Other studies have documented the presence of these organisms as part of the *L. pertusa* microbiome and discussed their metabolic potential (Kellogg et al. 2017, Galkiewicz et al. 2011, Neulinger et al. 2008), and this study has been able to confirm through direct observation some of these metabolic pathways, including chitin degradation (*Pantoea, Pseudoalteromonas, Pseudomonas*, *Vibrio*), sulfur reduction (*Pantoea*, *Pseudoalteromonas*), nitrogen fixation (*Vibrio),* nitrate reduction (*Pantoea, Pseudomonas*, *Vibrio*), fermentative metabolism (*Enterococcus, Photobacterium, Pseudoalteromonas*), and anaerobic growth (*Enterococcus, Vibrio*). Urease activity (*Pantoea, Pseudoalteromonas, Pseudomonas*) was also documented and is an important pathway for nitrogen recycling by the coral holobiont. These metabolic pathways are consistent with literature on type strains of these genera (Galkiewicz et al. 2011, Bowman & McMeekin 2015, Farmer et al. 2015, Grimont et al. 2015, Palleroni 2015) and offer a more complete picture of some of the functions of the *L. pertusa* holobiont.

Evidence from this study may offer support for a *Lophelia*-specific microbiome. *Alteromonadales* (to which *Pseudoalteromonas* belongs) may be part of a conserved core microbiome among Lophelia corals in Atlantic and GOM (Kellogg et al. 2017). As in the culture-based study of *L. pertusa* associated bacteria from the Gulf of Mexico, the majority of isolates in culture in this study of Atlantic *Lophelia* corals were identified as *Pseudoalteromonas*, and a smaller proportion of isolates were identified as *Photobacterium* and *Vibrio* species (Galkiewicz et al. 2011). Phylogenetic analysis of Atlantic and GOM *Pseudoalteromonas* isolates indicates some genetic separation of isolates from the different locations, but very little genetic variation overall. This separation could be connected to sample location, or it could be the result of differences in enrichment media. The high proportion of *Pseudoalteromonas* isolates taken from *Lophelia* corals in both locations are in alignment with evidence of some conserved microbial associates among *L. pertusa* coral populations (Galkiewicz et al. 2011, Kellogg et al. 2017). In both studies, *Pseudoalteromonas* species dominated among culturable isolates. Interestingly, some *Pseudoalteromonas tetraodonis* species cultured from other coral species have been shown to aid in protection against pathogenic *Vibrio* species (Torres et al. 2016), suggesting their role in antimicrobial production and protection of their coral host from attack by pathogens. Taken together this suggests that *Pseudoalteromonas* species may be an important part of a conserved *Lophelia* microbiome and may play a role in coral health.

Comparison of antibiotic resistance of isolates from the Atlantic and GOM indicates some alignment. Susceptibility of *Photobacterium* to tetracycline is consistent with isolates from the GOM (Galkiewicz et al. 2011). The majority of GOM *Pseudoalteromonas* isolates were resistant to Penicillin but were all clinically susceptible to tetracycline. This is in contrast to most *Pseudoalteromonas* isolates from the Atlantic, which were similarly resistant to penicillin but

also resistant to tetracycline. Antibiotic resistance may indicate exposure to certain antibiotics that could (theoretically) be produced by other associated microbes and may be involved in protecting coral from pathogens. The similarities and differences in resistance patterns among associates of *L. pertusa* from the Atlantic and GOM may suggest some location-specific differences. More generally, these results could point to some element of *Lophelia* coral health in these locations that is yet to be known.

CONCLUSION

This study has added to knowledge about the *Lophelia pertusa* holobiont through culture-dependent analysis of bacterial isolates and their ability to use various metabolic pathways involved in carbon and nitrogen cycling, including nitrogen fixation, urea hydrolysis, nitrate reduction, chitin degradation, and general carbon turnover. There is much still to be learned about microbial mediation in coral nutrition and health in the deep ocean. Future research may seek to tie isolates from this study to in situ diversity measures to determine if these organisms are, in fact, closely associated with *L. pertusa* corals and whether they are metabolically active within the functioning holobiont. While the presence and activity of diazotrophic bacteria has been presumptively supported by this study, more work can be done to confirm these results and to uncover additional microbial symbionts that are involved in other aspects of the nitrogen cycle, namely nitrification, additional steps in denitrification, and ammonification. Further studies of carbon fixation in *L. pertusa* are needed to better understand what microorganisms are involved and what inorganic substrates they are using as energy sources, including nitrogen-, sulfur-, iron-, phosphorus-based compounds. Finally, more work can be done to investigate antibiotic activities of the *Lophelia* holobiont. All of these areas are importantly relevant to understanding the function of the coral holobiont in nutrition, nutrient cycling, and overall coral health.

Conservation and recycling of carbon, nitrogen, and other nutrients by the *L. pertusa* holobiont are likely to be key aspects of its ecophysiology that allows it to grow in the deep ocean. This has important ecological implications. *Lophelia pertusa* and other CWC species are foundation species that form both the structural and trophic basis for entire ecosystems (Cordes

et al. 2008, van Oevelen et al. 2009). These ecosystems serve as important spawning, nursery, and breeding habitat for many fishes and invertebrates, and they also create carbon sinks, places where carbon gets taken up and stored in biomass (van Oevelen et al. 2009, White et al. 2012). Physical and physiological stress induced by changes in the environment resulting from human activities (trawling, dredging, oil drilling) and climate change (ocean acidification) could influence host-microbe interactions, potentially altering the ability of *L. pertusa* to conserve and recycle limiting nutrients. This has the potential to impact the ability of these corals to survive and sustain ecosystems, subsequently removing important habitat for other marine organisms and disrupting a globally relevant carbon sink.

REFERENCES CONSULTED

Altschul et al. (1990) *Journal of Molecular Biology* 215: 403-410

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, *6*(2), 71–79. https://doi.org/10.1016/j.jpha.2015.11.005

Bourne, D. G., Morrow, K. M., & Webster, N. S. (2016). Insights into the coral microbiome: Underpinning the health and resilience of reef ecosystems. *Annual Review of Microbiology*, *70*(1), 317–340. https://doi.org/10.1146/annurev-micro-102215-095440

Bowman, John P., & McMeekin, T. A. (2015). Pseudoalteromonas. *Bergey's Manual of Systematics of Archaea and Bacteria*, 1–22. https://doi.org/10.1002/9781118960608.gbm01098

Boyle-Yarwood, S. A., Bottomley, P. J., & Myrold, D. D. (2008). Community composition of ammonia-oxidizing bacteria and archaea in soils under stands of red alder and Douglas fir in Oregon. *Environmental Microbiology*, *10*(11), 2956–2965. https://doi.org/10.1111/j.1462-2920.2008.01600.x

Cappuccino, J. G., & Sherman, N. (2014). *Microbiology : A laboratory Manual* (10th ed.). Glenview, IL: Pearson Education, Inc.

Cárdenas, A., Rodriguez-R, L. M., Pizarro, V., Cadavid, L. F., & Arévalo-Ferro, C. (2011). Shifts in bacterial communities of two Caribbean reef-building coral species affected by white plague disease. *The ISME Journal*, *6*(3), 502–512. https://doi.org/10.1038/ismej.2011.123

Casamayor, E. O., Schafer, H., Baneras, L., Pedros-Alio, C., & Muyzer, G. (2000). Identification of and Spatio-Temporal Differences between microbial assemblages from two neighboring sulfurous lakes: Comparison by microscopy and denaturing gradient gel

electrophoresis. *Applied and Environmental Microbiology*, *66*(2), 499–508.
https://doi.org/10.1128/aem.66.2.499-508.2000

Chimetto, L. A., Brocchi, M., Thompson, C. C., Martins, R. C. R., Ramos, H. R., & Thompson, F. L. (2008). Vibrios dominate as culturable nitrogen-fixing bacteria of the Brazilian coral *Mussismilia hispida*. *Systematic and Applied Microbiology*, *31*(4), 312–319.
https://doi.org/10.1016/j.syapm.2008.06.001

Coles, S. L., and Strathman, R. (1973). Observations on coral mucus "floc" and their potential trophic significance. *Limnology and Oceanography*. 18, 673–678.
https://doi.org/10.4319/lo.1973.18.4.0673

Cordes, E. E., McGinley, M. P., Podowski, E. L., Becker, E. L., Lessard-Pilon, S., Viada, S. T., & Fisher, C. R. (2008). Coral communities of the deep Gulf of Mexico. *Deep Sea Research Part I: Oceanographic Research Papers*, *55*(6), 777–787.
https://doi.org/10.1016/j.dsr.2008.03.005

Costello, M. J., McCrea, M., Freiwald, A., Lundälv, T., Jonsson, L., Bett, B. J., . . . Allen, D. (2005). Role of cold-water *Lophelia pertusa* coral reefs as fish habitat in the NE Atlantic. *Cold-Water Corals and Ecosystems*, 771–805. https://doi.org/10.1007/3-540-27673-4_41

Crandall, J. B., & Teece, M. A. (2011). Urea is a dynamic pool of bioavailable nitrogen in coral reefs. *Coral Reefs*, *31*(1), 207–214. https://doi.org/10.1007/s00338-011-0836-1

Davis, E., Sloan, T., Aurelius, K., Barbour, A., Bodey, E., Clark, B., . . . Wildschutte, H. (2017). Antibiotic discovery throughout the Small World Initiative: A molecular strategy to identify biosynthetic gene clusters involved in antagonistic activity. *MicrobiologyOpen*, *6*(3).
https://doi.org/10.1002/mbo3.435

Farmer, J. J., III, Michael Janda, J., Brenner, F. W., Cameron, D. N., & Birkhead, K. M. (2015). *Vibrio*. *Bergey's Manual of Systematics of Archaea and Bacteria*, 1–79. https://doi.org/10.1002/9781118960608.gbm01078

Fisher, C. R., Demopoulos, A. W. J., Cordes, E. E., Baums, I. B., White, H. K., & Bourque, J. R. (2014). Coral communities as indicators of ecosystem-level impacts of the Deepwater Horizon spill. *BioScience*, *64*(9), 796–807.

Frias-Lopez, J., Zerkle, A. L., Bonheyo, G. T., & Fouke, B. W. (2002). Partitioning of Bacterial Communities between Seawater and Healthy, Black Band Diseased, and Dead Coral Surfaces. *Applied and Environmental Microbiology*, *68*(5), 2214–2228. https://doi.org/10.1128/aem.68.5.2214-2228.2002

Gaby, J. C., & Buckley, D. H. (2012). A Comprehensive Evaluation of PCR Primers to Amplify the nifH Gene of Nitrogenase. *PLoS ONE*, *7*(7), e42149. https://doi.org/10.1371/journal.pone.0042149

Galkiewicz, J. P., Pratte, Z. A., Gray, M. A., & Kellogg, C. A. (2011). Characterization of culturable bacteria isolated from the cold-water coral *Lophelia pertusa*. *FEMS Microbiology Ecology*, *77*(2), 333–346. https://doi.org/10.1111/j.1574-6941.2011.01115.x

Grimont, P. A. D., & Grimont, F. (2015). *Pantoea*. *Bergey's Manual of Systematics of Archaea and Bacteria*, 1–14. https://doi.org/10.1002/9781118960608.gbm0115

Hudzicki, J. (2009). Kirby-Bauer disk diffusion susceptibility test protocol. *American Society for Microbiology*. Cited 2019 Nov 10. Available from: www.asmscience.org

Kellogg, C. A., Goldsmith, D. B., & Gray, M. A. (2017). Biogeographic Comparison of *Lophelia*-associated bacterial communities in the western Atlantic reveals conserved core microbiome. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.00796

Kellogg, C. A., Piceno, Y. M., Tom, L. M., DeSantis, T. Z., Gray, M. A., Zawada, D. G., & Andersen, G. L. (2013). Comparing bacterial community composition between healthy and white plague-like disease states in *Orbicella annularis* using PhyloChipTM G3 microarrays. *PLoS ONE*, *8*(11), e79801. https://doi.org/10.1371/journal.pone.0079801

Kellogg, C. A., Lisle, J. T., & Galkiewicz, J. P. (2009). Culture-Independent characterization of bacterial communities associated with the cold-water coral *Lophelia pertusa* in the northeastern Gulf of Mexico. *Applied and Environmental Microbiology*, *75*(8), 2294–2303. https://doi.org/10.1128/aem.02357-08

Khripounoff, A., Caprais, J., Le Bruchec, J., Rodier, P., Noel, P., & Cathalot, C. (2014). Deep cold-water coral ecosystems in the Brittany submarine canyons (Northeast Atlantic): Hydrodynamics, particle supply, respiration, and carbon cycling. *Limnology and Oceanography*, *59*(1), 87–98. https://doi.org/10.4319/lo.2014.59.1.0087

Kramer, K. J., & Muthukrishnan, S. (1997). Insect chitinases: molecular biology and potential use as biopesticides. *Insect Biochemistry and Molecular Biology*, *27*(11), 887–900. https://doi.org/10.1016/s0965-1748(97)00078-7

Littman, R., Willis, B. L., & Bourne, D. G. (2011). Metagenomic analysis of the coral holobiont during a natural bleaching event on the Great Barrier Reef. *Environmental Microbiology Reports*, *3*(6), 651–660. https://doi.org/10.1111/j.1758-2229.2010.00234.x

Littman, R. A., Bourne, D. G., & Willis, B. L. (2010). Responses of coral-associated bacterial communities to heat stress differ with *Symbiodinium* type on the same coral host. *Molecular Ecology*, *19*(9), 1978–1990. https://doi.org/10.1111/j.1365-294x.2010.04620.x

Lu, S., Cai, W., Zhang, X., Li, X., Huang, H., Zhang, F., & Zhang, J. (2015). Black coral as a new environmental recorder: The lead profiles in coral skeletons over the past century. *Marine Pollution Bulletin*, *101*(1), 226–231. https://doi.org/10.1016/j.marpolbul.2015.09.058

Maxwell, S. (2005). An aquatic pharmacy: the biomedical potential of the deep sea. *Journal of Marine Education.21*(4), 31–32.

Meistertzheim, Anne.-L., Lartaud, F., Arnaud-Haond, S., Kalenitchenko, D., Bessalam, M., Le Bris, N., & Galand, P. E. (2016). Patterns of bacteria-host associations suggest different ecological strategies between two reef building cold-water coral species. *Deep Sea Research Part I: Oceanographic Research Papers*, *114*, 12–22. https://doi.org/10.1016/j.dsr.2016.04.013

Middelburg, J. J., Mueller, C. E., Veuger, B., Larsson, A. I., Form, A., & Oevelen, D. (2015). Discovery of symbiotic nitrogen fixation and chemoautotrophy in cold-water corals. *Scientific Reports*, *5*(1). https://doi.org/10.1038/srep17962

Mortensen, P. B., Hovland, M., Brattegard, T., and Farestveit, R. (1995). Deep water bioherms of the scleractinian coral *Lophelia pertusa* (L.) at 64°N on the Norwegian shelf: structure and associated megafauna. *Sarsia* 80, 145–158. https://doi.org/10.1080/00364827.1995.10413586

Mortensen, P. B. (2001). Aquarium observations on the deep-water coral *Lophelia pertusa* (L., 1758) (scleractinia) and selected associated invertebrates. *Ophelia*, *54*(2), 83–104. https://doi.org/10.1080/00785236.2001.10409457

Mueller, C. E., Larsson, A. I., Veuger, B., Middelburg, J. J., & van Oevelen, D. (2014b). Opportunistic feeding on various organic food sources by the cold-water coral *Lophelia pertusa*. *Biogeosciences*, *11*(1), 123–133. https://doi.org/10.5194/bg-11-123-2014

Murthy, N. and Bleakley, B. (2012). Simplified method of preparing colloidal chitin used for screening of chitinase-producing microorganisms. *The Internet Journal of Microbiology 10*(2).

Muscatine L, Porter JW (1977) Reef corals-mutualistic symbioses adapted to nutrient-poor environments. *Bioscience* 27: 454–460.

National Center for Biotechnology Information (NCBI) [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; (1988). Cited 2020 Jan 15. Available from: https://www.ncbi.nlm.nih.gov/

Neulinger, S. C., Jarnegren, J., Ludvigsen, M., Lochte, K., & Dullo, W.-C. (2008). Phenotype-specific bacterial communities in the cold-water coral *Lophelia pertusa* (Scleractinia) and their implications for the coral's nutrition, health, and distribution. *Applied and Environmental Microbiology*, *74*(23), 7272–7285. https://doi.org/10.1128/aem.01777-08

Palleroni, Norberto J. (2015). *Pseudomonas. Bergey's Manual of Systematics of Archaea and Bacteria*, 1. https://doi.org/10.1002/9781118960608.gbm01210

Rädecker, N., Pogoreutz, C., Voolstra, C. R., Wiedenmann, J., & Wild, C. (2015). Nitrogen cycling in corals: the key to understanding holobiont functioning? *Trends in Microbiology*, *23*(8), 490–497. https://doi.org/10.1016/j.tim.2015.03.008

Reshef, L., Koren, O., Loya, Y., Zilber-Rosenberg, I., & Rosenberg, E. (2006). The Coral Probiotic Hypothesis. *Environmental Microbiology*, *8*(12), 2068–2073. https://doi.org/10.1111/j.1462-2920.2006.01148.x

Ritchie, K. (2006). Regulation of microbial populations by coral surface mucus and mucus-associated bacteria. *Marine Ecology Progress Series*, *322*, 1–14. https://doi.org/10.3354/meps322001

Rix, L., de Goeij, J. M., Mueller, C. E., Struck, U., Middelburg, J. J., van Duyl, F. C., … van Oevelen, D. (2016). Coral mucus fuels the sponge loop in warm- and cold-water coral reef ecosystems. *Scientific Reports*, *6*(1). https://doi.org/10.1038/srep18715

Roberts and Hirshfield (2004) Deep-sea corals: out of sight, but no longer out of mind. *Frontiers in Ecology and the Environment* 2(3): 123-130. https://doi.org/10.1890/1540-9295(2004)002[0123:DCOOSB]2.0.CO;2

Roberts, J. M. (2006). Reefs of the Deep: The Biology and Geology of Cold-Water Coral Ecosystems. *Science*, *312*(5773), 543–547. https://doi.org/10.1126/science.1119861

Rohwer, F., Seguritan, V., Azam, F., & Knowlton, N. (2002). Diversity and distribution of coral-associated bacteria. *Marine Ecology Progress Series*, *243*, 1–10. https://doi.org/10.3354/meps243001

Rosenberg, E., & Falkovitz, L. (2004). The *Vibrio shiloi Oculina patagonica* model system of coral bleaching. *Annual Review of Microbiology*, *58*(1), 143–159. https://doi.org/10.1146/annurev.micro.58.030603.123610

Schöttner, S., Hoffmann, F., Wild, C., Rapp, H. T., Boetius, A., & Ramette, A. (2009). Inter- and intra-habitat bacterial diversity associated with cold-water corals. *The ISME Journal*, *3*(6), 756–759. https://doi.org/10.1038/ismej.2009.15

Soetaert, K., Mohn, C., Rengstorf, A., Grehan, A., & van Oevelen, D. (2016). Ecosystem engineering creates a direct nutritional link between 600m deep cold-water coral mounds and surface productivity. *Scientific Reports*, *6*(1). https://doi.org/10.1038/srep35057

Swofford, D. L. (2003). PAUP4.0. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts. http://phylosolutions.com/paup-test

Silvestro, D., & Michalak, I. (2011). raxmlGUI: a graphical front-end for RAxML. *Organisms Diversity & Evolution*, *12*(4), 335–337. https://doi.org/10.1007/s13127-011-0056-0

Svitil, A. L., Chadhain, S., Moore, J. A., & Kirchman, D. L. (1997). Chitin degradation proteins produced by the marine bacterium *Vibrio harveyi* growing on different forms of chitin. *Applied and Environmental Microbiology*, *63*(2), 408-13.

Thompson, J. R., Rivera, H. E., Closek, C. J., & Medina, M. (2015). Microbes in the coral holobiont: partners through evolution, development, and ecological interactions. *Frontiers in Cellular and Infection Microbiology*, *4*. https://doi.org/10.3389/fcimb.2014.00176

Torres, M., Rubio-Portillo, E., Antón, J., Ramos-Esplá, A. A., Quesada, E., & Llamas, I. (2016). Selection of the N-Acylhomoserine Lactone-degrading bacterium *Alteromonas stellipolaris* PQQ-42 and of its potential for biocontrol in aquaculture. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.00646

TSD-99. (n.d.). Bicarbonate-buffered synthetic *Crenarchaeota* medium. Retrieved May 24, 2019, from https://www.atcc.org/products/all/TSD-99.aspx#culturemethod

van Bleijswijk, J. D. L., Whalen, C., Duineveld, G. C. A., Lavaleye, M. S. S., Witte, H. J., & Mienis, F. (2015). Microbial assemblages on a cold-water coral mound at the SE Rockall Bank (NE Atlantic): interactions with hydrography and topography. *Biogeosciences*, *12*(14), 4483–4496. https://doi.org/10.5194/bg-12-4483-2015

van de Water, J. A. J. M., Melkonian, R., Voolstra, C. R., Junca, H., Beraud, E., Allemand, D., & Ferrier-Pagès, C. (2016). Comparative Assessment of Mediterranean Gorgonian-Associated Microbial Communities Reveals Conserved Core and Locally Variant Bacteria. *Microbial Ecology*, *73*(2), 466–478. https://doi.org/10.1007/s00248-016-0858-x

van Oevelen, D., Mueller, C. E., Lundälv, T., & Middelburg, J. J. (2016). Food selectivity and processing by the cold-water coral *Lophelia pertusa*. *Biogeosciences*, *13*(20), 5789–5798. https://doi.org/10.5194/bg-13-5789-2016

van Oevelen, D., Duineveld, G., Lavaleye, M., Mienis, F., Soetaert, K., & Heip, C. H. R. (2009). The cold-water coral community as hotspot of carbon cycling on continental margins: A food-web analysis from Rockall Bank (northeast Atlantic). *Limnology and Oceanography*, *54*(6), 1829–1844. https://doi.org/10.4319/lo.2009.54.6.1829

Vijayalakshmi, S., & Jawahar, S. (2011). Screening of marine bacterial extracts for antimicrobial activity. *Journal of Ecotoxicology & Environmental Monitoring, 21*(1), 83-86.

Wagner, H., Purser, A., Thomsen, L., Jesus, C. C., & Lundälv, T. (2011). Particulate organic matter fluxes and hydrodynamics at the Tisler cold-water coral reef. *Journal of Marine Systems*, *85*(1–2), 19–29. https://doi.org/10.1016/j.jmarsys.2010.11.003

Wang, Q, G. M. Garrity, J. M. Tiedje, and J. R. Cole. 2007. Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. *Appl Environ Microbiol.* 73(16):5261-5267; doi: 10.1128/AEM.00062-07 [PMID: 17586664]

Wegley, L., Edwards, R., Rodriguez-Brito, B., Liu, H., & Rohwer, F. (2007). Metagenomic analysis of the microbial community associated with the coral *Porites astreoides*. *Environmental Microbiology*, *9*(11), 2707–2719. https://doi.org/10.1111/j.1462-2920.2007.01383.x

Yakimov, M. M., Cappello, S., Crisafi, E., Tursi, A., Savini, A., Corselli, C., . . . Giuliano, L. (2006). Phylogenetic survey of metabolically active microbial communities associated with the deep-sea coral *Lophelia pertusa* from the Apulian plateau, Central

Mediterranean Sea. *Deep Sea Research Part I: Oceanographic Research Papers*, *53*(1), 62–75. https://doi.org/10.1016/j.dsr.2005.07.005

Yoshioka, Y., Tanabe, T., & Iguchi, A. (2017). The presence of genes encoding enzymes that digest carbohydrates in coral genomes and analysis of their activities. *PeerJ*, *5*, e4087. https://doi.org/10.7717/peerj.4087

APPENDIX A. MICROBIOLOGICAL MEDIA RECIPES

Medium for Ammonia Oxidizers (Modified from ATCC medium TSD-99)

Composition per liter:

Component	Amount
NaCl	26g
$MgSO_4 \cdot 7H_2O$	5g
$MgCl_2$	5g
$CaCl_2 \cdot 2H_2O$	1.5g
KBr	0.1g
KH_2PO_4	0.4g
$NaHCO_3$ (1M solution)	3 ml
FeCl EDTA (15.5 mM)	35ml
Trace element solution	1 ml
NH_4Cl (1M solution)	0.2ml
Alpha Glutaric Acid (100mM solution)	0.1ml

Trace element solution:
Composition per 100.00 mL:

Component	Amount
HCl (12M)	8.33 ml
H_3BO_3	30mg
$MnCl_2 \cdot 4H_2O$	100mg
$NiCl_2 \cdot 6H_2O$	24mg
$CuCl_2 \cdot 2H_2O$	2mg
$ZnSO_4 \cdot 7H_2O$	144mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	26.26mg

Preparation of trace element solution: Add components to 12M HCl and mix thoroughly. *Note: 12M HCl solution was used in place of 12.5M HCl solution. Molecular concentration was adjusted by adding volume.* $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ was used in place of $Na_2MoO_4 \cdot 2H_2O$.

Additional ammonium was not a concern as this medium was made to target ammonia oxidizing microbes.

Preparation of Medium**:** Add components distilled/deionized water and bring to volume of 1.0L. Mix thoroughly. Distribute into tubes or flasks. Autoclave for 30 min at 15 psi pressure-121°C. Note: for solid media, first add and dissolve 8g gellan gum and 1g CaCl into volume of distilled/deionized water.

Medium for Chitin Degraders (Modified from Murthy and Bleakley, 2012)

Composition per liter:

Component	Amount
NaCl	26g
K_2HPO_4	0.7g
KH_2PO_4	0.3g
$MgSO_4 \cdot 5H_2O$	0.5g
$FeCl_3$	25 µl
$ZnSO_4$	0.001g
$MnCl_2$	0.001g
Colloidal chitin solution	100ml

Preparation of colloidal chitin: Treat 20g of crushed chitin flakes with 150 ml of 12M HCl in a 1000ml sterile beaker under a chemical fume hood at room temperature (~25°C). Add chitin slowly, stirring continuously with a glass pipette for 5 minutes followed by stirring for 1 minute at 5 minute intervals for 60 minutes. Treat mixture with 2 liters of chilled deionized water to allow colloidal chitin to precipitate. Incubate overnight at 4°C. Pass solution through two layers of coffee filter paper. Pass approximately 3 L of deionized water through chitin trapped in filter paper to neutralize filtrate (test that pH has risen to ~7.0). Dispose of filtrate. Press colloidal chitin trapped in filter to remove excess moisture and place in 100 ml beaker. Use in preparation of chitin medium. (Solution can be stored in refrigerator for up to 48 hours.)

Preparation of Medium: Add components distilled/deionized water and bring to volume of 1.0L. (Use 900ml deionized water to account for 100 ml volume of colloidal chitin solution.) Mix thoroughly. Distribute into tubes or flasks. Autoclave for 30 min at 15 psi pressure-121°C. Note: for solid media, first add and dissolve 8g gellan gum and 1g CaCl into volume of distilled/deionized water.

Medium for Oligotrophic Heterotrophic Marine Organisms (General)

Composition per liter:

NaCl …………………………………………………………………………….. 26g

R2B stock solution ………………………………………………………….... 10ml

Preparation of R2B stock solution: Add 3.6g of R2B to 100 ml of distilled/deionized water. Mix thoroughly.

Preparation of Medium: Add components distilled/deionized water and bring to volume of 1.0L. Mix thoroughly. Distribute into tubes or flasks. Autoclave for 30 min at 15 psi pressure-121$_{o}$C. Note: for solid media, first add and dissolve 8g gellan gum and 1g CaCl into volume of distilled/deionized water. Note: for solid media, first add and dissolve 8g gellan gum and 1g CaCl into volume of distilled/deionized water.

APPENDIX B. SUPPLEMENTAL DATA

Table B1. Binary Key for Heatmap and PCA Analysis.

Trait	Description and Binary Key
Colony size	Diameter; 0 = < 1mm; 1 = 1+ mm
Colony consistency 1	0 = opaque; 1 = translucent
Colony consistency 2	0 = shiny; 1 = dull
Colony edge	0 = circular/entire; 1 = undulate
Colony elevation	0 = flat; 1 = raised
Cell size (length)	Length; 0 = < 1 µm; 1 = 1+ µm
Catalase	Enzyme activity; 0 = negative; 1 = positive
Oxidase	Enzyme activity; 0 = negative; 1 = positive
Motility	0 = negative; 1 = positive
Anaerobic growth	Growth; 0 = negative; 1 = positive
4C	Growth; 0 = negative; 1 = positive
10C	Growth; 0 = negative; 1 = positive

25C	Growth; 0 = negative; 1 = positive
37C	Growth; 0 = negative; 1 = positive
0% salt	Growth; 0 = negative; 1 = positive
10% salt	Growth; 0 = negative; 1 = positive
12.5% salt	Growth; 0 = negative; 1 = positive
15% salt	Growth; 0 = negative; 1 = positive
pH 3	Growth; 0 = negative; 1 = positive
pH 4	Growth; 0 = negative; 1 = positive
pH 5	Growth; 0 = negative; 1 = positive
pH 9-11	Growth; 0 = negative; 1 = positive
Glucose fermentation	Metabolic activity; 0 = negative; 1 = positive
Sucrose fermentation	Metabolic activity; 0 = negative; 1 = positive
Saccharose fermentation	Metabolic activity; 0 = negative; 1 = positive
Lactose fermentation	Metabolic activity; 0 = negative; 1 = positive
Sulfur reduction to H2S	Metabolic activity; 0 = negative; 1 = positive
Acetoin production	Metabolic activity; 0 = negative; 1 = positive
Urea hydrolysis	Metabolic activity; 0 = negative; 1 = positive
EMB	Metabolic activity (fermentation); 0 = negative; 1 = positive
Nutrient agar	Metabolic activity; 0 = negative; 1 = positive
Starch hydrolysis	Metabolic activity; 0 = negative; 1 = positive
Lipid hydrolysis	Metabolic activity; 0 = negative; 1 = positive
Casein hydrolysis	Metabolic activity; 0 = negative; 1 = positive
Gelatin hydrolysis	Metabolic activity; 0 = negative; 1 = positive
DNase	Metabolic activity; 0 = negative; 1 = positive
Nitrate reduction to nitrite	Metabolic activity; 0 = negative; 1 = positive

Chitin degradation	Metabolic activity; 0 = negative; 1 = positive
AR 1	Antibiotic resistance; 0 = susceptible; 1 = resistance to 1 antibiotic
AR 2+	0 = susceptible; 1 = resistance to 2 or more antibiotics
AR 3+	0 = susceptible; 1 = resistance to 3 or more antibiotics