ABSTRACT

Title of Thesis: TWO MARINE SPONGES, *LENDENFELDIA CHONDRODES* AND *HYMENIACIDON HELIOPHILA*, AND THEIR MICROBIAL SYMBIONTS: ROLES IN MARINE PHOSPHORUS CYCLING.

Lauren Elise Jonas, Master of Science, 2021

Thesis directed by: Dr. Russell T. Hill, Professor, Institute of Marine and Environmental Technology, University of Maryland Center for Environmental Science

Marine sponges have emerged as major players within coral reef biogeochemical cycles, facilitating intake and release of carbon, nitrogen, and phosphorus. The majority of studies have investigated the role of sponges in transforming dissolved carbon and nitrogen; however, the same breadth of insights has not been extended to phosphorus. This study uses ^{32}P-labeled orthophosphate and ATP to determine that two marine sponges, *Lendenfeldia chondrodes* and *Hymeniacidon heliophila*, both rapidly take up ambient dissolved inorganic phosphate and dissolved organic phosphorus. Subsequent genetic analysis and chemical extraction showed that sponge symbionts store phosphorus in the form of energy-rich polyphosphate (poly-P). *L. chondrodes*, a sponge from oligotrophic habitats and with a microbiome dominated by cyanobacterial symbionts, stores more phosphorus as

poly-P (6–8%) than *H. heliophila* (0.55%), a eutrophic sponge with low cyanobacterial abundance. This work adds new insights to the roles of the sponge holobiont in cycling the crucial element, phosphorus.

TWO MARINE SPONGES, *LENDENFELDIA CHONDRODES* AND *HYMENIACIDON HELIOPHILA*, AND THEIR MICROBIAL SYMBIONTS: ROLES IN MARINE PHOSPHORUS CYCLING.

by

Lauren Elise Jonas

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2021

Advisory Committee:
Professor Russell Hill, Chair
Associate Professor Yantao Li
Associate Professor Sairah Malkin

Dedication

To my parents, for buying a house in the woods and sending me to science camp.

Acknowledgements

I would like to first acknowledge the support of my family, who nurtured my passion for science and have always encouraged me to do what makes me happy. Thank you, Mom, Dad, Louisa, Sam, Alex, Courtney, Caroline, Anna, and my (soon-to-be) little niece for your love and support.

I would like to offer an enormous amount of thanks to my advisor Dr. Russell Hill. If he had not taken a chance on me, I would have never gone down this path toward my career in microbiology. His support was invaluable during my master's thesis and for that I will always be grateful. Thank you to my committee members Dr. Sairah Malkin and Dr. Yantao Li for their guidance and insights that have made my research stronger. I would like to thank Steve Rodgers and Chris Tollini for their invaluable help with my tank system. Thank you to Dr. Tsvetan Bachvaroff not only your general openness and support for all graduate students, but also for helping me develop a bioinformatic pipeline for community analysis that I will carry with me into my PhD research. Thank you to Pacific East Aquaculture for my various orders of *L. chondrodes* and for assistance in keeping them alive. I want to acknowledge the help of Dr. Fan Zhang, Sam Major, Dr. Hanzhi Lin, Amanda Lawrence, Hunter Hughes, and my awesome interns, Sophie LeClaire and Xavi Geisen.

A big thank you to Dr. Jan Vicente and Leah Shirizuru for their time dedicated to me at the Hawai‘i Institute of Marine Biology. From just three weeks, I gained a caring mentor and remarkable friend. I want to thank my professors from the Smithsonian Tropical Research Institute who gave me the best fieldwork experience I

could imagine and solidified my obsession with marine sponges. I would like to thank Erik Corbitt in his help with radiation safety and for being a great next-door neighbor. Thank you to my magical roommate, Abigail, who reminds me every day of who I want to be, and to my remarkable roommate, Katie, who makes me incredibly happy and brings peace to my days. There is no chance I would have made it through without the relentless support of Ana Sosa, Daniela Tizabi, and Tori Agnew. You three are true gems in my life. Finally, thank you to my lifelong friend and sister, Claire Murphy, for growing with me since day one.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

High microbial abundance (HMA)

Low microbial abundance (LMA)

Ankyrin-repeat proteins (ARPs)

Anaerobic ammonia oxidation (anammox)

Total dissolved phosphorus (TDP)

Dissolved organic phosphorus (DOP)

Dissolved inorganic phosphate (DIP)

Particulate organic phosphorus (POP)

Dissolved organic nitrogen (DON)

Dissolved organic carbon (DOC)

Particulate organic carbon (POC)

Particulate organic matter (POM)

Phosphate/Orthophosphate (Pi, PO_4^{3-})

Soluble Reactive Phosphorus (SRP)

Soluble non-reactive phosphorus (SNP)

Polyphosphate (polyP)

Polyphosphate kinase (PPK)

Polyphosphate kinase gene (*ppk*)

Exopolyphosphatase (PPX)

Exopolyphosphatase gene (*ppx*)

Polycyclic aromatic hydrocarbons (PAHs)

BioAnalytical Services Laboratory (BAS Lab)

Institute of Marine and Environmental Technology (IMET)

Disintegrations per minute (DPM)

Technical Data Sheet (TDS)

4’6-diamidino-2-phenylindole (DAPI)

National Center for Biotechnology Information (NCBI)

Constraint-based Multiple Alignment Tool (COBALT)

Méthodes et algorithmes pour la bio informatique (MAB)

Phosphate regulon (Pho regulon)

Carbon dioxide (CO_2)

Atmospheric nitrogen (N_2)0

Ammonium ($NH4^+$)

Nitrite ($NO2^-$)

Nitrate ($NO3^-$)

Chapter 1: Introduction

1.1 Marine sponges, their abundance, diversity, and crucial ecosystem function

1.1.1 Sponge distribution is worldwide and, in many ecosystems, sponges are rich in abundance and diversity.

Marine sponges are widely dispersed, sessile filter-feeders of the marine benthos and can play an important role in nutrient cycling and trophic transfer. Their morphology, species richness, and biomass dominance contribute to sponges being one of the most abundant life forms within diverse ecosystems, including tropical and sub-tropical reef ecosystems. In some reefs, like those found in the Caribbean, sponges often exceed corals and algae in species number (Diaz & Rutzler 2001). This colonization success may be due to sponges' remarkable morphological capacity. While many sponges dominate reef cover with visually striking shapes such as tubes, spheres, and barrels, many sponges remain cryptic either at depth (Thomson 1869; Carter 1874) or within reef crevices (Jackson & Winston 1982; Richter & Wunsch 1999; Richter et al. 2001; de Goeij et al. 2013). In fact, it has been suggested that we have yet to fully characterize numerous species of sponges that are concealed under rocks, in caves, or beneath the coral framework that was once thought to be uninhabited (Wulff & Buss 1979; Timmers et al. 2020).

Sponges have a very broad geographical distribution. They live across all salinity gradients, thriving in marine ecosystems, estuaries, and freshwater systems such as lakes, rivers, and fjords. A remarkable diversity of sponges has been found

across varying depth gradients, ranging from euphotic, shallow waters to the deepest freshwater lakes, where sponges have been found to occupy 44% of the benthos (Itskovich et al. 2015). Finally, sponges flourish across extreme temperature gradients, having been found at deep hydrothermal vent systems and at both the Arctic and Antarctic (Fricke et al. 1989; Sarà et al. 1992; McClintock et al. 2005; Schander et al. 2009; Dinn & Leys 2018).

1.1.2 Marine sponges feed by pumping water and provide vital ecosystem services.

Along with their widespread distribution (both around the world and within their specific habitats), sponges' physiology places them in a central role within the marine energy web. Sponges are active filter-feeders; they feed on particles and nutrients by pumping water through their tissue using specialized flagellated cells called choanocytes (Grant 1825; Vogel 1974, 1977). The internal structure and cell types of a typical demosponge are illustrated in Figure 1.1. As they pump, sponges are able to filter a volume of seawater several thousand-fold their body volume every 24 hours (Reiswig 1971, 1974). They filter this influx of water and remove 80%–90% of particles in the size range from 0.2 to 50 μm (Pile et al. 1996), which is primarily composed of bacterioplankton and picoplankton (Maldonado et al. 2010). As this water passes through the sponge body, nutrients in the water are chemically transformed. These processes are facilitated by microorganisms that live endosymbiotically with sponges (Section 1.2). As a result of their active pumping and their role in nutrient cycling, sponges are key players in benthic-pelagic coupling and their pumping greatly influences all major biogeochemical cycles within coral reefs.

One can imagine sponges acting similarly to the human cardiovascular system. Like the cardiovascular system pumps blood and nutrients throughout the body, sponges pump water and nutrients throughout the reef.

Marine sponges' role as nutrient cyclers is arguably their most widespread contribution to reefs and will be the focus of this research; however, sponges also provide a wide array of other ecological services. Sponges serve as a food source for certain fish known as spongivores (Randall & Hartman 1968 and references therein; Meylan 1988). They also provide habitat and refuge to many species of fish and invertebrates with the latter often living inside of sponge tissue (Wendt 1985). These "infauna" can be found inside sponges in high numbers, for example, 11,463 specimens (76 species) were found living in just 15 sponge individuals from the Antarctic (Kersken et al. 2014). Marine sponges' services span beyond their immediate ecosystem for they have been extensively utilized as a source of bioactive compounds, some of which have been used as antimicrobials, antivirals, antifungals, and anticancer treatments in humans (Kobayashi & Ishibashi 1993; Faulkner et al. 2000; Kobayashi 2000; Lee et al. 2001; Piel et al. 2004; Piel 2006).

It is important to emphasize that sponges are not acting alone while providing these broad services. Zilber-Rosenberg, Rosenberg, and their colleagues have asserted that that multicellular organisms—across all eukaryotic kingdoms—should no longer be considered autonomous entities, but instead as a partnership between the organism and its microbial symbionts (Zilber-Rosenberg & Rosenberg 2008). This partnership has been termed the "holobiont" and is increasingly accepted (McFall-Ngai et al. 2013; Bordenstein & Theis 2015; Pita et al. 2018; Dittami et al. 2021). This relatively

new concept could not be truer for marine sponges that can live in association with strikingly abundant and diverse microorganisms. In high microbial abundance (HMA) sponges, microbes can comprise up to *ca.* 35% of the biomass (Webster & Thomas 2016; Pita et al. 2018) although this microbial abundance is much lower in low microbial abundance (LMA) sponges.

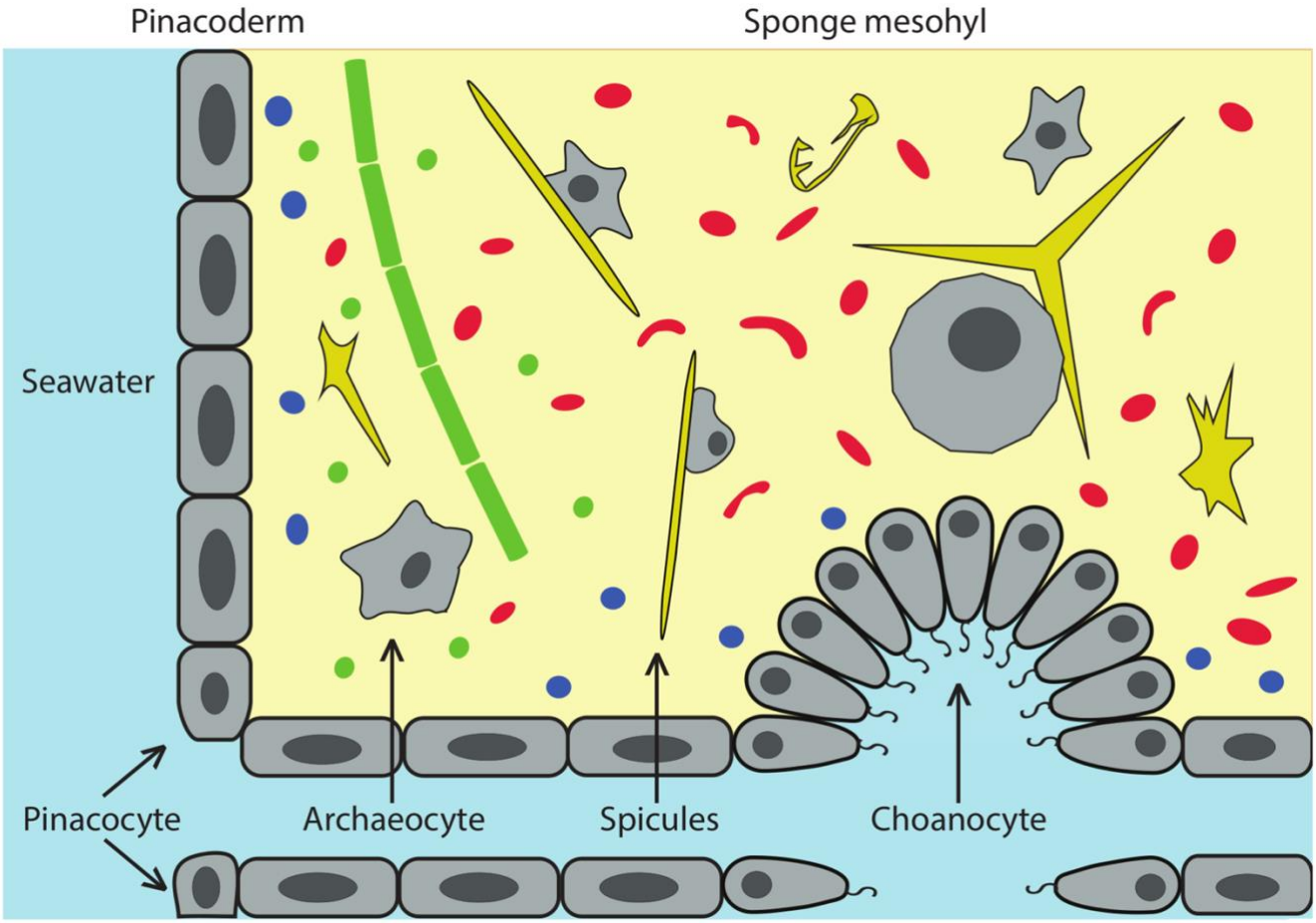


Figure 1.1. An illustration of the internal structure of a typical demosponge and the microbial symbionts. Major sponge cell (grey) types include pinacocytes, archaeocytes, and choanocytes. Through the choanocyte chambers, microorganisms from seawater are transported to the sponge mesohyl, where they are either engulfed by archaeocyte cells as food source or evade phagocytosis and become symbionts in their hosts. Diverse symbiotic microorganisms exist in the mesohyl of many demosponges. Representative microbial symbionts are cyanobacteria (green), archaea (blue) and heterotrophic bacteria (red). Siliceous spicules (yellow) act as structural skeletons to support the morphology of demosponges. Figure provided by Zhang, Jonas et al. 2019 with permission.

1.2 The microbial symbionts of marine sponges

1.2.1 Sponge-associated bacterial communities: complex, yet often consistent

Many sponges exist as a complex union between animal and microbe. Due to the fact that marine sponges may be the most primitive metazoans, their associations with microorganisms are quite possibly the oldest animal-microbial symbiosis on earth (Slaby & Hentschel 2017). The abundance of sponge microbial symbionts varies sufficiently to classify sponges into two groups: high microbial abundance (HMA) sponges that host a microbial density 3–4 orders of magnitude greater than the surrounding water and low microbial abundance (LMA) sponges that contain the same order of magnitude as the surrounding seawater (Hentschel et al. 2003; Taylor et al. 2007a; Gloeckner et al. 2014). The large collection of research dedicated to characterizing numerous sponge holobionts has revealed that marine sponges often harbor complex assemblies of bacteria, algae, fungi, and archaea which can constitute up to *ca.* 35% of the animal's biomass (Webster & Thomas 2016; Pita et al. 2018). There seems to be a theme of consistency when comparing the microbiomes of various sponges. In many cases, there is a high similarity among sponge-associated bacterial communities in different sponge species. Also, within a particular sponge species, microbial communities are generally very stable over time and geographic range (Montalvo & Hill 2011). Among sponges of different taxonomies and geographic locations, the 16S rRNA gene sequences from their symbionts are more related to one another than to those of any other origin (Hentschel et al. 2002). Sponge communities can be highly conserved and species-specific. When comparing bacterial communities in two closely related species of *Xestospongia* from different

oceans, Montalvo and Hill found the symbiotic communities were remarkably similar although still formed clusters showing species-specific divergence (Montalvo & Hill 2011). The theme of sponges containing highly diverse, yet species-specific communities has been repeatedly validated with many studies finding novel bacteria that have been found only within sponges (Lopez et al. 1999; Webster et al. 2001; Hentschel et al. 2002). Of 32 sponges collected from various oceans, each sponge species contained different bacterial species. However, the bacterial species found within all sponges were more closely related to each other than to other marine bacteria (Schmidt et al. 2012). This study and others reveal that sponges harbor bacterial symbionts from more than 30 phyla, including a candidate phylum of bacteria, Poribacteria, that are found exclusively in sponges (Fieseler et al. 2004; Taylor et al. 2007a; Sipkema et al. 2009, Lafi et al. 2009; Schmidt et al. 2012; Yang et al. 2019). However, there is some evidence showing that some bacteria classified as ‘sponge-specific' occur more widely outside of sponge hosts than previously thought, having been found in low abundances in seawater and other organisms like corals (Taylor et al. 2013). Other studies have found that sponge community structures are subject to shift during changes in temperature (Simister et al. 2012), pH (Morrow et al. 2012; Ribes et al. 2016), or both (Lesser et al. 2016).

1.2.2 The functions of sponge symbionts

Microorganisms face two fates within the sponge body: they are either consumed as food or are able to avoid phagocytosis and live as symbionts. Sponges have been shown to preferentially capture planktonic bacteria (Yahel, Eerkes-

Medrano & Leys 2006; Hanson et al. 2009; Maldonado et al. 2010; McMurray et al. 2016) with microbes 1−10 μm in diameter captured most efficiently (Pile et al. 1996; Turon et al. 1997; Ribes et al. 1999; Yahel et al. 2007). Sponges also are known to feed on viruses (Hadas et al. 2006). Sponges have been long shown to differentiate between symbionts and bacteria when filtering seawater (Wilkinson et al. 1984), indicating that microorganisms have several strategies to avoid phagocytosis by the host. A ubiquitous sponge symbiont, "*Candidatus* Synechococcus spongiarum", lacks certain genes for the complete biosynthesis of the O-antigen in lipopolysaccharide, which may contribute to resistance of both engulfment and lysis by cyanophages (Lerouge & Vanderleyden 2002; Burgsdorf et al. 2015). Phagocytic engulfment has also been thwarted by sponge symbionts able to express eukaryotic-like, ankyrin-repeat proteins (ARPs) (Thomas et al. 2010; Fan et al. 2012; Liu et al. 2012; Nguyen et al. 2014). Further, these ankyrin protein genes can be introduced to the bacteria through lysogenic conversation by phages known as "ankyphages" (Leigh 2019). Microorganisms that are not directly consumed as food are able to persist for long periods and provide a wide array of benefits to the sponge hosts. For example, the secondary metabolites produced by sponge symbionts provide defense against predators (Chanas et al. 1997; Pawlik et al. 1995; Becerro et al. 2003; Rhode et al. 2015), fouling organisms (Sears et al. 1990; Okino et al. 1995; Bovio et al. 2019) and pathogenic bacteria (Thakur et al. 2003; Pawlik 2011; Flórez et al. 2015). Symbiont-produced chemicals also help sponges colonize and compete for space within an often competitive and crowded reef (Bryan 1973; Turon 1996; Thacker et al. 1998; Wang et al. 2012, 2015).

Symbionts contribute to sponge nutrition (Wilkinson & Garrone 1980; Weisz et al. 2007; Erwin & Thacker 2008). They provide essential vitamins to sponges including vitamin B_1, B_2, B_6, B_7, and B_{12} (Hallam et al. 2006; Thomas et al. 2010; Liu et al. 2011; Siegl et al. 2011) and can acquire limiting nutrients through diverse metabolic strategies. Sponges often host photoautotrophic microbes that can supply fixed carbon and nitrogen from atmospheric CO_2 and N_2 respectively (Wilkinson & Fay 1979; van Duyl et al. 2008; Fiore et al. 2010; Weisz et al. 2010). Also found within the sponge holobiont are microbes capable of nitrification, denitrification, anaerobic ammonia oxidation, sulfate oxidation, and sulfate reduction (Zhang et al. 2019). The benefits of these alternate metabolic strategies extend beyond the holobiont as these microbially-mediated nutrient cycles contribute to the biogeochemistry of entire ecosystems.

1.3 Microbially-mediated nutrient cycling in marine sponges

1.3.1 Carbon, nitrogen, and sulfur cycling by the sponge holobiont

Because of their widespread abundance, ability to filter large volumes of water, and their association with metabolically diverse microbes, sponges are key organisms in recycling remineralized nutrients (carbon, nitrogen, sulfur, and phosphorus) for re-use by the benthic and pelagic community (Figure 1.2). The sponge holobiont quickly metabolizes both particulate organic carbon (POC) and dissolved organic carbon (DOC) through filtration of the surrounding water column (Reiswig 1971; Vogel 1977; Pile et al. 1997; Gili & Coma 1998; Ribes, Coma & Gili 1999; Yahel, Sharp & Marie 2003; Hadas, Shpigel & Ilan 2009; Leys et al. 2011).

This carbon acquisition through heterotrophic feeding may be influenced by filtration rates or the density of bacterial symbionts (de Goeij et al. 2008b). Interestingly, the sponge holobiont differentiates between coral-derived and algal-derived dissolved organic carbon with the latter source being incorporated into sponge biomass at higher rates (Rix et al. 2017). This carbon is then cycled back into the water column through the shedding of choanocyte cells (particularly by encrusting sponges living in reef cavities) where it is consumed by detritivores and passed up the food web (de Goeij et al. 2013). This "sponge loop" hypothesis has yet to be validated for large, emergent sponges who may prioritize production of biomass over production of particulate detritus (McMurray et al. 2018). However, in this case, carbon is still transferred to higher trophic levels through predation by, for example, spongivorous fish.

Every known mechanism of nitrogen transformation (nitrogen fixation, nitrification, denitrification, and anaerobic ammonia oxidation (anammox)) has been detected in sponges, and nitrogen cycling between sponges and their symbionts exists in a tightly coupled web (Maldonado et al. 2012; Zhang et al. 2019). Further, the sponge holobiont can act as both a source and a sink of bioavailable nitrogen to the surrounding reef ecosystem. In several cases, sponges obtain much of their nitrogen from atmospheric fixation (Wilkinson & Fay 1979; Wilkinson, Summons & Evans 1999; Shieh & Lin 1994) with cyanobacterial symbionts playing a large role (Mohamed et al. 2008c; Zhang et al. 2014). Research suggests that HMA sponges may rely more heavily on biological N_2 fixation and LMA sponges may obtain nitrogen by filter feeding and dissolved nitrogen uptake (Weisz et al. 2007). Nitrogen

cycling is not ubiquitous in sponges and not all sponges contribute to the nitrogen biogeochemistry on reefs; however, microbial nitrifiers are found within reef sponges and both ammonia and nitrate are commonly released into ambient water (Diaz & Ward 1997; Jimenez & Ribes 2007; Southwell, Popp & Martens 2008; Southwell et al. 2008). *Chondrilla nucula* on its own has been shown to contribute 50% to 120% of the nitrate needed to sustain primary production in a coral reef (Corredor et al. 1988). Sponge symbionts also contribute to efflux of nitrogen out of the reef through anammox and denitrification facilitated by anaerobic bacterial and archaeal symbionts. It must be noted that a large amount of research has been dedicated to both *in situ* nitrogen cycling within sponges and to sequencing the genes responsible for nitrogen fixation (*nifD, nifK, nifH*), ammonia oxidation (*amoA*), and denitrification (*napA, narG, nirK, nirS, cnorB, qnorB, nosZ, narK*) (Zhang et al. 2019 and references therein).

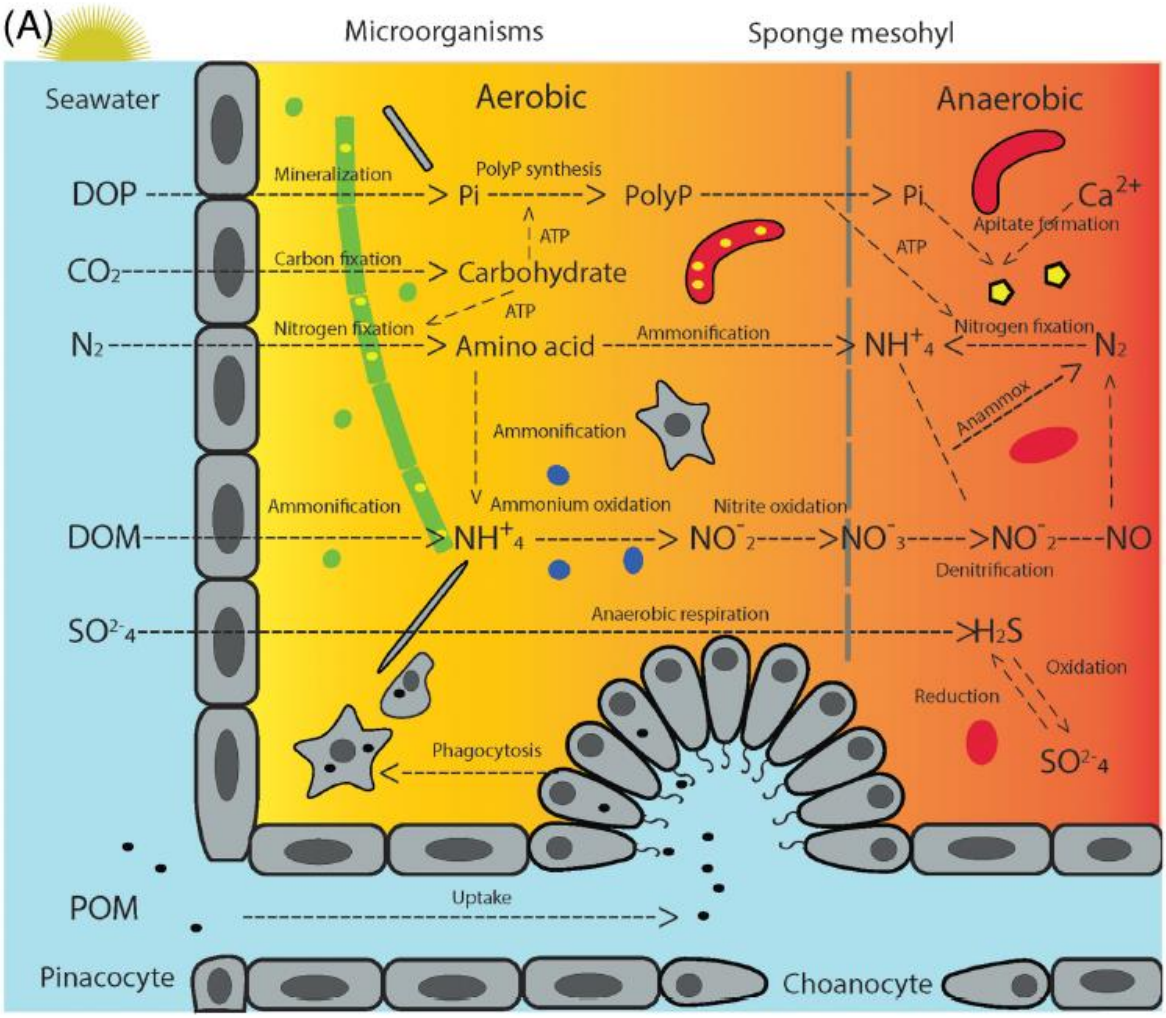
Although the body of work on sulfur cycling mediated by the sponge holobiont is significantly less than that of carbon and nitrogen, sponges do contribute greatly to sulfur cycling in marine environments. Sulfur oxidation and reduction occur within anoxic spaces of sponges by specialized symbionts that utilize sulfur compounds (Hoffman et al. 2005; Mohamed et al. 2008c). Further, sulfate-reducing bacteria have been found in many sponges and genome analyses reveal genes encoding for sulfatases, enzymes involved in SO_4^{2-} ester biosynthesis and cleavage (Imhoff & Trüper 1976; Schumann-Kindel et al. 1997; Manz et al. 2000; Hoffmann et al. 2005; Hoffmann et al. 2006; Taylor et al. 2007a; Kamke et al. 2013). Evidence suggests that likely both sulfur oxidation and reduction occur simultaneously within

sponges in order to detoxify sulfide (Hoffmann et al. 2005; Taylor et al. 2007a). This hypothesis is supported by both sulfur-oxidizing and sulfur-reducing bacteria having been found in the same sponge as well as a gene responsible for oxidation and reduction, *aprA* (Meyer & Kuever 2008). Sponges have been able to greatly expand their metabolic capacity and facilitate major biogeochemical transformations of carbon, nitrogen, and sulfur; however, the same breadth of insights has not yet been extended to another crucial element, phosphorus.

1.3.2 Phosphorus and marine sponges

Phosphorus is an essential macronutrient that is an important building block of DNA, RNA, ATP and the phospholipid membrane bilayer in cells. At the same time, it is regarded as the ultimate limiting element within marine systems over geological time (Redfield 1960; Van Cappellen & Ingall 1994; Toggweiler 1999; Tyrell 1999; Benitez-Nelson 2000). It has not yet been determined whether sponges act as sinks or sources of phosphorus, and their primary role in phosphorus biogeochemistry is not well-studied (Zhang et al. 2019). There have been a handful of studies that have measured phosphate efflux from sponges. Phosphate was released by the sponges *Dysidea avara*, *Chondrosia reniformis* and *Agelas oroides*, suggesting that sponges may be important suppliers of inorganic phosphate for free-living marine microbes or other sponge holobionts (Ribes et al. 2012). Another study found that sponge endosymbionts, specifically actinomycetes, are capable of solubilizing tricalcium phosphate, an inorganic phosphorus source, and potentially make it bioavailable to the sponge holobiont (Sabarathnam et al. 2010).

There is a clear research gap in determining the role of sponges in phosphorus biogeochemistry. Only a few papers, including one published by the Hill laboratory in 2015 (Zhang et al. 2015), have focused solely on microbially mediated phosphorus transformation in sponges. These pioneering papers provided the framework for the hypotheses of this thesis research and will be discussed in more depth in the following section (Section 1.5)



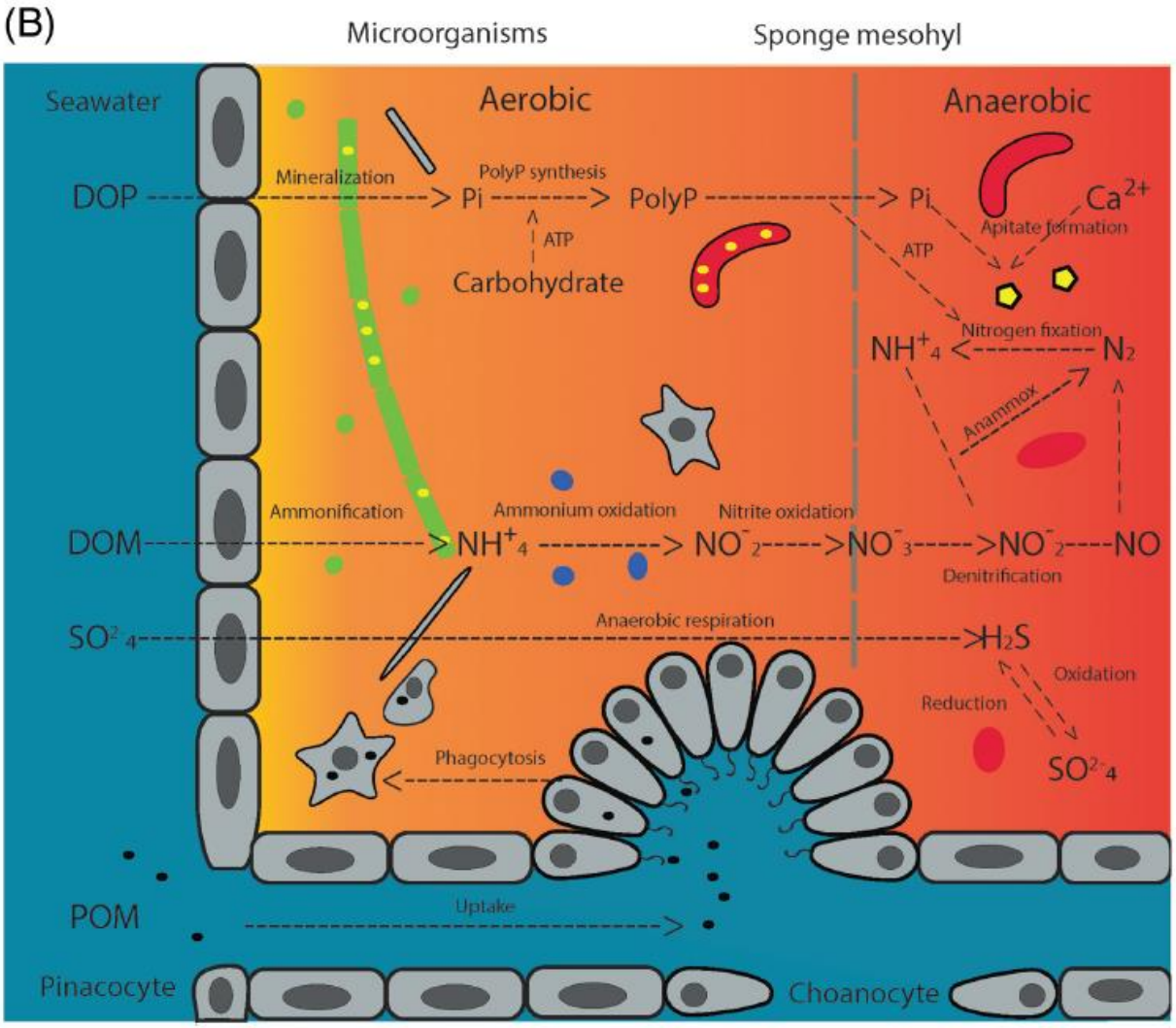


Figure 1.2. Conceptual diagram of microbially mediated nutrient cycles in sponge mesohyl during daylight **(A)** and night **(B)**. Color gradient (yellow to red) background indicates O_2 availability in sponge mesohyl, with yellow representing the most oxic state and red representing the most anoxic state. Microbial symbionts are divided in three groups: cyanobacteria (green), archaea (blue) and heterotrophic bacteria (red). PolyP granules (yellow) accumulated in microbial cells can contribute to the formation of apitate minerals (yellow pentagon) inside sponges. Sponge cells (grey) are involved in the uptake of DOM and POM from the surrounding seawater.

Abbreviations: Dissolved organic phosphorus (DOP); Dissolved organic nitrogen (DON); Particulate organic matter (POM-illustrated as black dots); Phosphate (Pi); Polyphosphate (PolyP). Figure provided by Zhang, Jonas et al. 2019 with permission.

1.4 A closer look at marine sponges and the phosphorus cycle

1.4.1 Phosphorus is simultaneously crucial and limiting

Phosphorus is transformed between a variety of inorganic and organic forms by microorganisms primarily within oligotrophic eutrophic environments and is a key factor in photosynthesis, carbon sequestration, and nitrogen fixation (Redfield 1960; Van Cappellen & Ingall 1994; Toggweiler 1999; Tyrell 1999; Benitez-Nelson 2000; Paytan & McLaughlin 2007; Karl 2014). While it is the 11th most abundant element on Earth, the majority of phosphorus is mineralized within the lithosphere (Karl 2014). Physical and biochemical erosion of the lithosphere contributes the largest input of bioavailable phosphorus to marine systems by rivers and runoff (Föllmi 1996). Within the marine environment, phosphorus is considered to be the ultimate limiting element (Redfield 1960; Broecker & Peng 1982). The major forms of phosphorus within the upper water column are dissolved inorganic phosphate (DIP) also known as orthophosphate (P_i)[1] and dissolved organic phosphorus (DOP), with P_i being the preferred phosphorus source for microorganisms (Orchard e al. 1999). The hydrolysis of DOP by cyanobacteria generates more bioavailable P_i, enhancing primary production and CO_2 uptake (Kolowith et al. 2001; Beneitez-Nelson &

[1] Orthophosphate (Pi) is also operationally measured as “Soluble Reactive Phosphorus” (SRP).

Buesseler 1990).

Within coral reefs, bioavailable phosphorus is delivered through upwelling of phosphorus-enriched seawater or by vertically migrating picoplankton (Karl et al. 1992; Karl & Tien 1997). P_i has also been found to make up the majority of the phosphorus pool (80-83%) within reef sediments (Entsch et al. 1983). Concentrations of P_i within the reef water column vary based on geographic location and time of year, but has been found ranging from 10 nM – 2.6 µM and is most commonly recorded below 0.5 µM (Entsch et al. 1983; Sorokin 1992; den Haan et al. 2016; Godinot et al. 2013; D'Angelo & Wiedenmann 2014; Silbiger et al. 2018). DOP concentrations usually are similar to that of P_i, but DOP often dominates the total dissolved phosphorus in the open ocean (Karl & Yanagi, 1997; Orrett & Karl, 1987). Terrestrial runoff after heavy rainfall can cause nutrient pulses of both nitrogen and phosphorus, causing pelagic algal blooms in which filamentous cyanobacteria have been shown to have high uptake rates of P_i (den Haan et al. 2016). The proliferation of cyanobacteria, microalgae and macroalgae stimulated by a eutrophic event can alter the benthic communities of coastal ecosystems (Valiela et al. 1997). Within the benthos, sponges may contribute to inputs of phosphorus through their pumping as they can couple pelagic and shallow waters. Sponges also have been shown to influence the availability of phosphorus. Genetic and chemical analyses by two main studies have shown that marine sponges have the capabilities to sequester large amounts of phosphorus in the form of polyphosphate (Zhang et al. 2015; Ou et al. 2020).

1.4.2 Polyphosphate in marine sponges

Polyphosphate (poly-P) is a multi-chained linear or circular compound comprised of tens to hundreds of phosphate molecules linked by phosphoanhydride bonds and is well-studied as a major energy storage compound in almost all organisms (Kornberg, Rao & Ault-Riché 1999). Poly-P is also essential for other cellular functions, including, but not limited to the formation of biofilms, motility, and cell persistence (Brown & Kornberg 2004). Poly-P in the marine world has been linked to geologic phosphorus sequestration as it facilitates the formation of calcium phosphate minerals (Diaz et al. 2008). Further, recent metagenomic studies have suggested the potential importance of poly-P to the metabolism of microorganisms, especially within nutrient depleted waters (Temperton et al. 2011; Martin et al. 2014). There seems to be an inverse relationship between phosphorus availability and poly-P accumulation by microorganisms. A relatively larger proportion of total phosphorus was found to be stored as poly-P in organisms from seawater containing the least amount of P_i (Martin et al. 2014).

Poly-P within the sponge body was brought to light by Zhang et al. (2015), who found through extraction, fluorescent spectroscopy, and SEM that the bacterial symbionts of three sponges, *Xestospongia muta*, *Ircinia strobilina*, and *Mycale laxissima* produce poly-P. These three sponges are abundant in the reef environment of the Florida Keys. Further, poly-P comprises 25–40% of the total P in sponge tissue (Zhang et al. 2015; reviewed by Colman 2015). It was also discovered that a specific filamentous cyanobacterial symbiont accumulated poly-P (Figure 1.3). Further insights into poly-P biosynthesis can be inferred by the detection of the gene (*ppk*)

that encodes for polyphosphate kinase, the bacterial enzyme responsible for polymerization of phosphates. Five *ppk* sequences were amplified and confirmed to be expressed in metabolically-diverse bacterial symbionts of *X. muta, I. strobilina,* and *M. laxissima* (Zhang et al. 2015). Five years later, a study identified 30 sequences from *ppk* gene libraries of eight sponge holobionts from the South China Sea (Ou et al. 2020). This study interestingly found that poly-P enrichment rates were higher for sponges living in oligotrophic environments, which is consistent with the findings of Martin et al. 2014. Poly-P has also been found in a freshwater sponge, *Ephydatia muelleri*, with concentrations shown to change during exposure to polluted water (Imsieke et al. 1996).

It is still unknown what fate phosphorus stored as poly-P meets within the sponge body. Zhang et al. (2015) detected evidence for the formation of apatite, a calcium phosphate mineral, which may be deposited into sediment thus making sponges a potential phosphorus sink. However, it is possible that P_i could be cleaved from poly-P, allowing the symbionts to utilize the large energy release (as commonly seen within algal systems and microbes in wastewater treatment plants (McMahon & Read 2013)). Subsequently, sponge pumping would release labile P_i back into the environment. It is hypothesized that during periods of active sponge pumping (thus high oxygen content within sponge tissue), symbionts produce poly-P. When the sponge stops pumping, poly-P faces two fates: (1) it can be quickly depleted and used for energy or (2) the phosphorus is combined with ions such as calcium and locked away as mineralized apatite (Zhang et al. 2015). In the case of released P_i, sponges would serve as a source of phosphorus, which would be quickly consumed by

phosphorus-limited microorganisms in seawater. Conversely, in the case of apatite formation and deposition, sponges could serve as a sink (Colman 2015).

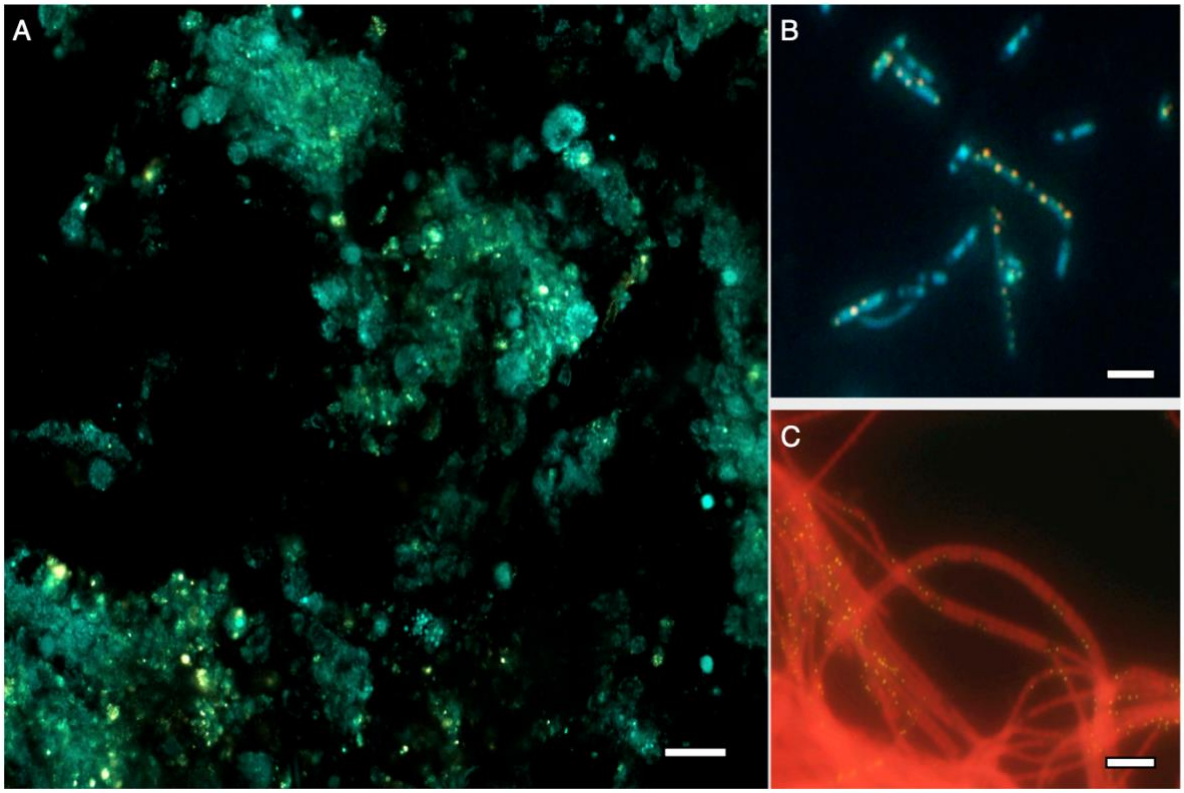


Figure 1.3. Yellow fluorescent granules observed in DAPI-stained samples. (A) *X. muta* tissue section under a confocal fluorescent microscope. (Scale bar: 20 μm.) (B) Bacterial cell fraction of the sponge *X. muta* under an epifluorescent microscope. (Scale bar: 1 μm.) (C) Enrichment culture of the filamentous cyanobacterial *Leptolyngbya* isolated from sponge *I. stroblina* tissue. (Scale bar: 1 μm.). Provided with permission by PNAS. Zhang F., Blasiak L. C., Karolinm J. O., Powell, R. J. , Geddes, C. D., Hill, R. T. (2015). Phosphorus sequestration in the form of polyphosphate by microbial symbionts in marine sponges. *Proc Natl Acad Sci*; 112: 4381–4386.

1.4.3 Research gap – Phosphorus tracing studies and the need for better understanding of phosphorus cycling in sponges

Compared to the breadth of research on carbon, nitrogen, and sulfur, there is little known about microbially mediated phosphorus transformations within sponges (Taylor et al. 2007a; Zhang et al. 2019). *In vivo* tracing studies are non-existent and will be crucial to understand the potential role of microbe-mediated mechanisms in phosphorus accumulation, release, and mineralization by marine sponges (Maldonado et al. 2012; Zhang et al. 2015). The major challenge in conducting phosphorus tracing studies is the lack of stable isotopes of phosphorus. Experimental tracing must be accomplished using radiolabeled isotopes ^{32}P or ^{33}P. As radioisotopes are not easily deployed in *in situ* experiments without meeting many regulatory and safety requirements, tracing studies must either be done in laboratory conditions (this study) or possibly by using the stable oxygen isotope ratio of phosphate, $\delta^{18}O_p$.

1.5 Focuses of this research

This research provides major insights into the role of sponges in the marine phosphorus cycle by conducting the first-ever tracing study on two sponges, *Lendenfeldia chondrodes* and *Hymeniacidon heliophila* using controlled aquarium systems and the addition of $^{32}P_i$ and γ-^{32P}ATP. The percent of phosphorus stored as poly-P was quantified in both sponges and five *ppk* gene sequences were amplified from bacterial symbionts. Considering the prevalence of sponges in phosphorus-limited reefs, the amount of phosphorus taken in by sponges and stored as poly-P is likely to have a profound effect on the phosphorus availability of these ecosystems.

This thesis supplies insights into a major research gap in sponge microbiology and is presented in three experimental chapters as detailed further in the following section.

1.5.1. *Lendenfeldia chondrodes, Hymeniacidon heliophila,* and their symbionts

All experimentation done throughout this research was done in parallel with two sponges, *Lendenfeldia chondrodes* from Palau and *Hymeniacidon heliophila* from the coastal bays of Maryland, USA. These two sponges provide an interesting contrast because *L. chondrodes* is found in a highly oligotrophic reef environment whereas *H. heliophila* occurs in coastal bays where nutrient (and phosphorus) concentrations are generally much higher. In light of the many genetic and metagenomic studies on sponges and their symbionts, I considered it to be important to first characterize these previously uncharacterized sponges. It is even more impactful to look at their microbiomes and attempt to culture viable isolates. However, because sponge-associated microorganisms remain largely uncultivated, culture-independent methodologies have been fundamental to gain genomic and putative functional information on the symbionts of both sponges. Identifying important taxa and their potential functions can be used to make inferences into whether certain sponges may store more phosphorus in the form of poly-P. In Chapter 2, sponge identities are confirmed with the 28S ribosomal RNA (28S rRNA) gene, the internal transcribed spacer (ITS), and the cytochrome oxidase I (COI) gene sequence barcoding. Sponge symbionts are isolated on three selective media: heterotrophs were plated on marine agar 2216 and autotrophs were cultured with carbon-free SN15 and marine BG11 media. Both approaches yielded cultured isolates

and uncultivated sponge communities were characterized using bacterial ribosomal (16S rRNA) gene sequencing.

1.5.2 Phosphorus tracing using ^{32}P

There are currently no studies that have identified in what form sponges obtain phosphorus. Phosphorus is most commonly found within marine systems as inorganic phosphate or organic phosphorus, either within living systems or as dissolved phosphorus such as dissolved ATP. Sponges must obtain phosphorus from the surrounding water through filtering because they cannot rely on microbially-mediated mechanisms such as fixation (as seen with carbon and nitrogen) because phosphorus has no inorganic atmospheric form. The lack of research regarding phosphorus inputs has largely been due to the fact that tracing phosphorus requires the usage of radiation and thus cannot easily be conducted in the field. In Chapter 3, with controlled laboratory experiments using ^{32}P, I determined two sources of phosphorus in both sponges' diets and calculated uptake rates.

1.5.3 Polyphosphate kinase (*ppk*) amplification and polyphosphate extraction

Following the findings of Chapter 3 identifying sources of phosphorus in the sponge diet, Chapter 4 provides insights into phosphorus storage in the form of poly-P. First, through amplification and cloning, polyphosphate kinase (*ppk*) was detected in both *L. chondrodes* and *H. heliophila*. Second, through chemical extraction and quantification using a colorimetric assay with malachite green, poly-P and total-phosphorus was extracted from sponge tissue. By calculating what percentage of phosphorus is stored as poly-P, I can infer and compare the potential role these two

sponges play in their respective ecosystems' phosphorus cycle. To my knowledge, only two studies have quantified poly-P within marine sponges. Chapter 3 provides five *ppk* gene sequences and poly-P profiles of two new sponges.

1.5.4 Overall conclusions and further directions

When discussing the energy dynamics of coral reef ecosystems, sponges have been historically ignored because many sponges have the tendency to occupy more inconspicuous parts of the benthos. Considering their worldwide distribution, filtering capabilities, and the taxonomic and metabolic diversity of their symbionts, marine sponges are central to marine biogeochemical cycling. However, their roles in phosphorus cycling is particularly understudied relative to that of carbon, nitrogen, and sulfur. Chapter 5 pulls insights from the three experimental chapters to expand on the PhD dissertation work of Zhang (2015) and the work of Ou et al. (2020) on marine sponge holobionts and their impact on phosphorus availability in marine ecosystems.

Chapter 2. Characterization of two geographically distinct sponges, *Lendenfeldia chondrodes* and *Hymeniacidon heliophila*, and their symbionts

2.1 Abstract

This chapter describes the bacterial communities associated with *L. chondrodes* and *H. heliophila* and how specific taxa may contribute to holobiont functioning and nutrient cycling. *L. chondrodes* was supplied by Pacific East Aquaculture in Mardela Springs, MD and *H. heliophila* was collected off Assateague Island in the Sinepuxent Bay by kayak and snorkel. Three sequencing projects were conducted to characterize these two sponges and their symbiotic communities. Using three primer sets that target three well-studied eukaryotic barcodes—the 28S ribosomal RNA (28S rRNA) gene, the internal transcribed spacer (ITS), and the cytochrome oxidase I (COI) gene—sponge identities were confirmed. Targeting the V3-V4 region of the 16S rRNA gene, bacterial communities were amplified within both sponges. Isolates were also cultured from both sponges and were identified through sequencing. Using three selective media, five microbes were isolated from *H. heliophila* and twelve microbes were cultured from *L. chondrodes*. Cultured autotrophic symbionts included *Glabratella* sp. from *H. heliophila*, and *Cyanobium* sp., *Pseudanabaena* sp., and a previously uncultured cyanobacterium from *L. chondrodes.* Heterotrophic isolates from both sponges were primarily members of the genera *Pseudoalteromonas* and *Bacillus.* Metagenomic analysis showed that *H. heliophila* hosted a microbial community dominated by the bacterial

phylum Proteobacteria, specifically the class Alphaproteobacteria. Within *L. chondrodes,* one species of Cyanobacteria, *Synechococcus spongarium,* made up ca. 68% of the entire bacterial community with Proteobacteria and Actinobacteria making up the remaining 30% and 2%, respectively. To understand how sponge holobionts may contribute to benthic biogeochemical cycles, it is paramount to carefully characterize potential key taxa in their symbiotic communities.

2.2 Introduction

2.2.1 *Hymeniacidon heliophila*

Hymeniacidon heliophila, commonly called "the sun sponge", is a marine demosponge found throughout the Atlantic Ocean, spanning from the Bay of Fundy in Canada to southern Brazil (Candelas & Candelas 1963; Wiedenmayer 1977; Ginn et al., 2000; Lerner et al. 2005; Muricy & Hajdu 2006; Turque et al. 2008; Weigel & Erwin 2016; van Soest et al. 2020; Turner 2020). *H. heliophila* exists in a wide range of habitats, often found within subtidal and intertidal zones (Weigel & Erwin 2016), between rocky and sandy bottoms (Muricy & Hajdu 2006), shallow hydrothermal vent ecosystems (Coelho et al. 2018), and pilings of offshore oil and natural gas drilling platforms in the northern Gulf of Mexico (Erwin et al. 2011). *H. heliophila* is an unusual sponge in that it is able to withstand extended exposure to air and as such, is commonly found within the intertidal zone (Weigel & Erwin 2016). The sponge can exist in many morphotypes; it is often a large, encrusting layer of soft tissue, or can be covered by sand with only the papillae being exposed (Ginn et al. 2000; Lôbo-Hajdu et al. 2004; Muricy & Hajdu 2006). In this study, *H. heliophila* was collected from the Sinepuxent Bay, an inland waterway that is part of the Maryland Coastal Bays ecosystem (Wazniak & Hall 2016). It is dominant within this estuarine ecosystem and is found at shallow depths (1 m or less) within the soft sediment and throughout patches of bay grasses. The common bay grass, *Ruppia maritima*, grows directly through the tissue of the sponge (Figure 2.2A). *H. heliophila* is the main species of sponge found in coastal areas of the southeastern Brazil (Monteiro & Muricy 2004), which are often contaminated, and *H. heliophila* has been shown to be

a pollution-tolerant species able to adapt to eutrophic environmental conditions (Turque et al. 2010).

H. heliophila has been studied in various contexts due to the fact that it is abundant, easy to collect and identify, and can withstand exposure to air (Muricy & Hajdu 2006). For example, it has been used in early studies regarding cell aggregation (Candelas & Candelas 1964; Costa et al. 2019), regeneration (Coutinho et al., 2017), and locomotion (Bond & Harris 1988). It has been used to enhance understanding of the complexities of animal multicellularity (Custódio et al. 2004; Costa et al. 2019) and as a representative of Porifera in studies focused on other phyla, such as cnidarians (Odorico & Miller 1997). *H. heliophila* has been a target for the discovery of novel marine natural products and has been shown to produce secondary metabolites (Sennet et al. 1990; Granato et al. 2000). For instance, *H. heliophila* has been found to contain the potent antioxidant, L-5-hydroxytryptophan, which can reduce apoptosis in human cells after exposure to short-wave UV light (Lysek et al. 2003). *H. heliophila* has also been the focus of antifouling studies and crude extracts from sponge tissue were recorded to defend the sponge against predation by sea urchins, hermit crabs, and fishes (Henrikson & Pawlik 1995; Ribeiro et al., 2010). Due to the fact that *H. heliophila* was found to be efficient in accumulating metals, the sponge may be used as an efficient way to monitor metallic pollution in waterways or as a tool for the bioremediation of electronic waste (Batista et al. 2014; Rozas et al. 2017). The sponge also serves as a proficient bioindicator of polycyclic aromatic hydrocarbons (PAHs) as two studies found that sponge PAH concentrations reflected that of local contamination (Batista et al. 2013; Pedrete et al. 2017). Thus *H.*

heliophila seems to be a good candidate for both biomonitoring and bioremediation programs in coastal areas prone to anthropological impacts such as pollution; although it has been hypothesized that increased pollution may negatively affect the chemical activity of *H. heliophila* (Batista et al. 2018).

Many organisms have been reported to coexist with *H. heliophila*. The sponge is host to polychaetes (Wilson 1979), the decorator crab, *Libinia dubia* (Stachowicz and Hay 2000) and the seagrass *Ruppia maritima* (this thesis). Rozas et al. (2011) isolated five different fungal strains from *H. heliophila* cells and nine from whole tissue samples. This is the first report of fungal symbionts isolated directly from sponge cells (Rozas et al. 2011). Three phyla of archaea (*Euryarchaeota*, *Crenarchaeota*, and *Parvarchaeota*) have been found within the *H. heliophila* holobiont, including ammonia-oxidizing archaea that have been hypothesized to increase sponge fitness within polluted environments (Turque et al. 2010; Weigel & Erwin 2016). A handful of studies have examined the bacterial communities of *H. heliophila* using metagenomic analyses of the 16S rRNA gene (Turque et al. 2010; Erwin et al. 2011; Weigel & Erwin 2016, 2017; Coelho et al. 2018) and this approach will be the focus of Section 2.2.2.

2.2.2 *Lendenfeldia chondrodes*

Lendenfeldia chondrodes is a demosponge that is commonly found in temperate environments and salt-water aquaria around the world. Belonging to the subclass Keratosa, the sponge does not contain spicules found in all other sponge subclasses and is historically difficult to characterize based on morphology

(Erpenbeck et al. 2012). *L. chondrodes* displays a high phenotypic plasticity in both shape and color and is often confused with other sponges such as *Collospongia auris* (Galitz et al. 2018). The shape of *L. chondrodes* can be low and encrusting, foliose (leaf-shaped), lamellate (plate-shaped), or cup shaped (Figure 2.1). The sponge's color has been recorded as purple, blue, green, and yellow-green (Ridley et al. 2005a; Galitz et al. 2018; Vargas et al 2020). The *L. chondrodes* individuals used in my thesis research were lamellate and purple (Figure 2.1C & D). The aquaculture supplier (Pacific East Aquaculture) from whom I bought the sponges gave the sponge the common name "photosynthetic blue-purple plating sponge".

Ecological research on *L. chondrodes* has been conducted primarily in the Pacific Ocean. Both massive, fan-shaped and thin, encrusting morphotypes were found north coast of Moorea, French Polynesia (Freeman & Easson 2016). This habitat is classified as having low sponge cover (<1% of benthos) from transects along 21 sites. Of the eight putative sponge species found, *L. chondrodes* (of both growth forms) was the only sponge found in areas well-exposed to sunlight, suggesting that the sponge may rely on phototrophy, consistent with the abundance of cyanobacterial symbionts and relatively high levels of chlorophyll *a*. *L. chondrodes* may be the only phototrophic sponge found in French Polynesia (Freeman & Easson 2016). *L. chondrodes* has also been described in the Republic of Palau as being yellow-green in coloration and having a smooth growth form like a spreading sheet with numerous vertical fingers (Ridley et al. 2005a). Intriguingly, some of the major research on *L. chondrodes* doesn't rely on collecting the sponges from the wild since they are common sponges in saltwater aquaria. In fact, the sponge is considered an

aquarium pest due to its rapid growth, resistance to removal, and ability to regenerate (Brümmer & Nickel 2003; Galitz et al. 2018). Where aquarium hobbyists see a pest, sponge biologists see potential (see Galitz et al. 2018), and I chose this sponge in hope that it would fare well in captivity within tank systems.

L. chondrodes has also been found to be a robust species in its natural environment. When testing the efficacy of transplantation of nursery-reared reef organisms into intertidal zones in Singapore, *L. chondrodes* was the most successful colonizer in terms of growth rate and mean survival time (Ng et al. 2015). Other studies on *L. chondrodes* have solely focused on extraction of natural products from the sponge. For example, an antifouling substance, epidioxy sterol, has been extracted from *L. chondrodes* tissue (Sera et al. 1999). A cyanobacterial symbiont of *L. chondrodes, Oscillatoria spongeliae* have also been of interest to natural products chemists due their production of both halogenated and brominated secondary metabolites (Unson 1994; Ridley et al. 2005b). The handful of studies on *L. chondrodes* that have focused on the sponge's symbiotic communities will be discussed in depth in Section 2.2.3.

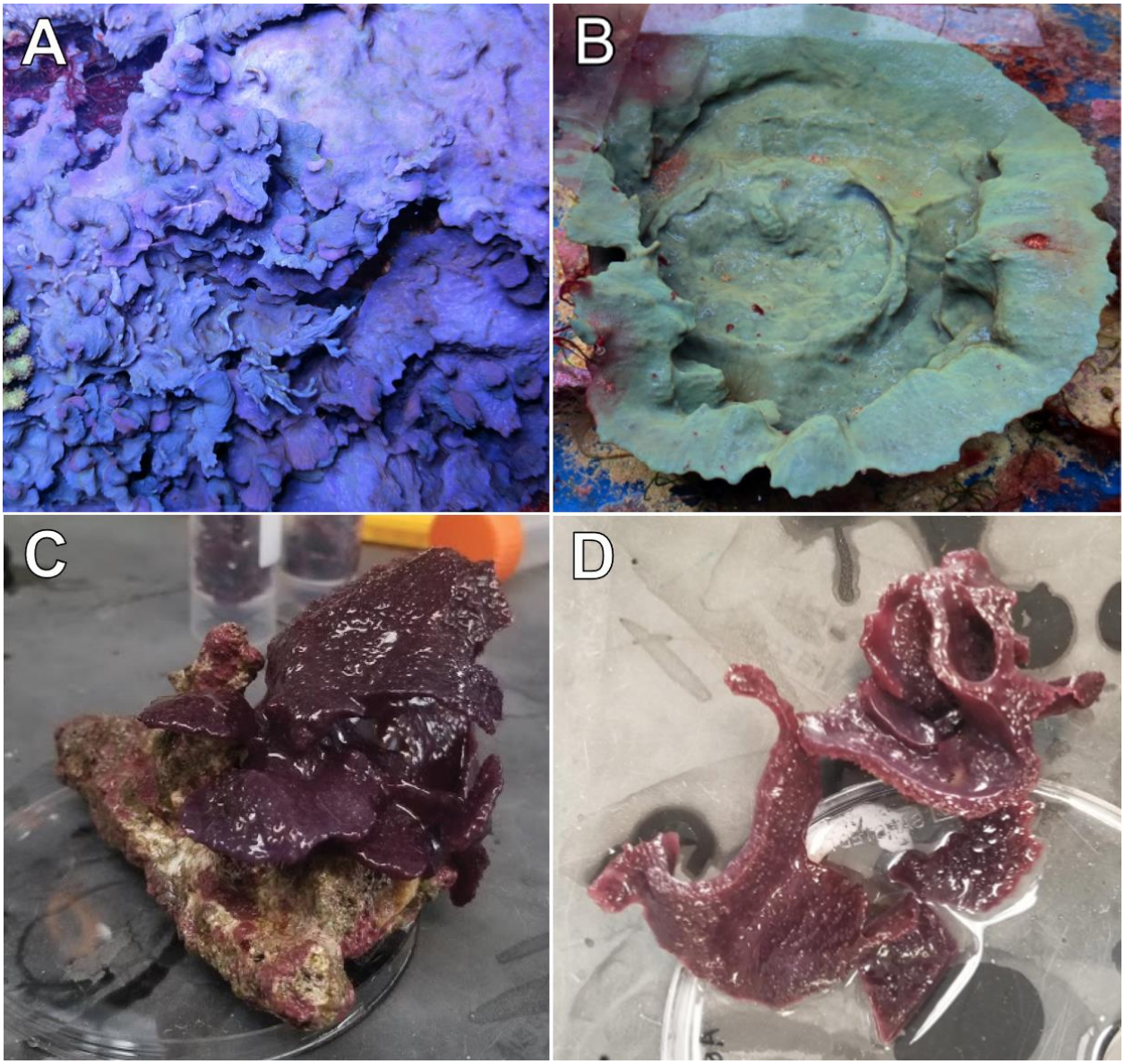


Figure 2.1 Morphotypes of *L. chondrodes*, all grown in aquaculture. (A) Foliose growth, two intermingled specimens of different blue-purple sponges, (B) cup shape, green color, (C) dark purple individual attached to aquarium rock, and (D) smooth, rubbery purple morphotype. A & B from Galitz et al. 2018 with permission, DOI: 10.7717/peerj.5586/fig-1. C & D from this study and provided by Pacific East

Aquaculture. For a sense of scale for the sponges in (C) and (D), the petri dish they are placed on is *ca*. 9 cm in diameter.

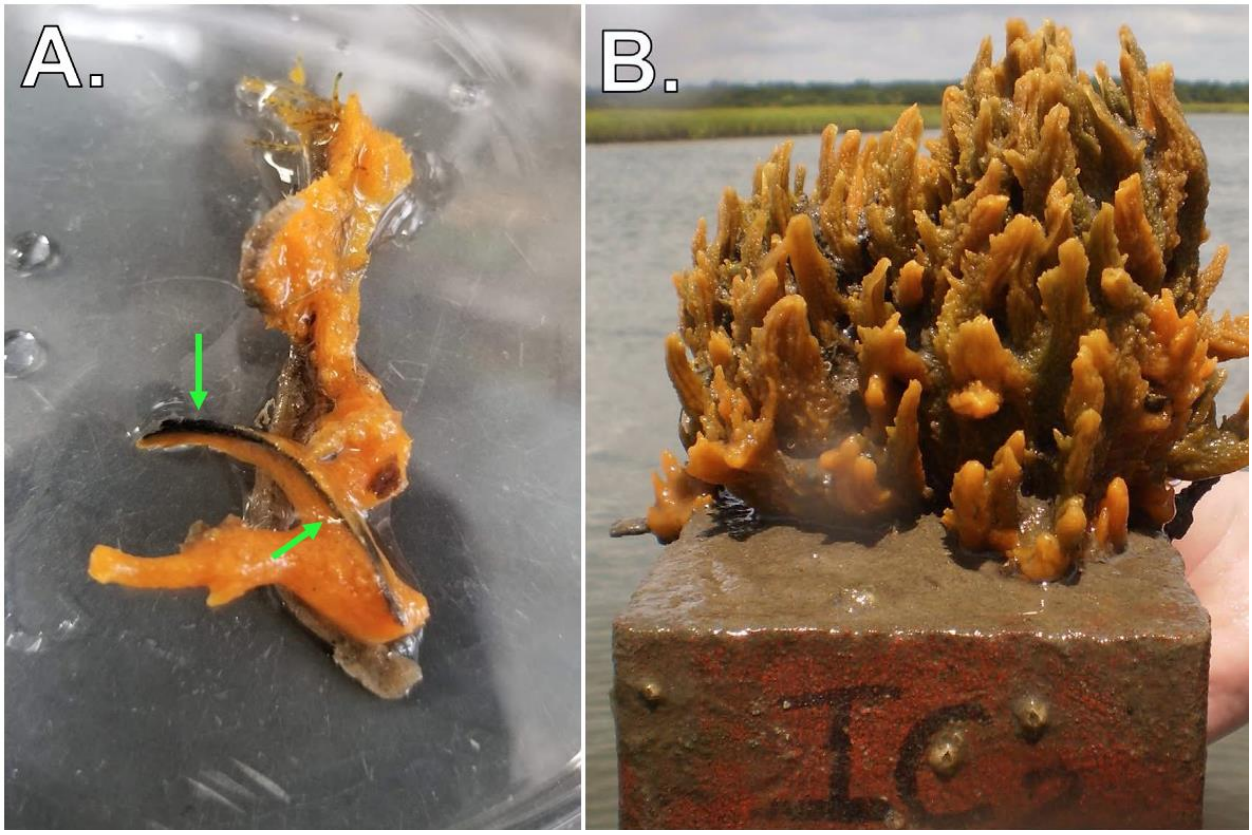


Figure 2.2 (A) *H. heliophila* (specimen pictured *ca*. 4 cm in length) collected from Sinepuxent Bay, green arrows indicate *R. maritima* growing through sponge tissue. (B) *H. heliophila* from Wilmington, North Carolina from Weigel & Erwin 2017 provided with permission https://www.nature.com/articles/srep43247/figures/1.

2.2.3 The bacterial communities of *H. heliophila* and *L. chondrodes*

Using clone libraries and Illumina MiSeq, symbiont communities have been examined from *H. heliophila* in Brazil (Turque et al. 2008, 2010), the Gulf of Mexico (Erwin et al. 2011), North Carolina (Weigel & Erwin 2016, 2017), and *Hymeniacidon* sp. in Taiwan (Coelho et al. 2018). These analyses have revealed dominant taxa across expansive geographic distances with Proteobacteria being the dominant bacterial phyla in all cases thus far. Two studies examining communities of *H. heliophila* in Brazil found a high diversity of both bacterial (Turque et al. 2008) and archaeal symbionts (Turque et al. 2010). Bacterial communities were made up of representatives of Alpha-, Beta- and Gamma- Proteobacteria, Actinobacteria, Cyanobacteria, and Firmicutes with Alphaproteobacteria clusters representing more than 50% of all clones (Turque et al. 2008). A subsequent study of microbial communities of sponges in the same habitat showed that pollution may influence the structure of the archaeal communities (Turque et al. 2010). When comparing sponge abundance and archaeal communities of three sponges (*H. heliophila, Paraleucilla magna*, and *Petromica citrina*) in two distinct environments, Guanabara Bay, a highly polluted estuary, and the nearby relatively unaffected Cagarras Archipelago, *H. heliophila* was found in a higher abundance within the polluted bay. Further, *H. heliophila* within the polluted waters was found to host ammonia-oxidizing archaea and may have acquired these symbionts in order to increase their resistance to eutrophication (Turque et al. 2010). *H. heliophila* bacterial communities have also been analyzed from individuals in the Gulf of Mexico. The communities again were found to be comprised predominantly of Alphaproteobacteria (63%- relative

abundance) and Gammaproteobacteria (20%) (Erwin et al. 2011). Further, more than half of all clones in this study (n = 68, 50.4%) matched with nearly identical sequence similarity to sequences derived from *H. heliophila* in Brazil (Turque et al. 2008). Further, the *H. heliophila* sponges in Brazil harbored more diverse communities than the sponges from the Gulf of Mexico. *H. heliophila* often has similar communities to that of a geographically distant but related sponge, *Hymeniacidon perlevis* (Alex et al. 2013). 76% of microbial communities retrieved from *H. perlevis* collected in Portugal were associated with *H. heliophila* sequences from the Gulf of Mexico (Erwin et al 2011). However, there was no sequence similarity with the *H. heliophila* communities from Brazil (Turque et al. 2008). Proteobacteria were again found to dominate the microbiome of *H. heliophila* in North Carolina, but habitat played a role in shaping these sponges' microbial assemblages (Weigel & Erwin 2016). *Proteobacteria* were more dominant in intertidal sponges than in subtidal sponges. Further, the subtidal *H. heliophila* communities displayed a larger proportion of Planctomycetes, Cyanobacteria, and Actinobacteria than the intertidal communities (Weigel & Erwin 2016). The following year the same group examined *H. heliophila* communities in the context of nitrogen cycling. Using a predictive metagenomic approach, they showed that the *H. heliophila* holobiont has the capability to contribute to various nitrogen transformations (Weigel & Erwin 2017). The core phyla in *H. heliophila* (dominated by Alphaproteobacteria) were shown to be affiliated with at least one gene involved in nitrogen cycling, and Proteobacteria may be the only phylum that has the potential to contribute to all nitrogen biogeochemical transformations (Weigel & Erwin 2017). Finally, the communities of *Hymeniacidon* sp. was examined in hydrothermal vent

systems in Taiwan (Coelho et al. 2018). The sequences had remarkable similarity to those found in the intertidal zone in North Carolina by Weigel and Erwin (2016). The potential link between sponges from these two geographically distant locations may be that both populations of sponges experience oxidative stress. The high tolerance of this sponge to oxidative stress could potentially stem from its production of the antioxidant, L-5-hydroxytryptophan (Lysek et al. 2003). Interestingly, no studies on *H. heliophila,* to my knowledge, have emphasized the role of cyanobacterial symbionts, even though cyanobacteria are present in *H. heliophila* communities and have been shown to provide important service to sponges (Usher 2008). There is one study that has reported a diverse assemblage of cyanobacterial symbionts in a related sponge, *H. perlevis* (Alex et al. 2012).

Compared to that of *H. heliophila*, there have been fewer studies analyzing the microbiomes of *L. chondrodes.* One group studying sponges in Palau found a consistent community of Alphaproteobacteria in *L. chondrodes* that best align to the genus *Rhodobacter* (Ridley et al. 2005a). The role of these Alphaproteobacteria within the holobiont is unclear, but Ridley et al. suggest they may help expand the metabolic diversity of sponges due to the *Rhodobacter* genus having been shown to be capable of aerobic and anaerobic anoxygenic photosynthesis (Gregor & Klug 1999; Allgaier et al. 2003), nitrogen fixation (Cantera et al. 2004), and denitrification (Schwintner et al. 1998). A recent study examined the shifts of the *L. chondrodes* microbiome in response to increased seawater temperature as a potential model for climate changes responses in this species (Vargas et al. 2020). The core bacterial community of *L. chondrodes* was comprised of Proteobacteria, Cyanobacteria,

Actinobacteria, Bacteroidetes, and Planctomycetes; Cyanobacteria had the highest abundance (48.75%) and Proteobacteria had the highest richness (431 OTUs) (Vargas et al. 2020). Heat stress did have an effect on the relative abundances of specific taxa with Proteobacteria and Planctomycetes becoming richer after heat treatment and Bacteroidetes and Cyanobacteria decreasing in abundance. Cyanobacterial symbionts are of utmost interest in *L. chondrodes* because this sponge presumably relies on nutrition from photosymbionts. The role of cyanobacteria in *L. chondrodes, H. heliophila*, and sponges in general will be the focus of the next section.

2.2.4 Functional roles of cyanobacterial symbionts of marine sponges

Cyanobacteria are one of the most common phyla of sponge-associated bacterial communities and have been shown to benefit the host through photosynthesis (Wilkinson 1987; Freeman & Thacker 2011), carbon fixation (Erwin & Thacker 2008a; Freeman et al. 2013), nitrogen cycling (Mohammed et al. 2008c; Fiore et al. 2010; Zhang et al. 2014; Ribes et al. 2015), and phosphorus cycling (Zhang et al. 2015). Cyanobacteria have been found to provide up to 50% of energy and 80% of carbon for reef sponges (Wilkinson 1983). Cyanobacteria are also key in providing nitrogen to sponges through atmospheric nitrogen fixation. The first molecular characterization of a diazotrophic community in sponges found cyanobacterial symbionts encoded for the *nifH* gene, one of three key genes needed for nitrogen fixation (Mohamed et al. 2008c). Cyanobacterial symbionts occur widely in geographically and genetically distant sponges, with *Oscillatoria spongeliae* and *Synechococcus spongiarum* being the most widespread (Usher 2008; Burgsdorf et al. 2015). *Synechococcus spongiarum* comprises at least 12 different subclades (Erwin et

al. 2012) and has been shown to be vertically transmitted from parent to offspring (Usher et al. 2001; Oren et al. 2005; Schmitt et al 2008: Webster et al. 2010). Typically, 30–50% of sponges within coral reef ecosystems contain cyanobacteria, but the dominance of "cyanosponges" can occasionally be as high as 80–90% (Wilkinson 1983,1992; Rützler 1990). Both sponges in this study have been previously shown to contain cyanobacteria (Turque et al. 2008; Ridley et al. 2005a; Freeman & Easson 2016; Vargas et al. 2020). Nutrient translocation by cyanobacterial and algal symbionts has been shown to occur within the sponge holobiont (Wilkinson 1979; Wilkinson & Fay 1979). Further, it is well-known that algal symbionts of marine invertebrates accumulate phosphorus from the surrounding water (as reviewed by Ferrier-Pagès et al. 2016). The first instance of phosphorus storage by sponges was detected in 2015 by the Hill laboratory both within sponge tissue and within isolated cyanobacterial symbionts (Zhang et al. 2015). The filamentous cyanobacterium, *Leptolyngbya* sp., stored poly-P that could be visualized by confocal microscopy as yellow fluorescent granules after DAPI-staining. However, it is still unknown whether this phosphate or energy is made available directly to the sponge host. It can be reasonably hypothesized that cyanobacteria provide host cells with bioavailable phosphorus, analogous to cyanobacterial symbionts providing photosynthetically fixed carbon to their hosts (Wilkinson 1983). The discovery that cyanobacterial symbionts are able to contribute phosphorus to the sponge diet would be one of the first discoveries of direct nutrient storage by bacterial symbionts for host cells (Colman 2015).

Cyanobacterial symbionts have been found to make up ca 5–9% of the

microbiome in *H. heliophila* (Weigel & Erwin 2016, 2017), although no studies have focused on the functional roles of these taxa. However, one study identified cyanobacteria associated with the related sponge *H. perlevis.* Electron microscopy and 16S rRNA gene sequencing identified the presence of *Synechococcus* sp. (47%), uncultured cyanobacteria, *Acaryochloris* sp., and *Xenococcus*-like morphotypes, with the latter two having been never reported in sponges (Alex et al. 2012). This study also found temporal variation of cyanobacteria assemblages when sampling *H. perlevis* in different seasons.

L. chondrodes has also been found to harbor a large abundance of cyanobacterial symbionts (Ridley et al. 2005a; Freeman & Easson 2016; Vargas et al. 2020). In addition to Alphaproteobacteria found within the communities of *L. chondrodes* in Palau, the sponges host the filamentous cyanobacteria, *O. spongeliae,* as well as *Synechocystis* sp. (Ridley et al. 2005a). Through fluorescence in situ hybridization experiments, the *Synechocystis* sp. were shown to be present in both the pinacoderm (surface epithelial tissue) and mesohyl, while *O. spongeliae* was found solely in the mesohyl (Ridley et al 2005a). While *O. spongeliae* is a common cyanobacterial symbiont of sponges (Usher 2008; Burgsdorf et al. 2015), *Synecocystis* sp. has only been found in a few sponges, although many species of *Synecocystis* are found within ascidians (Cox et al. 1985; Hernádez-Mariné et al. 1990). Interestingly, *O. spongeliae* and *Synechocystis* sp. were found in *L. chondrodes* that were collected a year apart, indicating they are consistent members of the bacterial community in this sponge (Ridley et al. 2005a). A later study examining symbionts of two other sponges, *Sarcotragus* and *Spongia* sp. found sequence similarities (ca. 97%) with the

Synechocystis sp. symbionts from Ridley et al. 2005a (Keesing et al. 2012). Of the two morphotypes of *L. chondrodes* examined in French Polynesia, the massive, fan-shaped sponges had higher photosymbiont abundance when compared to the thin, encrusting individuals (Freeman & Easson 2016). 16S rRNA gene and ITS sequencing studies again revealed both *O. spongeliae* and *Synechocystis* sp. symbionts, an assemblage that seems to be unique to *L. chondrodes* (Freeman & Easson 2016). This trend is given additional validity by the work of Vargas et al. (2020) who found cyanobacterial symbionts of *L. chondrodes* to be the highest in abundance within the bacterial community, but low in richness, meaning few certain cyanobacterial species (presumably *O. spongeliae* and *Synechocystis* sp.) dominate the *L. chondrodes* microbiome. The roles of cyanobacterial symbionts of both *L. chondrodes* and *H. heliophila* are of considerable interest in this thesis. Considering the plethora of research conducted on both sponges and the fact that both of their common names have to do with the sun—"Photosynthetic Blue-purple Plating sponge" (*L. chondrodes*) and "Sun-loving Sponge" (*H. heliophila*)—I hypothesized that these photosynthetic symbionts perform key functions in the sponges, especially in the context of phosphorus cycling.

2.2.4 Overview of this Chapter

The bacterial communities associated with *H. heliophila* and *L. chondrodes* were analyzed in this chapter. Both symbionts were able to be cultured in the laboratory and those only able to be characterized through metagenomic analysis were studied. To culture viable isolates, I used three selective media, although, it must be stated that one could spend years working to culture more sponge symbionts.

For example, it has been shown that previously unculturable isolates have been able to be cultured with the addition of antibiotics (Versluis et al. 2017), oligotrophic media (Sipkema et al. 2011), or with longer incubation times (Davis et al. 2005). As with most bacteria found on Earth, the majority of sponge symbionts remain uncultivated (Taylor et al. 2008; Thacker & Freeman 2012). I used culture-independent methodologies to characterize the sponge-associated community using Illumina MiSeq sequencing of the V3-V4 region of the 16S rRNA gene.

2.3 Methods

2.3.1 Sponge collection and processing

L. chondrodes was supplied by Pacific East Aquaculture in Mardela Springs, MD and *H. heliophila* was collected off of Assateague Island in the Sinepuxent Bay by kayak and snorkel at 1–1.5 m under the authority of an UMCES collection permit valid in the State of Maryland. Both sponges were cut into 1-cm^3 pieces and preserved in 95% ethanol for molecular analysis and 4% paraformaldehyde for histological examination. Sponges were also frozen at -80°C and lyophilized. To isolate symbiotic microorganisms, sponges were cut into 1-cm^3 pieces, washed with 70% ethanol, crushed with a mortar and pestle, diluted to 10^{-4} with sterile artificial seawater, and inoculated on three selective media: marine agar 2216 (heterotrophic isolation) (BD Biosciences[2]) and marine BG11 and SN15 (autotrophic isolation) (see chapter appendix for media recipes). From the bacterial colonies on marine agar 2216 plates, well-isolated colonies were sub-cultured onto marine agar 2216 and inoculated into marine broth 2216. Autotrophic cultures of marine BG11 and SN15 were sub-cultured with a 1:100 dilution every two weeks. Vials were kept at room temperature and were exposed to ambient light on a south-facing window sill.

2.3.2 DNA extraction, sequencing, and bioinformatic analysis

DNA extractions: DNA was extracted from sponge tissue (30 mg) by the AllPrep DNA/RNA Mini Kit (Qiagen) and DNA was extracted from culturable symbionts using the Ultraclean DNA Microbial Kit (Qiagen). DNA concentrations

[2] https://legacy.bd.com/europe/regulatory/Assets/IFU/Difco_BBL/212185.pdf

were checked by absorbance at 260 nm using a UV–visible spectrophotometer (NanoDrop, Thermo Scientific). All DNA samples were stored at -20°C. Six primer sets were used to identify sponges, culturable symbionts, and to sequence the sponge holobiont for community analysis (Table 2.1). Sponge identities were confirmed using two 28S rRNA gene primer sets, two ITS primer sets as described in Galitz et al. 2018, and universal COI primers as described by Folmer et al 1994. Cultured sponge symbiont DNA was amplified using the universal 16S rRNA gene primer set 27F/1492R and uncultured metagenomic DNA was amplified using V3-V4 rimer sequences (Table 2.1).

PCR: For confirming sponge identities, polymerase chain reactions were carried out in 25-µl total volume including 12.5 µl JumpStart REDTaq ReadyMix (Sigma Aldrich), 1 µl of each primer (initial concentration 10 µM), 30 ng of DNA template, and PCR-grade water. For the 28S rRNA gene and ITS primers, the PCR program consisted of an initial denaturation at 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute, with a final extension at 72°C for 5 minutes. For the COI gene primers, the PCR program consisted of an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 95°C for 1 minute, 40°C for 1 minute, and 72°C for 1.5 minutes, with a final extension at 72°C for 7 minutes. For 16S amplification, the PCR program consisted of 30 cycles of 95°C for 1 minute and 30 seconds, 55°C for 1 minute and 30 seconds, and 72°C for 1 minute and 30 seconds, with a final extension at 72°C for 7 minutes. PCR products were confirmed through gel electrophoresis on 1.5% TBE gels stained with ethidium bromide (10 mg/ml) and visualized under UV light.

Sequencing and Bioinformatic analysis: To assign taxonomy to sponges and culturable isolates, PCR products were sequenced by the BioAnalytical Services Laboratory (BAS Lab) at the Institute of Marine and Environmental Technology (IMET). Sequence chromatograms were analyzed with CLC Main Workbench, trimmed based on default quality parameters, and aligned to nearest relatives with NCBI BLASTn. For bacterial community analysis, DNA was sequenced on an Illumina paired-end platform (MiSeq 2 x 300) at the BAS Lab at IMET. *L. chondrodes* 1 samples were sequenced October 28, 2019. *H. heliophila* 1 & 2 and *L. chondrodes* 2 samples were sequenced on February 27, 2021. Sequence reads were analyzed using the QIIME2 pipeline and trimmed based on a Phred-quality score of 20 (99% base calling accuracy). Sequence quality control and Feature Table Construction was completed with DADA2 (offered as a plug-in with Qiime2). Taxonomy was assigned (≥ 99% similarity) using the Naive Bayes classifier trained from the newest Silva database (version 132) that had been trained on the V4 region of 16S (515F/806R).

Table 2.1 Primers used for the amplification of eukaryotic and prokaryotic barcode genes to identify sponges and symbionts. Within the

Primer Name	Nucleotide Sequence	Target Region	Organism(s)	Origin
RA2_keratose (forward)	**5' GRA TGG TTT AGT GAG ATC TT 3'**	**ITS**	***L. chondrodes***	**Galitz et al 2018**
ITS2.2_keratose (reverse)	**5' AAA TTC AGC GGG TAG YCT GG 3'**	**ITS**	***L. chondrodes***	**Galitz et al 2018**
RA2 (forward)	**5' GTC CCT GCC CTT TGT ACA CA 3'**	**ITS**	***L. chondrodes H. heliophila***	**Wörheide 1998**
ITS2.2 (reverse)	**5' CCT GGT TAG TTT CTT TTC CTC CGC 3'**	**ITS**	***L. chondrodes H. heliophila***	**Wörheide 1998**
28S-C2_keratose (forward)	**5' GAA AAG AAC TTT GRA RAG AGA GTC 3'**	**28S**	***L. chondrodes***	**Galitz et al 2018**
28S-D2_keratose (reverse)	**5' CCG TGT TTC AAG ACG GGT CGR ACG AG 3'**	**28S**	***L. chondrodes***	**Galitz et al 2018**
28S-C2 (forward)	**5' GAA AAG AAC TTT GRA RAG AGA GT 3'**	**28S**	***L. chondrodes H. heliophila***	**Chombard, Boury-Esnault & Tillier 1998**
28S-D2 (reverse)	**5' TCC GTG TTT CAA GAC GGG 3'**	**28S**	***L. chondrodes H. heliophila***	**Chombard, Boury-Esnault & Tillier 1998**

LCO1490 (forward)	**5' GGT CAA ATC ATA AAG ATA TTG G 3'**	**COI**	***H. heliophila***	**Folmer et al 1994**
HC02198 (reverse)	**5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3'**	**COI**	***H. heliophila***	**Folmer et al 1994**
27F (forward)	**5′ AGA GTT TGA TCM TGG CTC AG 3'**	**16S nearly full length**	**Sponge symbiont**	**Lane 1991; Stackebrandt & Liesack 1993**
1492R (reverse)	**5′ TAC GGY TAC CTT GTT ACG ACT T 3'**	**16S nearly full length**	**Sponge symbiont**	**Lane 1991; Stackebrandt & Liesack 1993**
V3-V4 16S (forward)	**5' TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG *CCT ACG GGN GGC WGC AG* 3'**	**V3-V4 region 16S**	**Sponge holobiont**	**Illumina**
V3-V4 16S (reverse)	**5' GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G*GA CTA CHV GGG TAT CTA ATC C* 3'**	**V3-V4 region 16S**	**Sponge holobiont**	**Illumina**

2.4 Results

2.4.1 Confirmation of the identities of *L. chondrodes and H. heliophila*

Using three eukaryotic barcodes—28S, ITS, & COI—sponge identities were confirmed. Fragments of the 28S rRNA and ITS genes were successfully amplified and matched with 100% identity to *L. chondrodes* (LR69935.1, LS974451.1). I used two primer sets for each barcode, one that was designed for keratose sponges and one that was not (Table 2.1). To confirm the identity of *H. heliophila*, I did not use the primers designed for keratose sponges as *H. heliophila* does not belong to this subclass. I originally used the 28S rRNA gene primer set, 28-C2 (forward) and 28S-D2 (reverse), which is the one that I had success with previously with *L. chondrodes.* However, the nearest BLAST alignment with this primer set was a segment of the 26S rRNA gene of *Hymeniacidon perlevis* (MF685334.1). I realized that there was no reference 28S rRNA gene sequence of *H. heliophila* in GenBank. I then used the same ITS primer set that I used for *L. chondrodes*, RA2-fwd and ITS2.2-rev, which aligned with ITS of *H. heliophila.* For confirmation, I used a primer set for the CO1 barcode gene, LCO1490 (forward) and HC02198 (reverse) and had a 100% identity alignment with a fragment of the CO1 gene in *H. heliophila* (EF519629.1)

2.4.2. Identification of the culturable microbial isolates from *L. chondrodes* and *H. heliophila*

Using the universal bacterial primer set 27F/1492R, culturable isolates were identified. Heterotrophic symbionts were isolated on marine agar 2216 and

autotrophic symbionts were isolated using marine BG11 and SN15. One autotrophic symbiont isolated from *H. heliophila* was identified as a benthic foraminiferan, *Glabratella* sp.; in this case the 16S rRNA gene fragment amplified is from mitochondria within the foraminiferan. Four heterotrophic symbionts were isolated from *H. heliophila*. These isolates are from the genera *Pseudoalteromonas, Bacillus*, *Flexibacter,* and *Simidua* (Table 2.2). Three autotrophic symbionts and nine heterotrophic isolates were cultured from *L. chondrodes* (Table 2.3). The three autotrophic isolates consisted of *Cyanobium* sp., a unicellular cyanobacterium and *Pseudanabaena* sp., a filamentous cyanobacterium, and a cultured cyanobacterium designated W_7, the closest relative of which is a previously uncultured cyanobacterium. Of the nine heterotrophic symbionts, two isolates are in the genus *Pseudoalteromonas* and seven belong to the genus *Bacillus*.

Table 2.2 Culturable heterotrophic and autotrophic isolates from *H. heliophila.* MA= marine agar 2216

SAMPLEID	NEAREST BLAST ALIGNMENT (ACCESSION #)	IDENTITY (%)	COVERAGE (%)	MEDIA	AUTOTROPHIC (A) OR HETEROTROPHIC (H)
Symb1	*Flexibacter* sp. (FJ457296.1)	97	95	MA2216	H
Symb2	*Pseudoalteromonas* sp. (MN889154.1)	99	99	MA2216	H
Symb3	*Bacillus* sp. (MK415704.1)	98	98.58	MA2116	H
Symb4	*Simiduia* sp. (NR_134172.1)	95	95	MA2216	H
AT_SN15_2	*Glabratella* sp. (JQ580013.1)	97	98	SN15	A

Table 2.3 Culturable heterotrophic and autotrophic isolates from *L. chondrodes*. MA= marine agar 2216

SAMPLE ID	NEAREST BLAST ALIGNMENT (ACCESSION #)	IDENTITY (%)	COVERAGE (%)	MEDIA	AUTOTROPHIC (A) OR HETEROTROPHIC (H)
W_5	*Cyanobium* sp. (KC695842.1)	99	69	SN15	A
W_6	*Pseudanabaena* sp. (JQ9273541.1)	98	98	SN15	A
W_7	Uncult. Cyanobacterium (KF741498.1)	98	98.58	SN15	A
LC_1	*Bacillus* sp. (MK634591.1)	99	99	MA2216	H
LC_2	*Pseudoalteromonas* sp. (MN889154.1)	99	99	MA2216	H
LC_3	*Bacillus* sp. (MN232164.1)	98	99	MA2216	H
LC_4	*Bacillus* sp. (KU721999.1)	99	99	MA2216	H
LC_5	*Pseudoalteromonas* sp. (MF359426.1)	99	99	MA2216	H
LC_7	*Bacillus* sp. (MT176570.1)	99	99	MA2216	H
LC_8	*Bacillus* sp. (KP7206001.1)	99	99	MA2216	H
LC_9	*Bacillus* sp. (MH921588.1)	99	99	MA2216	H
LC_10	*Bacillus* sp. (KJ534465.1)	100	99	MA2216	H

2.4.3 Molecular characterization of the microbiomes associated with *L. chondrodes* and *H. heliophila*

In total, 194 OTUs were recovered from *H. heliophila* and *L. chondrodes* duplicate samples, representing nine bacterial phyla (Table 2.4). *H. heliophila* (1), *Hh1*, and *H. heliophila* (2), *Hh2*, are identical DNA samples sequenced in duplicate. ~75% of *Hh1* and ~78% of *Hh2* sequences passed through the filtering step. 60% of both sequence data sets then passed the merging step, resulting in 17587 (*Hh1*) and 22971 (*Hh2*) merged paired reads (Table 2.4). After OTU picking, *Hh1* had 6631 non-chimeric OTUs and *Hh2* had 9172. Alpha rarefaction plotting showed that diversity of *H. heliophila* communities was captured at a sequencing depth of ~800, as seen as a plateau (Figure 2.3). Figure 2.4 shows both replicate communities of *H. heliophila*. For clarity when looking into the microbial community further, *Hh2* was selected based on the fact that this replicate recovered more OTUs than *Hh1*, although it must be noted that the relative abundance of these two replicates was remarkably similar, indicating that either replicate could have been chosen for further analysis (Figure 2.4).

L. chondrodes (1), *Lc1*, and *L. chondrodes* (2), *Lc2*, are each identical DNA samples sequenced in duplicate. However, *Lc1* was sequenced in October 2019 and *Lc2* was sequenced with *Hh1* and *Hh2* in February 2021. Both *Lc1* and *Lc2* passed through the same filtering and merge steps but had a larger discrepancy of sequence loss. ~23% of *Lc1* and ~74% of *Lc2* sequences passed through the filtering step. ~22% of *Lc1* and ~70% of *Lc2* passed the merging step, resulting in 92644 (*Lc1*) and

20363 (*Hh2*) merged paired reads (Table 2.4). After OTU picking, *Lc1* had 25672 non-chimeric OTUs and *Lc2* had 6563. Alpha rarefaction plotting shows that the diversity of *L. chondrodes* communities was approaching being captured at a sequencing depth of ~2000, as seen as a flattening curve in Figure 2.3. Because of this rarefaction analysis, all sponge sequences were rarified to an even depth of 2000. Regardless of sequencing differences, both *L. chondrodes* replicate communities look nearly identical (Figure 2.4). For clarity when looking into the microbial community further, *Lc1* was selected based on the fact that the sequencing round in October 2019 recovered more OTUs than *Lc2* in February 2019.

2.4.4 Microbial community diversity and composition of *L. chondrodes* and *H. heliophila*

The *H. heliophila* community included nine bacterial phyla, but only three were dominant: Proteobacteria (95%), Bacteroidetes (3%), and Cyanobacteria (1%) (Figure 2.5). Other phyla that were present but made up <0.2% of the community in *H. heliophila* were Spirochaetes, Planctomycetes, Firmicutes, Chlamydiae, and Verrucomicrobia. Proteobacteria dominate the *H. heliophila* bacterial community; within this phylum, the relative abundance of Alphaproteobacteria was the highest at 95% with Gammaproteobacteria making up the remaining ~ 5%. Deltaproteobacteria were also present but made up >0.5% of the Proteobacteria community. 95% of Alphaproteobacterial sequences were found to be Rhizobiaceae and ~1% were found to be Rhodobacteraceae. *L. chondrodes* had a less diverse assemblage of bacteria than *H. heliophila*, with three phyla being recovered: Cyanobacteria (68%),

Proteobacteria (30%), and Actinobacteria (2%). Cyanobacteria are the predominant taxa found in *L. chondrodes*, and interestingly, >99% of Cyanobacteria sequences are made up of one species, *Synechococcus spongarium*. Protobacteria communities consist of Alphaproteobacteria (53%), Gammaproteobacteria (45%), and Deltaproteobacteria (2%). SAR11 (~81%) dominates the Alphaproteobacteria, and Rhodobacteraceae makes up ~15%.

Table 2.4 Sequence quantities at trimming, filtering, and merging steps of pipeline using QIIME2 with merging of paired reads using DADA2 plugin.

Sponge	# of sequences after trimming	# of sequence after filtering	# of merged paired reads	# of OTUs
H. heliophila (1)	29326	22100	17587	6631
H. heliophila (2)	37867	29364	22971	9172
L. chondrodes (1)	417873	96250	92644	25672
L. chondrodes (2)	28795	21405	20363	6563

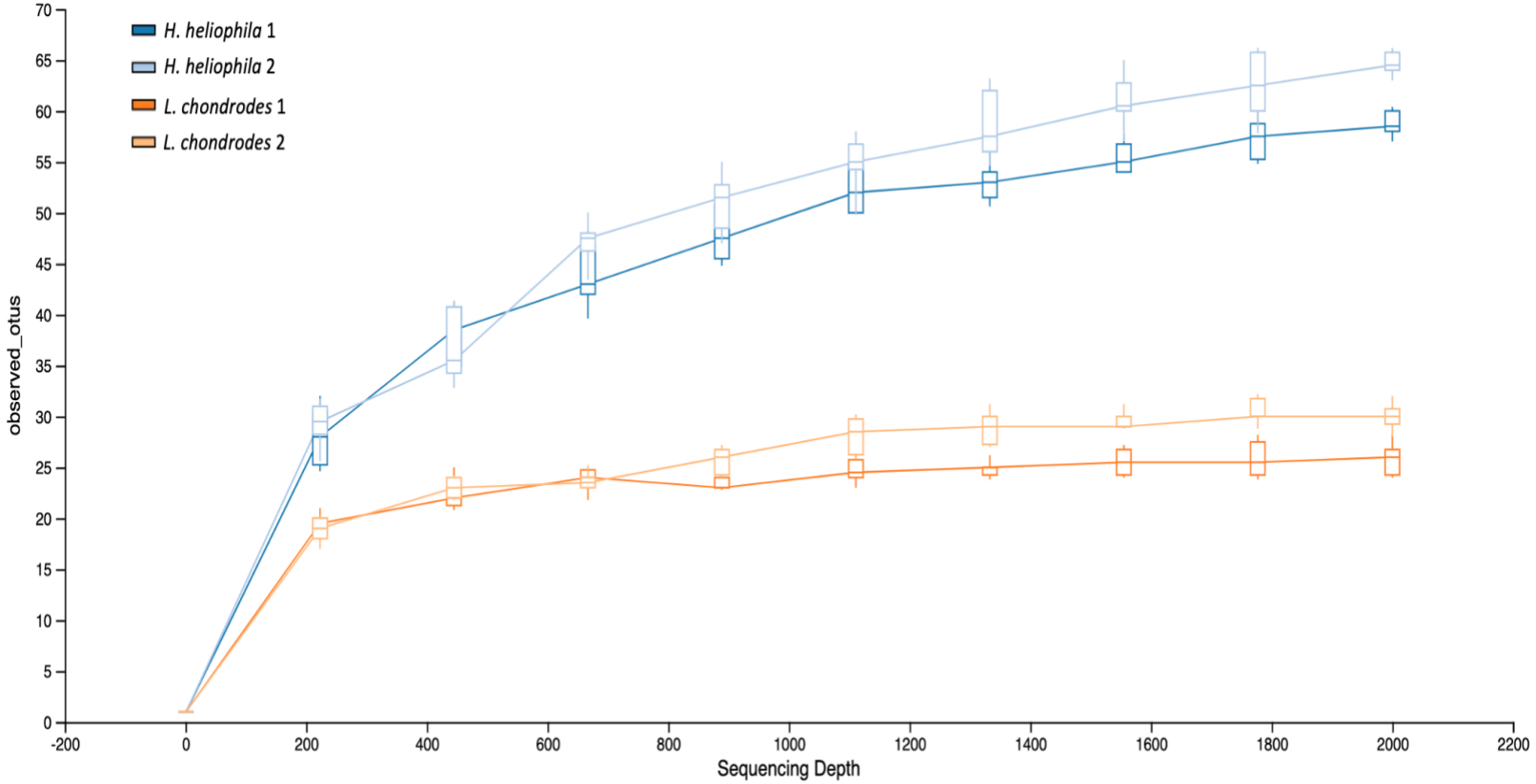


Figure 2.3 Alpha rarefaction curve showing the relationship between observed OTUs and sampling depth.

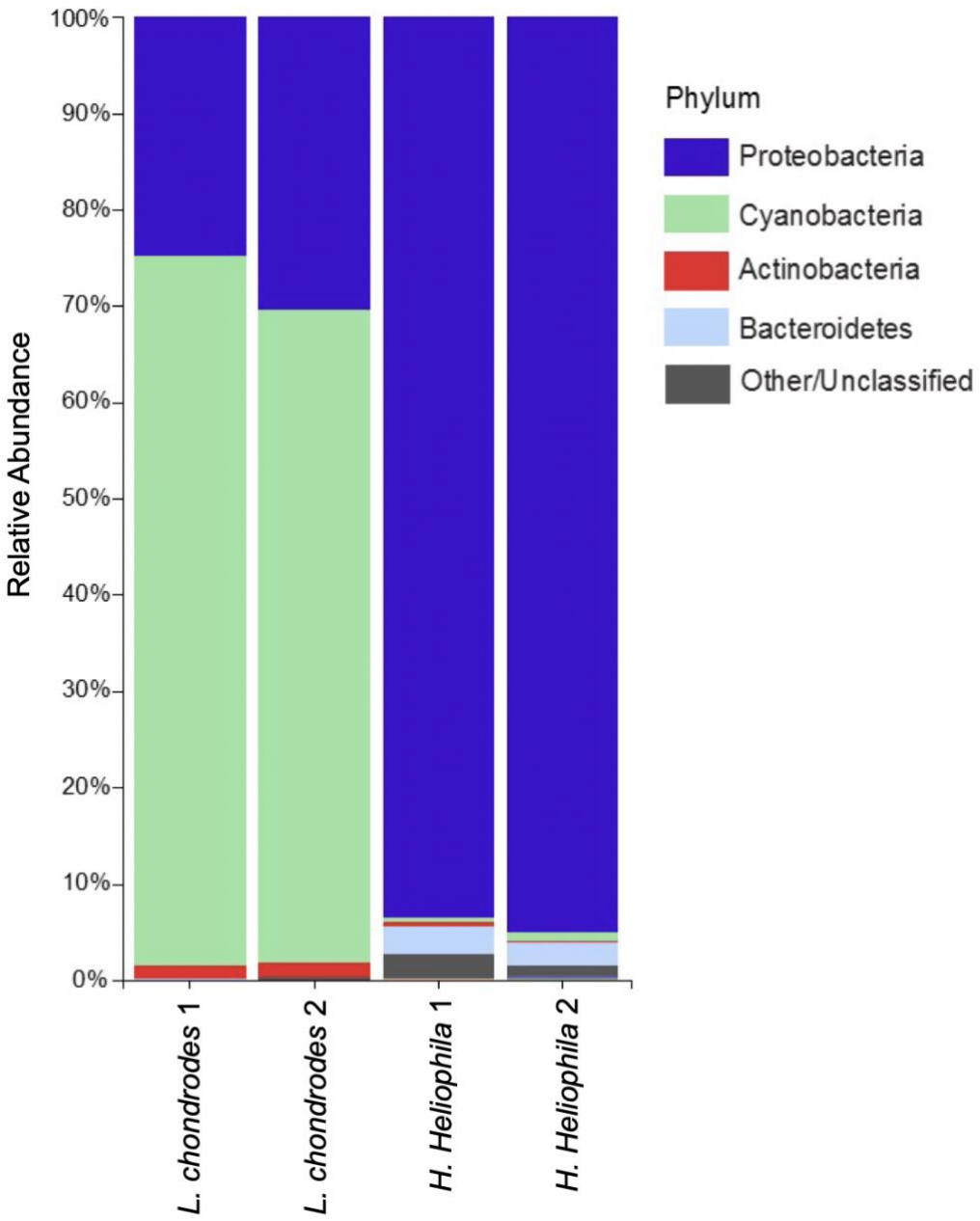


Figure 2.4 Relative abundance of phyla in communities of *L. chondrodes* and *H. heliophila* based on analysis of the V3/V4 region of 16SrRNA. Both sponge communities were sequenced in duplicate. Phyla not shown (<0.2% of the community in *H. heliophila*): Spirochaetes, Planctomycetes, Firmicutes, Chlamydiae, and Verrucomicrobia.

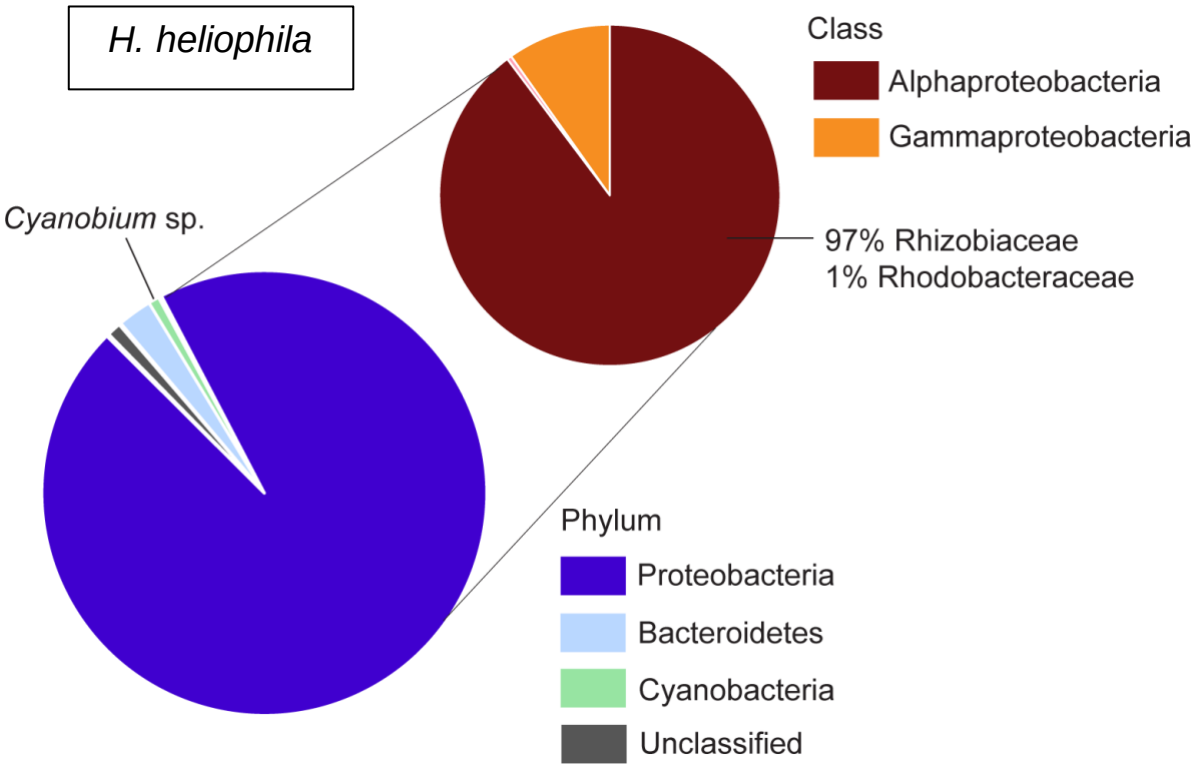


Figure 2.5. Relative abundance of *H. heliophila* (2) bacterial communities. Phylum level is comprised of Proteobacteria (95%), Bacteroidetes (3%), Cyanobacteria (1%), and Unclassified/other (1%). Phyla not shown (<0.2% of the community in *H. heliophila*): Spirochaetes, Planctomycetes, Firmicutes, Chlamydiae, and Verrucomicrobia. Cyanobacteria was comprised solely of *Cyanobium* sp. Protobacteria are broken down to class level and are comprised of Alphaproteobacteria (~90%), Gammaproteobacteria (~10%), and Deltaproteobacteria (not shown in legend, >0.5%). Rhizobiaceae and Rhodobacteraceae make up the Alphaproteobacteria, 97% and 1% respectively.

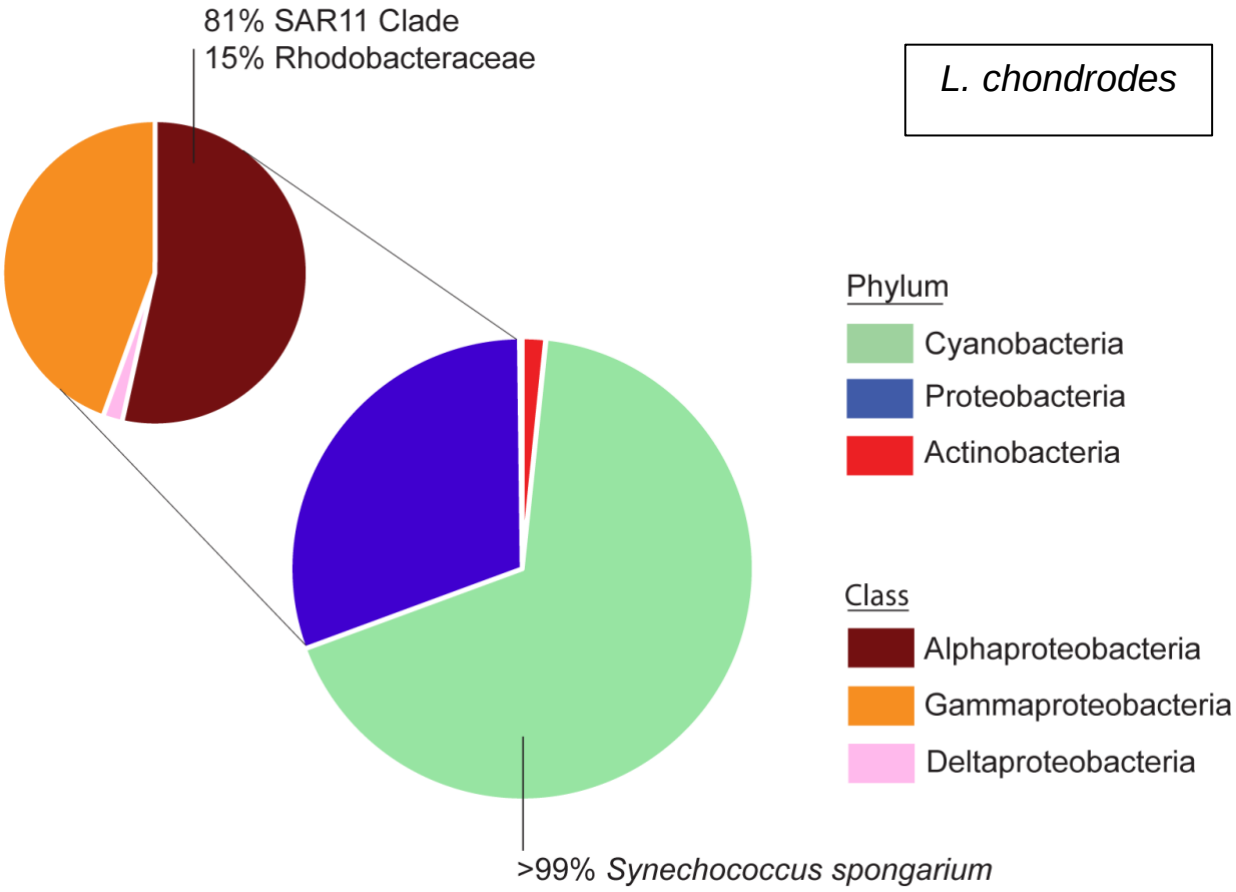


Figure 2.6. Relative abundance of *L. chondrodes* (1) bacterial communities. Phylum level is comprised of Cyanobacteria (68%), Proteobacteria (30%), and Actinobacteria (2%). >99% of Cyanobacteria sequences are made up of species *Synechococcus spongarium.* Protobacteria are broken down to class level and are comprised of Alphaproteobacteria (53%), Gammaproteobacteria (45%), and Deltaproteobacteria (2%). SAR11 and Rhodobacteraceae dominate the Alphaproteobacteria, making up 81% and 15% respectively.

2.5 Discussion

2.5.1 Analysis of the dominant microbial members of *H. heliophila*

Overall, *H. heliophila* hosted a microbial community dominated by the bacterial phylum Proteobacteria, specifically the class Alphaproteobacteria (Figure 2.5). Proteobacteria, (especially Alpha- Delta- and Gammaproteobacteria) are commonly the most abundant and diverse bacteria phylum in sponges (Hentschel et al. 2002; Webster et al. 2010; Schmitt et al. 2012; Easson & Thacker 2014) and many isolates of Alphaproteobacteria have been cultured from sponges (Webster & Hill 2001; Enticknap et al. 2006). Previously characterized symbiont communities of *H. heliophila* have had a comparable community structure to that found in this study, all being dominated by Alphaproteobacteria and including symbionts affiliated with Beta- and Gammaproteobacteria, and Cyanobacteria (Turque et al. 2008; Erwin et al. 2011; Weigel & Erwin 2016, 2017; Coelho et al. 2018). Some taxa (such as Firmicutes and Actinobacteria) are present in previously studied communities, but are not found in this study. Further, a previous study found *H. heliophila* to contain 48 bacterial phyla, compared to our study which found nine (Weigel & Erwin 2016). The most abundant phyla were Proteobacteria (67%), Planctomycetes (8%), and Cyanobacteria (5%) (Weigel & Erwin 2016), compared with my findings of Proteobacteria (95%), Bacteroidetes (3%), and Cyanobacteria (1%). When comparing subtidal vs. intertidal individuals of *H. heliophila*, Proteobacteria and Cyanobacteria displayed a significantly greater relative abundance in intertidal sponges (Weigel & Erwin 2016). However, the sponges from Sinepuxent Bay in this thesis are subtidal and Cyanobacteria made up only 1% of the community. Planctomycetes were found

within *H. heliophila* communities in this study, but at a relative abundance of <0.2% (Figure 2.4). This discrepancy is most likely due to sequencing differences as this study used different primers and bioinformatic analysis. While *H. heliophila* sponges from separate locations have been shown to have similar communities (Erwin et al. 2011), there has been evidence of varying relative abundances of dominant taxa. For instance, a recent investigation showed that many phyla (including Cyanobacteria) displayed significantly different abundances when sampled from the same intertidal zone in May and July of the same year (Weigel & Erwin 2017). In all, the composition of the communities of *H. heliophila* in this thesis have a bacterial structure generally consistent with those previously characterized. The same cannot be said for archaeal structure. Three studies have amplified archaeal symbionts from *H. heliophila* using archaeal-specific primers (Turque et al. 2010) or universal bacterial/archaeal primers (Weigel & Erwin 2016; Coelho et al. 2018); I used universal bacterial primers (Table 2.1) and did not amplify archaeal sequences from *H. heliophila*.

Some of the core bacteria in *H. heliophila* have been shown to exhibit diverse metabolic strategies that may benefit the sponge host, for example, Proteobacteria as nitrogen cycling taxa (Weigel & Erwin 2017). Using PICRUSt predicted average gene counts, Weigel and Erwin (2017) discovered among *H. heliophila* symbionts, there was potential for all known genes in the nitrogen cycle, with ammonia assimilation having the highest predicted gene count (Weigel & Erwin 2017, Figure 4). Proteobacteria was the dominant phylum within each nitrogen transformation. Within this study, the Alphaproteobacteria class was dominated (97%) by

Rhizobiaceae, a class containing many well-known diazotrophs in the root nodules of legumes (Zakhia & De Lajudie 2001). One species of interest that may contribute to phosphorus cycling in *H. heliophila* is a member of the SAR11 group—strain HIMB114. This strain was shown to rely on a component of the organic phosphorus pool to support growth as it took up DOP even when P_i was available in its media (Grant et al. 2019). However, this strain was found at low abundance within my *H. heliophila* communities, making up only ~0.4% of the entire community.

Another interesting taxon, *Cyanobium* sp., present in the *H. heliophila* community has been studied in the context of phosphate uptake. *Cyanobium* belongs to a clade of picocyanobacteria that are unable to fixation nitrogen but have been found to be a perennially dominant taxon in a eutrophic lagoon system where anthropogenic phosphate inputs are high (Albrecht et al. 2017; Berthold & Schumann 2020). *Cyanobium* sp. was found in both of the sponges in this study. This species was present in the metagenomic community of *H. heliophila* but was not cultured (Figure 2.5). The opposite is true for *L. chondrodes*, *Cyanobium* sp. having been cultured from sponge tissue, but not found within the community amplicon sequences (Table 2.3). Of the cultured symbionts of *H. heliophila* (Table 2.2), none are major players within microbially mediated nutrient cycles in sponges. For instance, while *Flexibacter* was found to be one of the dominant taxa in the microbial community of a biological phosphorus removal reactor, poly-P granules were accumulated in the other predominant bacteria (Beta- and Gamma- Proteobacteria), but not *Flexibacter* (Liu et al. 2001). Because the cultured isolates may be only minor players in the total

microbiomes, it is more meaningful to focus on the taxa revealed through metagenomic analysis when speculating about their potential roles in *H. heliophila*.

2.5.2 *L. chondrodes* hosts noteworthy cyanobacterial symbionts.

Of the twelve isolates cultured from *L. chondrodes*, three were autotrophic species of cyanobacteria (Table 2.3). As stated previously, *Cyanobium* sp. was cultured but did not show up in the metagenomic analysis. This incongruity could be due to many factors. For instance, extracting DNA from certain microbial cells can be very difficult. Some cells are harder to lyse and as such may be left out of the amplified DNA pool during PCR (Yuan et al. 2012; Sohrabi et al. 2016). Microbiome analysis may not accurately reflect total microbial communities due to bias resulting from "harder-to-lyse" species being captured with lower efficiency. Another organism that was cultured, but not detected in the metagenomic analysis was *Pseudanabaena* sp. *Pseudanabaena* sp. was found in the same eutrophic lake system as *Cyanobium* sp. (Albrecht et al. 2017). Similar to *Cyanobium* sp., *Pseudanabaena* sp. is a non-heterocystous filamentous species of cyanobacteria (Acinas et al. 2008). *Pseudanabaena* sp. likely contributes to the phosphorus cycling capacity of *L. chondrodes.* A *Pseudanabaena* species was found to be an efficient assimilator of phosphorus from wastewater, even within waters with a high N:P ratio (Liu & Vyverman 2015). Further, observation of *Limnothrix/Pseudanabaena* sp. cells with TEM showed a large accumulation of poly-P right before the cells underwent autolysis (Kitazaki et al. 2013). TEM images of *Pseudanabaena rutilus–viridis* also show some accumulation of poly-P granules, but the implications of this phosphorus

storage in this species has not yet been elaborated (Kling et al. 2012). *Ppk* has also been found within *Pseudanabaena* sp. and will be discussed further in Chapter 4 (Figure 4.2). Due to the fact that most sponge symbionts remain unable to be cultured, their functions have largely been inferred from metagenomic and metatranscriptomic analysis (Taylor et al. 2008; Thacker & Freeman 2012)

From my metagenomic analysis of *L. chondrodes*, *Synechococcus spongarium* is the dominant cyanobacterial symbiont, making up ~68% of the entire community (Figure 2.6). This high abundance, but low richness of cyanobacteria is consistent with the findings of Vargas et al. (2020). *S. spongarium* is a widespread taxon among sponge communities and is the largest sponge-specific cluster (Usher et al. 2004; Simister et al. 2012). Considering this species has not yet been amplified in a free-living state, it seems that it is solely vertically transmitted and is an important taxon passed down through various sponge lineages (Usher et al. 2005). Due to the fact that representatives of *S. spongiarum* across various geographical habitats show little genetic variation, most research has been focused on better understanding their phylogeny and that of other sponge-associated cyanobacteria (Usher et al. 2004; Oren et al. 2005; Steindler et al. 2005; Erwin & Thacker 2007, 2008b; Burgsdorf et al. 2015; Steinert et al. 2016; Slaby & Hentschel 2017). Another focus has been on understanding how sponges are able to discriminate between bacteria cells as a food source or as acquired symbionts. *S. spongiarum* lacks the O-antigen of LPS, which may enable the cells to remain symbionts instead of being lysed or engulfed as a food source (Lerouge & Vanderleyden 2002; Burgsdorf et al. 2015). Although cyanobacterial symbionts, most commonly *Synechococcus*, have been found to major

contributors of carbon to several different sponges (Erwin & Thacker 2007; Usher 2008), no studies to my knowledge have shown *S. spongarium* specifically contributing to the nitrogen or phosphorus budget of sponges. In fact, this symbiont may depend on the sponge host for nutrition as genes involved in the biosynthesis of methionine and spermidine were lost in *S. spongiarum* strain SH4 in the Red Sea sponge *Carteriospongia foliascens* (Gao et al. 2014). A SAR11 strain HIMB100 was the dominant member of SAR11 of the *L. chondrodes* communities; in fact, HIMB100 sequences made up ~10% of the entire bacterial community. However, no studies to my knowledge have conducted phosphorus intake studies with this particular strain.

After careful analysis of the culturable and uncultured taxa present within *H. heliophila* and *L. chondrodes*, I have singled out three bacteria that may contribute to enhanced phosphorus cycling: SAR11 strain HIMB114 in *H. heliophila*, *Pseudanabaena* sp. in *L. chondrodes*, and *Cyanobium* sp. found in both (sequenced from *H. heliophila*, cultured from *L. chondrodes*). While there are many other interesting bacteria within both communities, only those studied in the context of sponge functioning or nutrient cycling have been discussed in detail here.

Chapter 3: The first study of phosphorus uptake by marine sponges using ^{32}P isotope tracing

3.1 Abstract

Marine sponges thrive within oligotrophic coral reefs and it has long been of interest to understand how these organisms are able to obtain sufficient nutrients when the concentrations of dissolved carbon, nitrogen, and phosphorus in the surrounding water are so low. It is now known that sponge holobionts make important contributions to coral reef biogeochemical cycling by facilitating intake and release of carbon and nitrogen. However, sponges' role in phosphorus biogeochemistry has been hardly studied. I present here the first-ever phosphorus tracing study with marine sponges. I aimed to determine if sponges take up phosphorus from ambient dissolved inorganic phosphate (DIP), dissolved organic phosphorus (DOP), or both. Uptake of phosphorus by the sponges, *Lendenfeldia chondrodes* and *Hymeniacidon heliophila*, was determined by tracing with the radiolabeled isotope, ^{32}P, within a closed aquarium system over 78-hours. Specifically, ^{32}P-labeled phosphate ($^{32}P_i$) was used to trace uptake of DIP and ^{32}P-labeled ATP $\gamma^{32}P$[ATP] was used to trace uptake of DOP. Both *L. chondrodes* and *H. heliophila* took up DIP and DOP in all six experiments that I performed. For both sponges, ATP was taken up in larger amounts and at higher rates than P_i. ATP uptake rates were rapid within the first six hours, followed by a plateau. Ultimately, both sponges took in DOP at a higher rate, but DIP uptake was more consistent over time. This study is the first to show two sponge holobionts directly taking up DIP and DOP from the water column. Gaining a new

understanding of marine sponges' role in phosphorus biogeochemistry is an important step forward for interpreting the high biodiversity of organisms found within oligotrophic coral reefs.

3.2 Introduction

3.2.1 Forms of dissolved phosphorus and its nomenclature

Total dissolved phosphorus (TDP) is generally organized into two categories: dissolved inorganic phosphate (DIP) and dissolved organic phosphorus (DOP). Phosphorus is also present as particulate organic phosphorus (POP), either contained within living cells such as planktonic microorganisms or in the water column as detritus. DIP is commonly referred to as soluble reactive phosphorus (SRP) and includes PO_4^{3-} (orthophosphate, P_i), HPO_4^{2-}, and $H_2PO_4^-$. However, SRP is not a direct synonym for DIP. SRP is an operational definition that primarily includes orthophosphate, but may also include some DOP species. The DIP category can also include both insoluble, particulate phosphorus (apatite), non-reactive phosphorus (calcium phosphate, polyphosphate), or both (aluminum phosphate) (Burton 1973). DOP consists of non-reactive phosphorus molecules that are almost entirely bioavailable such as phosphorus present in DNA, RNA, and ATP. DOP also includes recalcitrant humic substances. DOP is often termed soluble non-reactive phosphorus (SNP). Again, this synonym is not necessarily congruent with DOP because inorganic molecules such as polyphosphates can fall under the category of SNP (Thomson-Bulldis & Karl 1998). For sake of clarity, I will use the DIP and DOP distinction between dissolved phosphorus species. Regardless of nomenclature, phosphorus is widely known to be the major limiting factor within many aquatic systems and in response, many techniques have been developed to attempt to characterize phosphorus fluxes in the ocean.

3.2.2 The current state of phosphorus tracing within aquatic systems

Research on phosphorus biogeochemistry in marine systems has largely been focused on the roles of planktonic microorganisms in the open ocean. Within the water column, microbes have developed strategies to capitalize on varying and often limited concentrations of phosphorus (Dyhrman et al. 2007). For example, both *Prochlorococcus* and *Synechococcus*—two ubiquitous microbes within phosphorus-limited waters—were found to substitute sulfur for phosphorus within their lipid membranes (Van Mooy et al. 2006). This sulfur substitution may be an important adaptation to free up phosphorus for other important cellular processes such as ATP synthesis. Phosphorus concentrations play a critical role in determining rates of primary production and community composition of ocean microorganisms, although there are several technical challenges in studying the various DIP and DOP species. One of the major challenges is that larger, organic molecules of phosphorus are not easily characterized by extraction or NMR analysis (Karl & Björkman 2002; Dyhrman et al. 2007). A second major challenge is the lack of a stable phosphorus isotope. This absence has led to a disparity between our understandings of phosphorus and other fundamental elements such as nitrogen and carbon that can be traced using $^{14}N/^{15}N$ and ^{12}C, respectively. However, researchers have be able to utilize the unstable radioisotopes of phosphorus, ^{32}P and ^{33}P, in tracing experiments.

^{32}P and ^{33}P are cosmogenic and enter the oceans predominantly as rainwater (Benitez-Nelson & Buesseler 1998) and both isotopes can act as potent tracers of phosphorus within the open ocean (Lal & Lee 1988, and subsequent studies). ^{32}P and ^{33}P can also be manufactured and these short-lived radionuclides can be utilized in

controlled laboratory experiments.[3] ^{32}P has most widely been used as a biochemical marker in marine ecotoxicology to provide insights into the impacts of radiation contamination. Both marine and freshwater bivalves are key indicator organisms and have been shown to accumulate high ^{32}P concentrations within their digestive glands after a controlled exposure (Vernon et al. 2018). A common approach to link xenobiotic exposures in waterways to detrimental health effects is ^{32}P-postlabeling analysis for the detection of carcinogen-DNA adducts in marine mammals, fish, and whales (Ray et al 1991; Stein et al. 1994; Reichert et al. 1999; Phillips & Arlt 2007). ^{32}P has additionally been used to study phosphorus assimilation efficiency and nutrition by feeding ^{32}P-spiked food to insect larvae (Giguere 1981), fish (Winpenny et al. 1998), and marine copepods (Reinfeld & Fisher 1991). One study used a combined approach with ^{14}C and ^{32}P to study the potential nutritional benefits between the coral *Millepora dichotoma* with the symbiotic barnacle, *Savignium milleporum* (Cook et al. 1991).

Most tracing studies using ^{32}P have been done on easily contained and controlled systems such as soil and water samples, root systems, plants, microbes, and tissues (reviewed by Smith et al. 2011). To study reef systems specifically, Atkinson and Smith (1987) added $^{32}P_i$ directly into the water of a reef flat and also into aquarium tanks with subsamples of the same reef water. Water was filtered to determine if suspended particulate matter took up significant amounts of ^{32}P. Phosphorus uptake rates from the aquarium subsamples were found to be proportional to the net rate of uptake measured in the field for the entire reef flat (Atkinson 1987a;

[3] ^{32}P half-life: 14.3 days; ^{33}P half-life: 25.4 days (Perkin Elmer)

Atkinson & Smith 1987). Similarly, coral (both living individuals as well as microbe-covered coral rubble and sand) was found to take in and release $^{32}P_i$ (Sorokin 1999). From the literature it can be inferred that the majority of phosphorus cycling research has been conducted with planktonic microorganisms primarily in the open ocean and in some cases, reef flats. There have been substantially fewer studies that have sought to enhance understanding of the processes whereby symbiotic microbes take up and release phosphorus. A handful of studies have used ^{32}P tracing to show that zooxanthellae symbionts of corals (D'Elia 1977; Deane & O'Brien 1981; Jackson & Yellowlees 1990) and foraminifera (Lee et al. 1980) are responsible for ambient P_i uptake. However, these studies primarily are conducted on isolated cultures as opposed to the full holobiont. There have been no studies of which I am aware that have utilized ^{32}P to conduct tracing studies through marine sponges and their associated symbionts.

3.3 Methods

3.3.1 Aquarium set-up and ^{32}P addition

Four aquarium tanks were used in total, three being exposed to radiation and one serving as a quarantine tank where sponges were acclimated for up to 7 days before experimentation. Three tanks were 37.8 l (commercial 10-gallon tanks) and one tank was 75.7 l (20 gallons). Each tank was placed inside a larger containment tank in accordance with radiation safety protocols to prevent spills. All tanks and equipment were kept within the laboratory's designated radiation area and labeled according to safety guidelines (Figure 3.1). Tanks were filled with artificial seawater obtained from the Aquaculture Research Center (ARC) at the Institute of Marine and Environmental Technology (IMET). Water volume was calculated from the measured water depth and tank dimensions. Each tank was exposed to ambient light and kept at 25.5°C with a submersible heater. Salinity was 33 ppt and 27 ppt for *L. chondrodes* and *H. heliophila* respectively. Before experimentation, a 50% water change was performed to minimize mineralized nutrient accumulation.

Radiolabeled P_i (carrier-free 10 mCi/ml, 1.094 µM $H_3{}^{32}PO_4$, Perkin Elmer) was used to measure DIP uptake by sponges. Depending on the volume of water in the tank, anywhere from 76–80 µl of the isotope was added to establish a final concentration of 1.36–3.58 µM. Radiolabeled adenosine 5'-triphosphate (10 mCi/ml, 3.33 µM [γ -32P], Perkin Elmer) was used to determine DOP uptake. Either 10 µl or 25 µl was added to establish a final concentration of 1.25–3.13 pM of radiolabeled phosphorus.

Either P_i or ATP was added to the tank and stirred gently. Two pairs of

gloves and safety glasses were worn and ^{32}P contamination was carefully checked with a Geiger-Muller counter to ensure safe experimentation. Small pieces of sponge tissue were excised (ca. 1 cm^3) from each of the three sponges at each time point, over a 78-hour time series. The sponge tissue samples were dried with a Kimwipe, weighed, and placed into scintillation vials containing 5 ml of Ecoscint H scintillation cocktail (National Diagnostics). The tank was then gently stirred for 10 seconds and water samples (1 ml) were collected and placed in 5 ml scintillation tubes with cocktail. Radiation was measured using a scintillation counter (Beckman LS6500) on automatic count settings for ^{32}P. To decontaminate radiation between experiments, tanks were left to sit for at least 18 weeks (ca. nine half-lives) and cleaned. Background radiation counts were taken before each experiment to ensure there was no residual radiation detectable.

3.3.2 ^{32}P Calculations

Inorganic phosphate: The concentration of pmol PO_4^{3-} g^{-1} sponge tissue/ ml^{-1} water was calculated using the specific activity of the isotope while accounting for radioactive decay. Disintegrations per minute (DPM) values were divided by sponge weights (DPM/g), 1 Curie (Ci) =$2.22*10^{12}$ DPM. DPM/g was divided by $2.22*10^{12}$ to convert DPM/g to Ci/g. To convert Ci/g to mmol PO_4^{3-} /g, values were divided by specific activity of the $H_3{}^{32}PO_4$ after the specific activity was adjusted for decay. The specific activity is specified by the supplier as of the isotope's calibration date. This was taken into consideration when calculating concentration in mass-dependent applications. The specific activity on any day prior to the calibration date can be

calculated using the decay table on the Technical Data Sheet (TDS) provided for each isotope.

Organic phosphorus: To convert Ci/g to mmol of ATP/g, values were divided by specific activity of the ATP [γ -32P] after the specific activity was adjusted for decay. The specific activity on any day *prior* to the calibration date can be calculated using the formula:

$$\mathrm{SA} = \frac{\mathrm{SA\ cal}}{\mathrm{Df} + \frac{\mathrm{SA\ cal} * (1 - \mathrm{Df})}{\mathrm{SA\ Theo}}}$$

The specific activity on any day *after* the calibration date can be calculated using the formula:

$$\mathrm{SA} = \frac{\mathrm{Df}}{\frac{1}{\mathrm{SA\ cal}} - \frac{(1 - \mathrm{Df})}{\mathrm{SA\ Theo}}}$$

Where: SA cal = 3000 Ci/mmol (from TDS), Df = fraction of radioactivity (decay chart, TDS), and SA theo = 9120 Ci/mmol.

Uptake Rates: Two time intervals are used to display rates of uptake by both sponges. The first six hours represents short-term uptake and the remaining 78 hours represents long-term uptake. Uptake rates were calculated as follows:

$$\frac{\Delta\ \mathrm{pmol\ per\ g\ sponge}}{\Delta\ \mathrm{time}}$$

This equation represents the average rate of change of phosphorus intake over either the short- or long-term time periods. The uptake rates are expressed as pmol PO_4^{3-} g^{-1} hr^{-1}. All statistical significance was determined through one-way ANOVA.

This study is the first of its kind using short-lived ^{32}P to trace phosphorus uptake by two marine sponge holobionts. ^{32}P was added either as $^{32}P_i$ to determine rates of inorganic phosphate uptake as or as $\gamma^{32}P$[ATP] to determine rates of organic phosphorus uptake. Sponges were first acclimated to aquarium tanks that mimicked their natural environments, but it must be noted that keeping sponges in aquaculture does present some challenges. For instance, sponge symbiont communities in some sponges have been found to shift after introduction into aquaculture (Mohamed et al. 2008a, 2008b).

Figure 3.1 Example of tank set up within the Hill laboratory designated radiation area. Three individuals of *H. heliophila* in a 37.8 l tank sitting within an empty containment tank labeled with radiation safety tape. A submersible heater kept water temperature at 25.5°C.

3.4 Results

3.4.1 Inorganic phosphate tracing with $^{32}P_i$

$^{32}P_i$ was found within the tissue of both *L. chondrodes* and *H. heliophila* at significantly higher concentrations than that of the surrounding water (ANOVA $p < 0.01$) (Figure 3.1), based on comparing the counts present in grams of sponge tissue and milliliters of water. The initial phosphate concentration in the experimental tanks was higher than concentrations of phosphate normally found in the natural environment. Phosphate concentrations within reefs are generally below 0.5 µM but can reach 31-fold higher during nutrient pulses after storms and increased terrestrial runoff (Atkinson 1987a; Charpy 2001; den Haan et al. 2016). Several studies have used a similar range of phosphate concentrations in controlled aquarium systems to examine the effects of elevated phosphate concentrations on coral health: 0.3–0.9 µM (low), 2–2.3 µM (medium), and 3–5.3 µM (high) (Ferrier-Pagès et al. 2000; Koop et al. 2001; Renegar & Riegl 2005). Due to radiation contamination of the tanks in my experiments, it was only safe to determine nutrient concentrations during the first round (July 2019), the second round (August 2019), and the fifth round (July 2020). The phosphate concentrations in our experimental tanks were 4.8 µM, 6.6 µM, and 8.0 µM (Table 3.1). Further, the concentration of the ^{32}P inoculum was approx. 1000-fold lower than that of the experimental tanks (1.25–3.58 pM). This low concentration of added $^{32}P_i$ was ample to trace uptake rates because ^{32}P is an energetic beta emitter which can be easily detected at low concentrations, which are safer to work with. The ^{32}P thus acts as an excellent tracer; it is naturally taken up by

the sponges along with the other unlabeled phosphate and allows us to calculate uptake rates and determine which forms of phosphorus are consumed by sponges.

To calculate uptake rates, my time series analysis was broken into two categories. Short-term uptake is classified as the first six hours of sampling and long-term uptake comprises the remaining 78 hours, during which sponges and water were sampled every 12 hours. P_i uptake in the short and long term can be seen in Table 3.2. In the first round of experimentation in July 2019, the time series was extended beyond 78 hours (Figure 3.5).

Table 3.1. Details regarding six rounds of experimentation with ^{32}P performed in 2019 and 2020. Three sponge individuals were used per round and serve as biological replicates. Tanks of two sizes were used. In Round 5, P_i and ATP tracing was done simultaneously in two tanks containing three *H. heliophila* individuals in each tank. *Initial phosphate concentration (μM) was calculated only during rounds

Round	Date	Sponge	Initial Phosphate Concentration (μM)*	^{32}P Isotope	Concentration of ^{32}P inoculum (pM)
1	July 2019	*L. chondrodes*	4.8	Pi	3.58
2	August 2019	*L. chondrodes*	6.6	Pi	1.43
3	December 2019	*L. chondrodes*	n.a.	ATP	1.25
4	January 2020	*L. chondrodes*	n.a.	ATP	3.13
5	July 2020	*H. heliophila*	8.0	Pi & ATP	1.36, 1.36
6	September 2020	*H. heliophila* *R. maritima* (plant) *H. heliophila* & *R. maritima*	n.a.	ATP	1.36, 1.36, 1.36

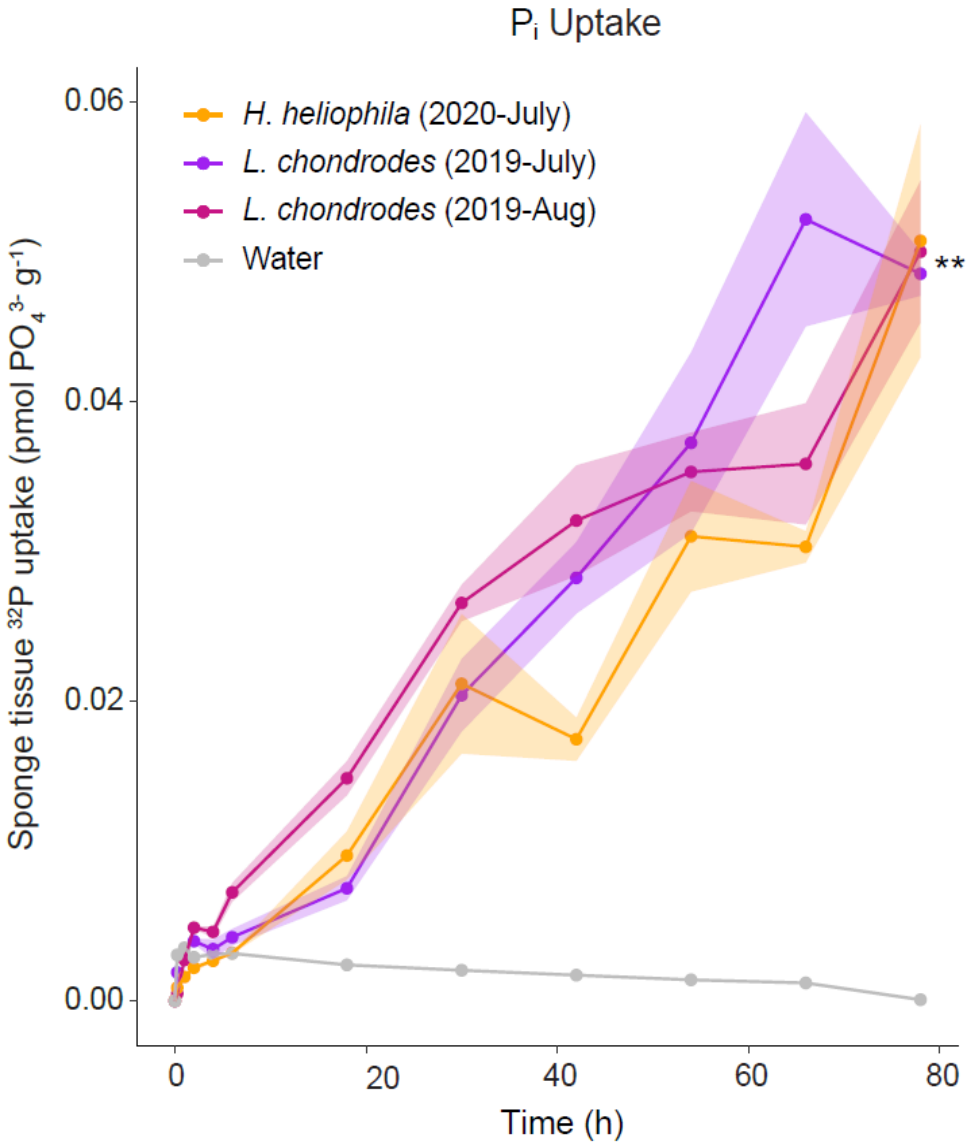


Figure 3.2 Average P_i uptake of biological replicates of *L. chondrodes* from July 2019 ( ), *L. chondrodes* from August 2019 ( ), *H. heliophila* from July 2020 ( ), and aquarium water ( ) over 78 hours. X-axis shows time in hours and Y-axis shows uptake of $^{32}P_i$ in pmol per gram of sponge tissue and per ml of water. Standard error is shown as corresponding-colored ribbons. Standard error has been calculated for the water but is too small to appear at this scale. At all time points, all three sponges contained significantly higher concentrations of P_i compared to surrounding water (**ANOVA $p < 0.01$).

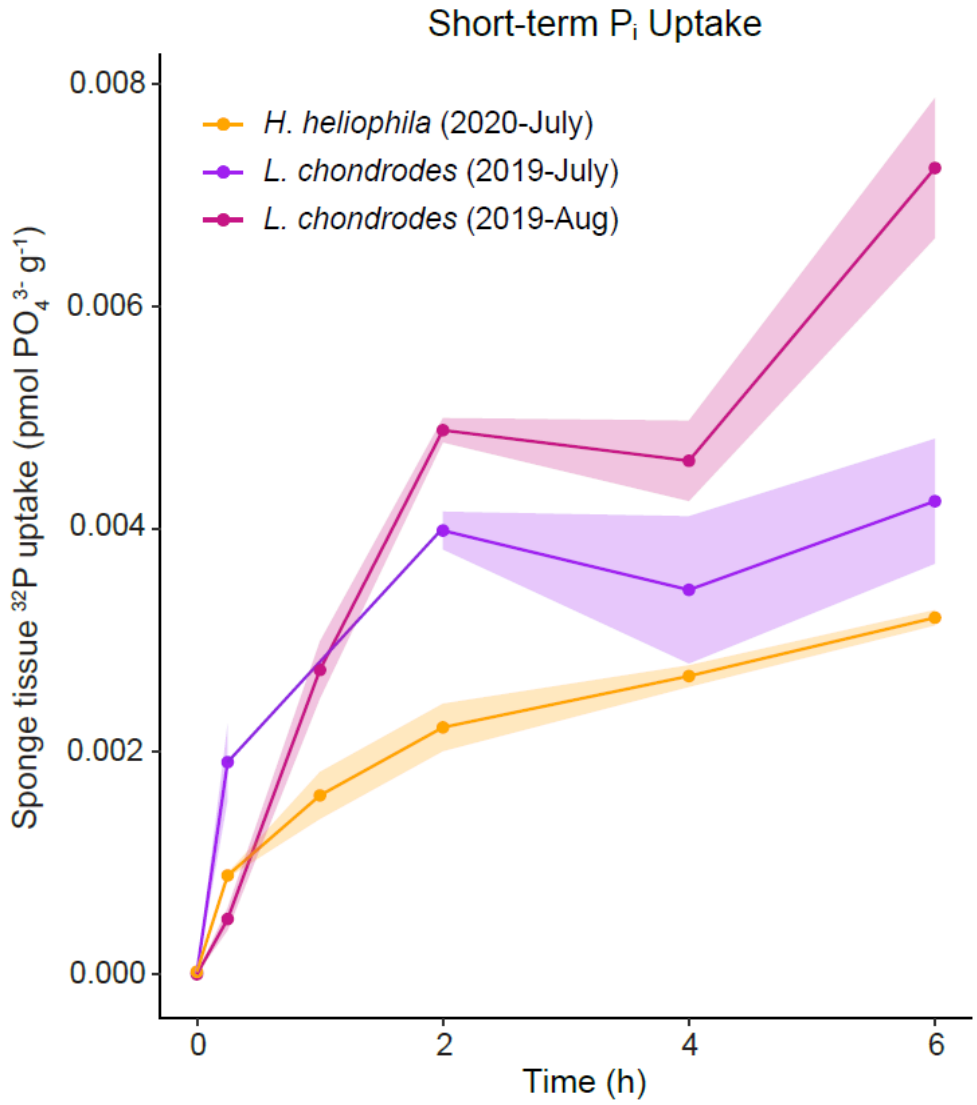


Figure 3.3 Average P_i uptake of biological replicates of *L. chondrodes* from July 2019 ( ), *L. chondrodes* from August 2019 ( ), and *H. heliophila* from July 2020 ( ) over the first six hours of experiment (short-term). X-axis shows time in hours and Y-axis shows uptake of $^{32}P_i$ in pmol per gram of sponge tissue. Standard error is shown as corresponding-colored ribbons.

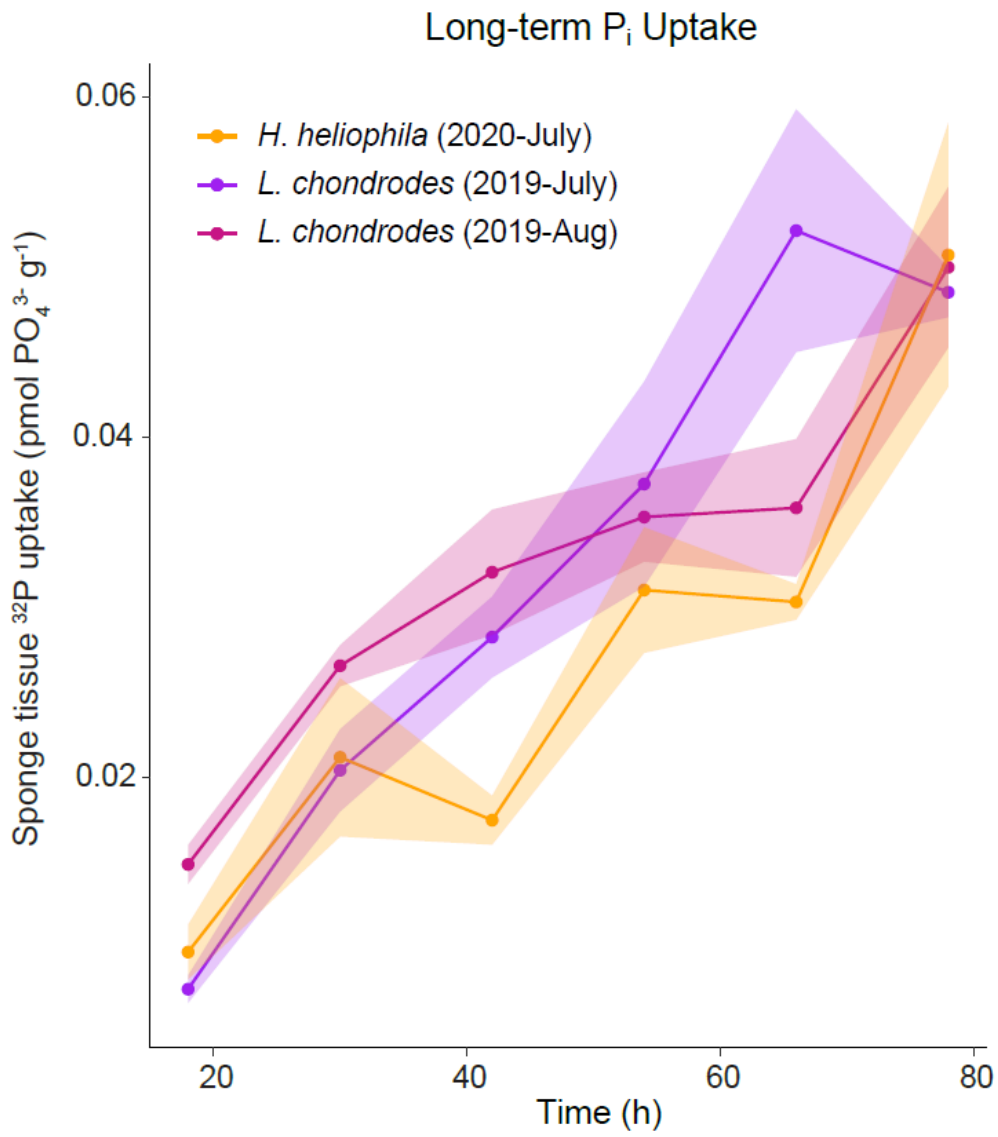


Figure 3.4 Average P_i uptake of biological replicates of *L. chondrodes* from July 2019 ( ), *L. chondrodes* from August 2019 ( ), and *H. heliophila* from July 2020 ( ) over the remaining 78 hours (long -term). X-axis shows time in hours and Y-axis shows uptake of $^{32}P_i$ in pmol per gram of sponge tissue. Standard error is shown as corresponding-colored ribbons.

Table 3.2 Short-term and long-term P_i uptake rates in *L. chondrodes* and *H. heliophila* (n = 3). Uptake rates are expressed as fmol PO_4^{3-} g^{-1} hr^{-1}.

Sponge	**Short-term uptake rate of P_i (fmol g^{-1} h^{-1})**	**Long-term uptake rate of P_i (fmol g^{-1} h^{-1})**
L. chondrodes (2019-July)	1.21	0.585
L. chondrodes (2019-Aug)	0.707	0.683
H. heliophila (2020-July)	0.530	0.683

Both *L. chondrodes* and *H. heliophila* rapidly take up $^{32}P_i$ from the water column immediately upon addition of the isotope. *L. chondrodes* takes up more P_i at a slightly higher rate (1.21 and 0.707 fmol g^{-1} h^{-1}) compared to *H. heliophila* (0.530 fmol g^{-1} h^{-1}) (Table 3.2). There is also a difference between rounds using the same sponge species. *L. chondrodes* from August 2019 took up P_i at a higher rate than *L. chondrodes* from July 2019. *L. chondrodes* and *H. heliophila* continued to take up P_i for the remaining 78 hours of the time series. The uptake rates from both sponge species became more similar to one another past the six-hour time point. No sponge takes up P_i at a significantly higher rate than the other. In fact, *L. chondrodes* from July 2019 had the same P_i uptake rate of ca. 0.7 fmol g^{-1} h^{-1} as *H. heliophila* from December 2019. Data from all three rounds show no sign of plateau, indicating that the sponges may continuously take up P_i well past the 78-hour time point. In fact, *L. chondrodes* from July 2019 continuously took up beyond 78 hours when the time series for that round was extended to three weeks (Figure 3.5).

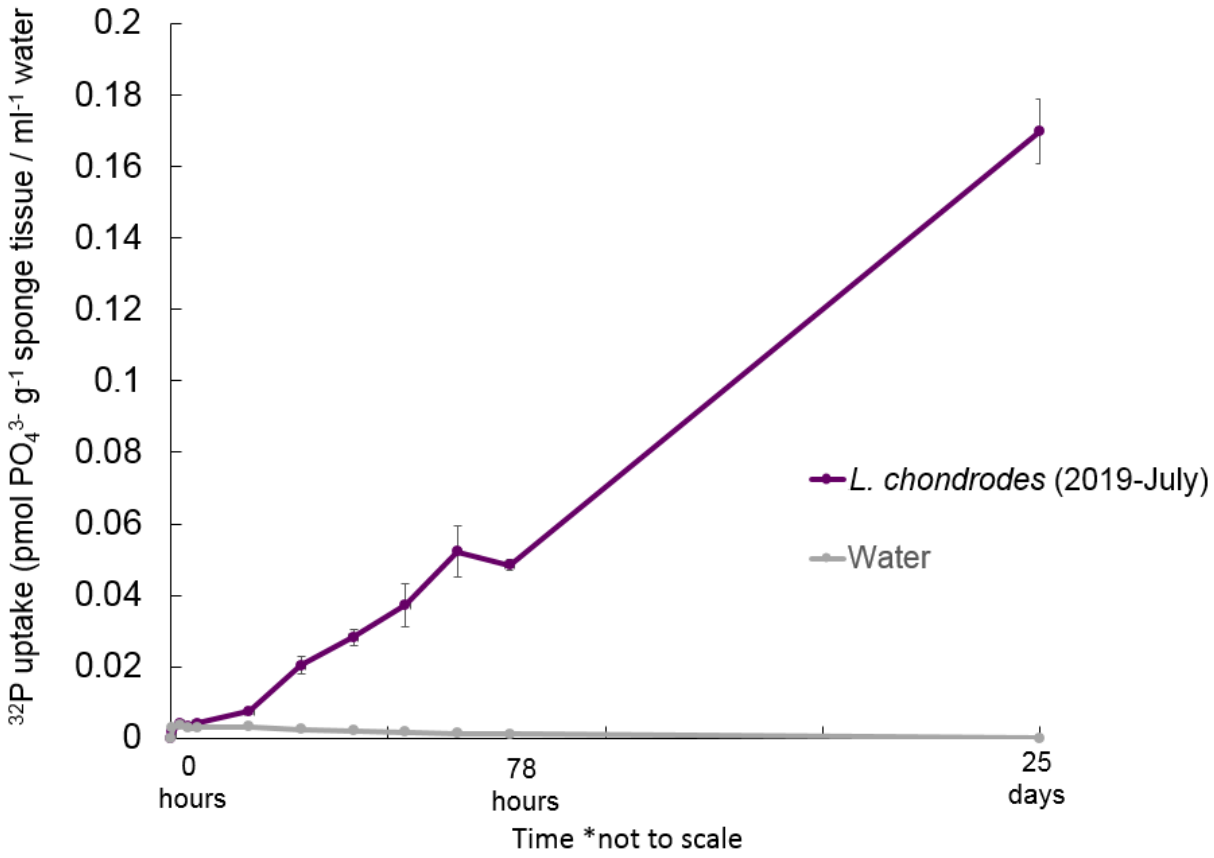


Figure 3.5 P_i uptake by *L. chondrodes* in Round 1 (July 2019). Sponges and water were sampled in triplicate 25 days after the initial inoculation of $^{32}P_i$. X-axis shows time (not to scale), and y-axis shows uptake of P_i in pmol per gram of sponge tissue and per ml of water. Radioactive decay was accounted for when calculating uptake. Error bars show standard error of the mean. Standard error has been calculated for the water but is too small to appear at this scale.

3.4.2 Organic phosphorus tracing with γ^{32}P[ATP]

When comparing grams of tissue to milliliters of water, γ^{32}P[ATP] was found within the tissue of both *L. chondrodes* and *H. heliophila* at significantly higher concentrations than that of the surrounding water (ANOVA $p < 0.01$) (Figure 3.6). Using ^{31}P-NMR analysis, studies have shown that phosphorus esters comprise the majority of high–molecular weight DOP in the ocean (75%) and in tidal salt marshes (phosphomonoesters: 61%, phosphodiesters: 31%) (Kolowith et al. 2001; Bell et al. 2020). Thus, ATP (a phosphomonoester) is an appropriate tracer for DOP uptake for both *L. chondrodes* in coral reefs and *H. heliophila* in coastal bays.

Similar to the DIP tracing rounds, the initial phosphate concentrations within experimental tanks were higher than phosphate concentrations normally found within the natural environment. Phosphate concentrations were not determined during the December 2019 and January 2020 rounds due to potential residual radiation within the tanks. Concentrations were measured in July 2020 and found to be ca 8.0 µM (Table 3.1). It is assumed that the phosphate concentrations are ca. 6.0-8.0 µM for the other experimental rounds. This study did not determine the concentrations of DOP in the tanks before experimentation. The concentration of the γ^{32}P[ATP] inoculum (1.36 pM) was more than 1000-fold lower than that of the initial phosphate and thus serves as an effective tracer for it does not interfere with natural uptake. The time series analysis was again broken into two categories. Short-term uptake is classified as the first six hours of sampling and long-term uptake comprises the remaining 78 hours, during which sponges and water were sampled every 12 hours. In the rounds of

experimentation from December 2019 and January 2020, the time series was extended beyond 78 hours (Figure 3.9).

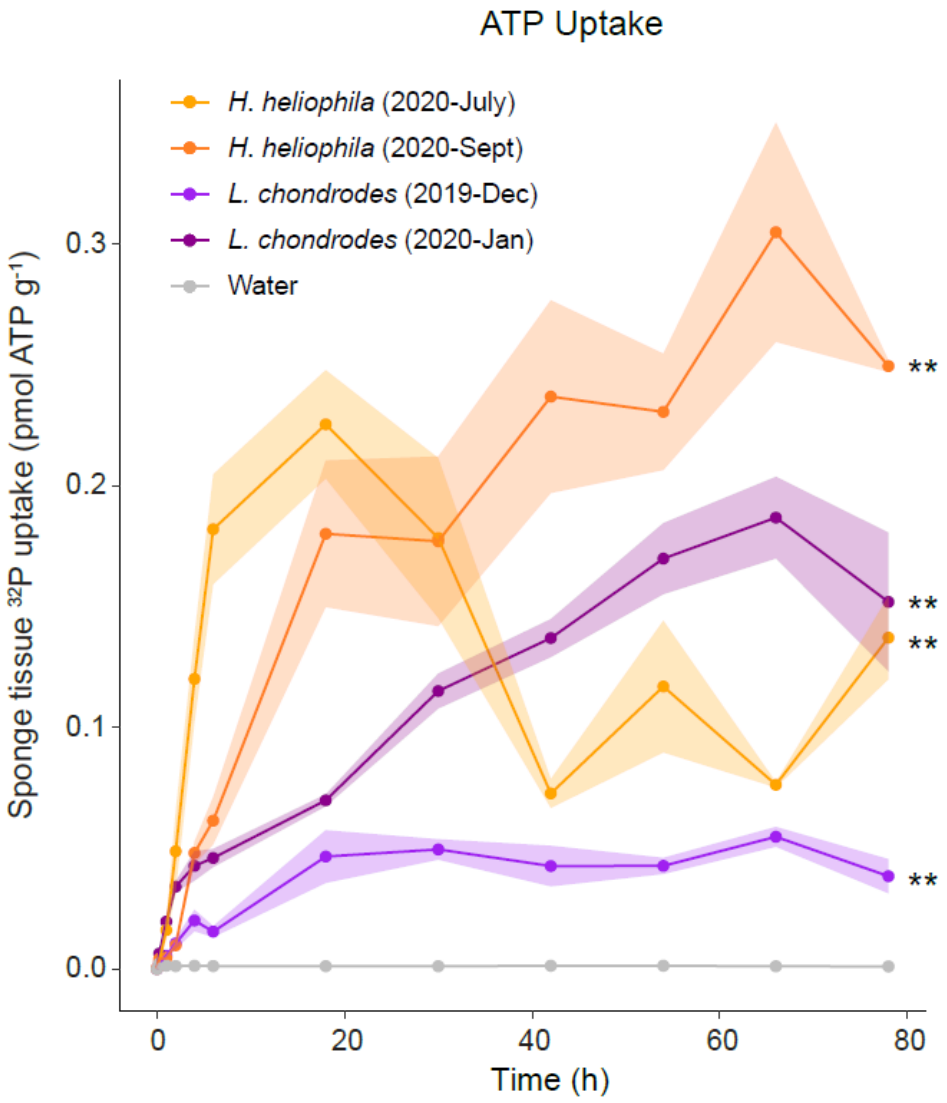


Figure 3.6 Average ATP uptake of biological replicates of *L. chondrodes* from December 2019 (), *L. chondrodes* from January 2020 (), *H. heliophila* from July 2020 (), *H. heliophila* from September 2020 (), and aquarium water ( ) over 78 hours. X-axis shows time in hours and Y-axis shows uptake of ^{32}ATP in pmol per gram of sponge tissue and per ml of water. Standard error is shown as corresponding-colored ribbons. Standard error has been calculated for the water but is too small to appear at this scale. At all time points, all four sponges contained significantly higher concentrations of P_i compared to surrounding water (**ANOVA $p < 0.01$).

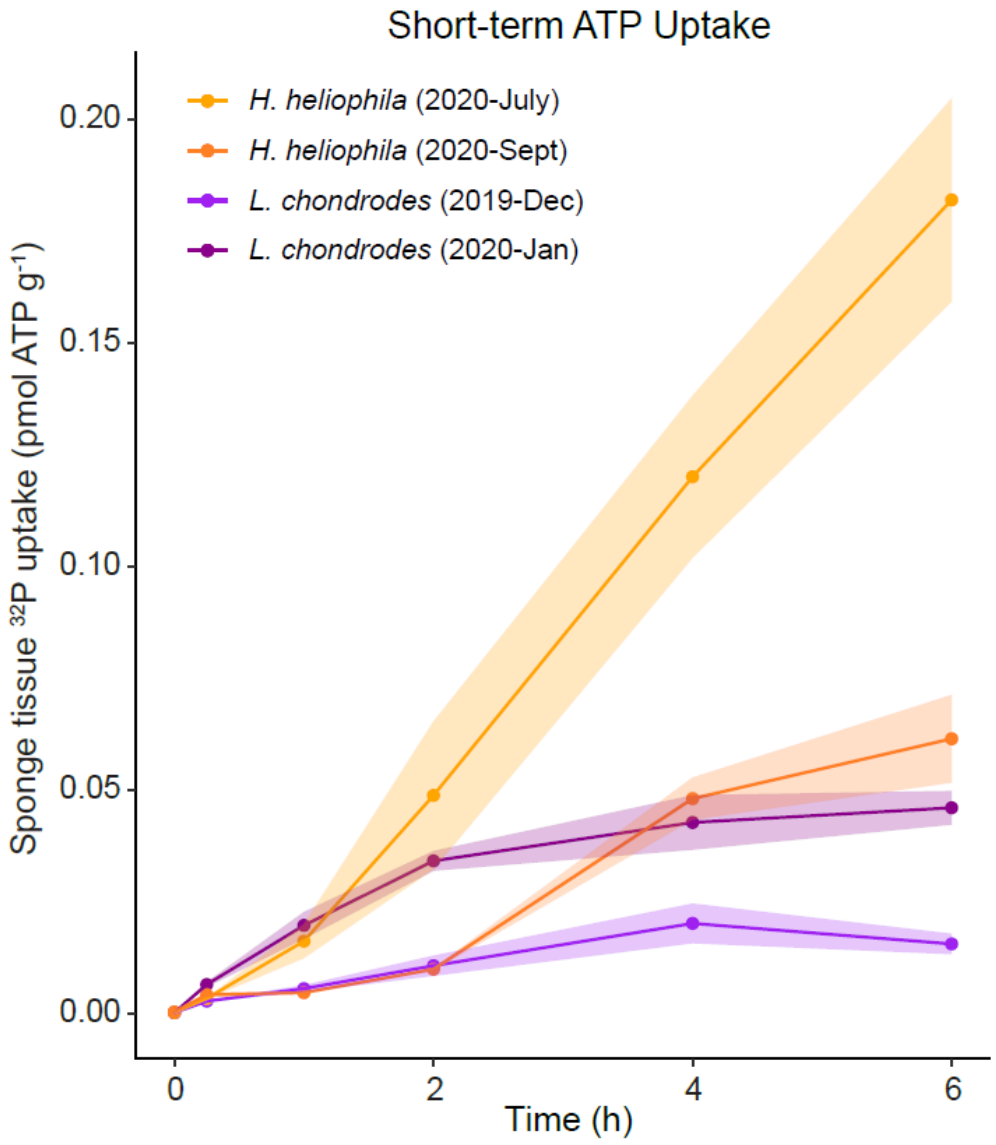


Figure 3.7 Average ATP uptake of biological replicates of *L. chondrodes* from December 2019 ( ), *L. chondrodes* from January 2020 ( ), *H. heliophila* from July 2020 ( ), *H. heliophila* from September 2020 ( ) over first six hours (short-term). X-axis shows time in hours and Y-axis shows uptake of ^{32}ATP in pmol per gram of sponge tissue. Standard error is shown as corresponding-colored ribbons.

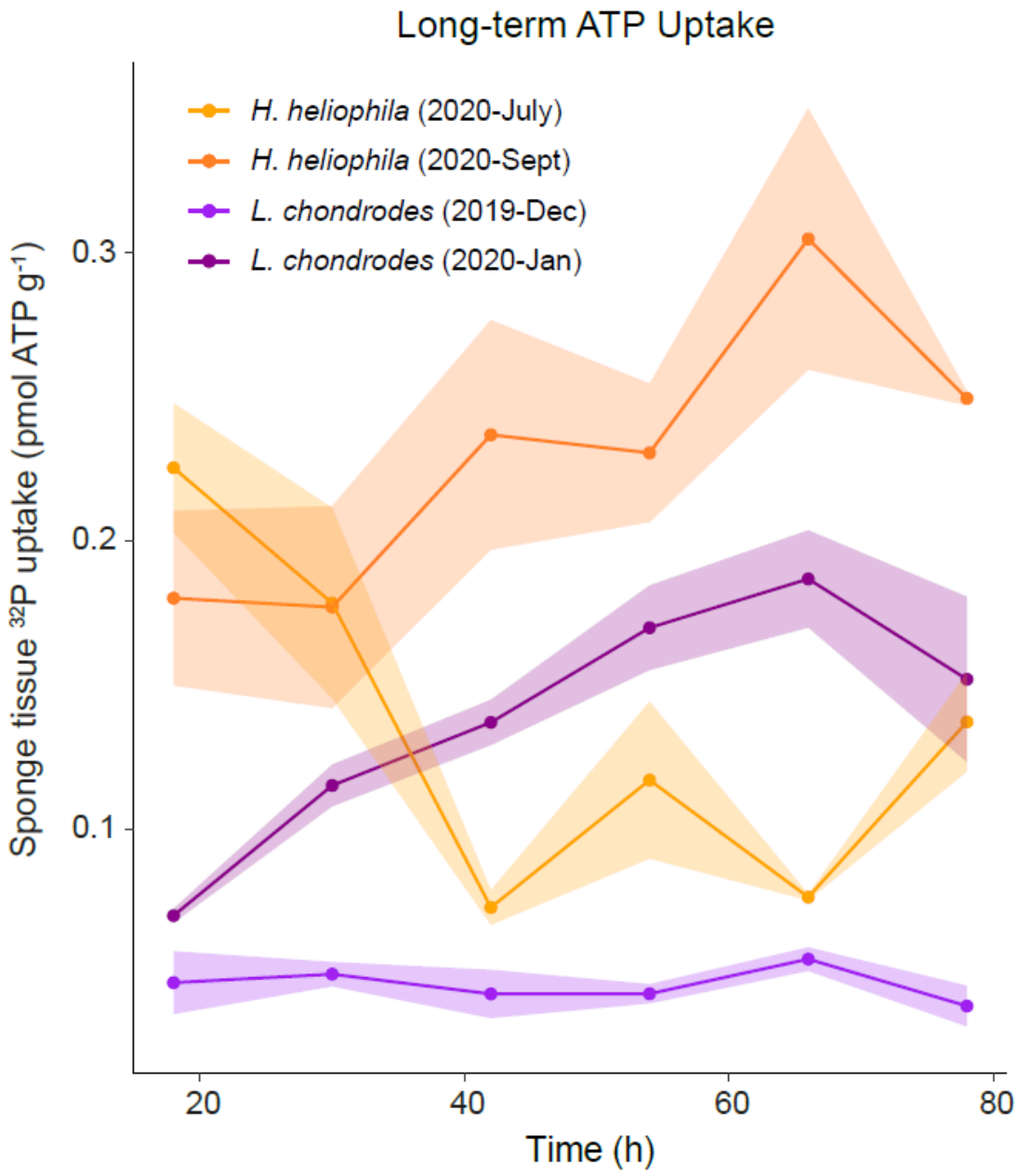


Figure 3.8 Average ATP uptake of biological replicates of *L. chondrodes* from December 2019 (—●—), *L. chondrodes* from January 2020 (—●—), *H. heliophila* from July 2020 (—●—), *H. heliophila* from September 2020 (—●—) over remaining 78 hours (long-term). X-axis shows time in hours and Y-axis shows uptake of ^{32}ATP in pmol per gram of sponge tissue. Standard error is shown as corresponding-colored ribbons.

Table 3.3 Short-term and long-term ATP uptake rates in *L. chondrodes* and *H. heliophila* (n = 3). Uptake rates are expressed as fmol PO_4^{3-} g^{-1} hr^{-1}.

Sponge	Short-term uptake rate of ATP (fmol g^{-1} h^{-1})	Long-term uptake rate of ATP (fmol g^{-1} h^{-1})
L. chondrodes (2019-Dec)	2.57	-0.135
L. chondrodes (2020-Jan)	7.64	1.37
H. heliophila (2020-July)	30.3	-1.47
H. heliophila (2020-Sept)	10.2	1.16

L. chondrodes and *H. heliophila* both took up organic phosphorus in the form of ATP over the first six hours with *H. heliophila* taking up more overall and at a faster rate. The changing scale of the Y-axis must be emphasized when comparing P_i intake with that of ATP (Figure 3.6 vs. Figure 3.2). In the short-term, *H. heliophila* took up ATP at higher rates than *L. chondrodes*, with *H. heliophila* from July 2020 having the highest uptake rate of 30.3 fmol g^{-1} h^{1}. *H. heliophila* from July 2020 also had a significantly higher uptake rate than *H. heliophila* from September 2020 even though the ATP concentrations of those rounds (Round 5 & 6) were identical (Table 3).

L. chondrodes and *H. heliophila* continued to take up ATP for the remaining 78 hours of the time series, albeit at lower uptake rates. While *H. heliophila* individuals from July 2020 had the highest short-term uptake rate, their long-term uptake rate changed to -1.47 fmol g^{-1} h^{-1}. Partial tissue necrosis of these sponge individuals was observed at the 18-hour time point where pmol ATP values drop, indicating these sponges may have been dying. The other uptake rates of *L.*

chondrodes and *H. heliophila* began to level out at the 18-hour mark. Short-term uptake of ATP was more rapid followed by a leveling out over the remaining 78 hours. Although when the time series was extended beyond 78 hours (Figure 3.9), the sponges continue to show uptake of ATP when sampled at 18 days and 23 days. However, there is no significant difference in uptake at 78 hours, 18 days, and 23 days with both *L. chondrodes* from December 2019 and from January 2020.

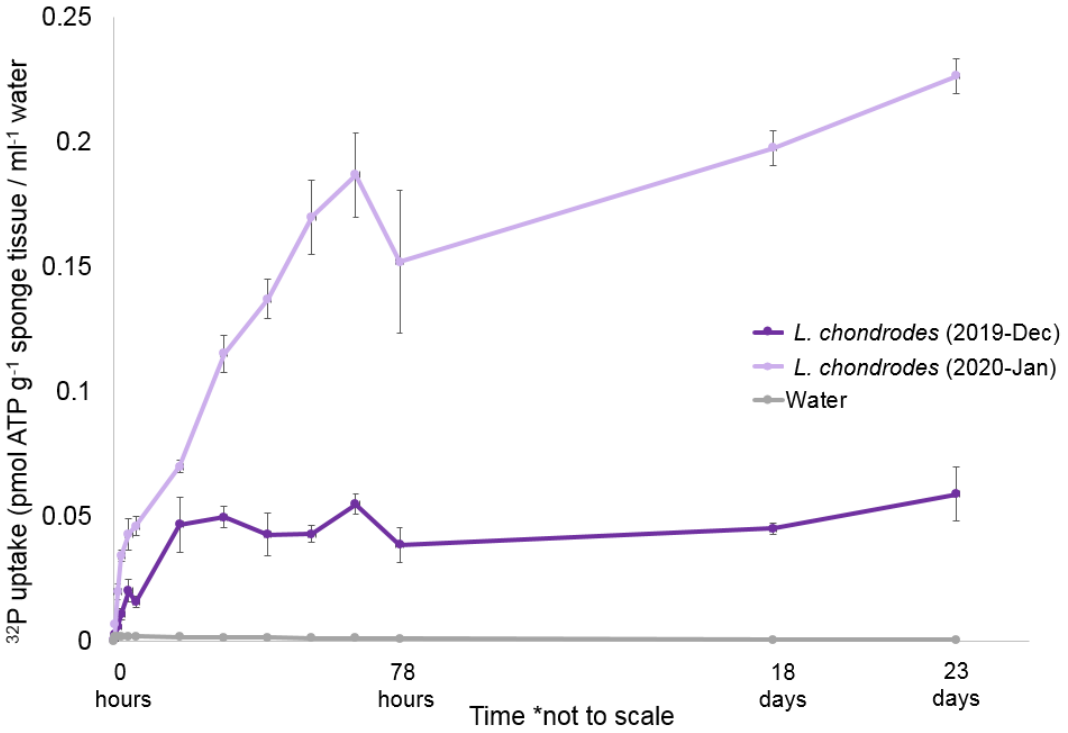


Figure 3.9. ATP uptake by *L. chondrodes* in Round 3 (December 2019) and Round 4 (January 2020). Sponges and water were sampled in triplicate 18 and 23 days after the initial inoculation of ^{32}ATP. X-axis shows time (not to scale), and y-axis shows uptake of ATP in pmol per gram of sponge tissue and per ml of water. Radioactive decay was accounted for when calculating uptake. Error bars show standard error of the mean. Standard error has been calculated for the water but is too small to appear at this scale.

3.5 Discussion

3.5.1 Comparative DIP and DOP uptake rates between *L. chondrodes* and *H. heliophila*

Both *L. chondrodes* and *H. heliophila* took up DIP in the form of P_i and DOP in the form of ATP in all six cases of experimentation. Both sponges consistently took up P_i over 78 hours at similar rates of uptake. Sponges took up more ATP at higher rates of uptake than that of P_i. ATP uptake rates were higher over the first six hours, followed by a plateau or potential release. In all, both sponges took up organic phosphorus at a higher rate, but inorganic uptake was more consistent over time.

Although DIP and DOP concentrations within the marine systems are highly variable, both are most commonly range from 0.05 – 0.5 µM (Atkinson 1987b; Charpy 2001). DOP concentrations in seawater can reach as high as 1 µM (Schaffelke et al. 2012) and DIP can reach ca. 2.5 – 3 µM; however, these instances of DIP influx from terrestrial runoff are transient and may only affect barrier reefs closer to land (den Haan et al. 2016; Silbiger et al. 2018) This study's initial phosphorus concentrations were higher than what is naturally found within the marine environment. This was due to the fact the phosphate concentrations are naturally higher within aquarium tanks due to waste breakdown within a small and enclosed tank (Blanchard 2014). Further, I did not want the sponges to be starved of phosphorus before determining uptake rates. The phosphate concentrations in this study (ca. 5-8 µM) show that sponges will readily take up phosphorus from the water even in times of excess. Interestingly, the rates of ATP uptake were higher than that of P_i even when there was a surplus of phosphate already present within the water.

This result indicates that sponges may readily take up DOP at significantly higher rates than DIP in reef systems regardless of either form's availability. This is especially interesting because sponges and their symbionts are assumed to take in DIP at higher efficiencies than DOP as the latter is more energetically expensive (Björkman & Karl 1994). I hypothesize that strain HIMB114 from the SAR11 group in *H. heliophila* could contribute to higher DOP cycling in this sponge. Strain HIMB114 was reported to rely on DOP to support despite the availability of DIP (Grant et al. 2019). However, no conclusive insights can be made on the importance of this particular strain in *H. heliophila* because it was only found to make up 0.4% of the bacterial community.

DIP uptake has been studied in several marine invertebrates with microbial symbionts, although only a handful have used ^{32}P. Uptake rates for these studies are calculated as the rate of phosphate depletion from seawater (Pomeroy & Kuenzler, 1969). Zooxanthellae symbionts of the anemone *Aiptasia pallida* took up P_i under times of severe starvation (Muller-Parker et al. 1990). Algal symbionts (most notably the dinoflagellate *Symbiodinium* sp.) of clams, jellyfish, and coral have also been found to take up phosphate across various concentration gradients (D'Elia 1977; Belda et al. 1993; Todd et al. 2006; Godinot et al. 2009). To my knowledge, only two studies have measured phosphate intake by sponges (Yahel et al. 2007; Ribes et al 2012). These two studies use a method known as In-Ex, in which a tube is positioned next to the sponge osculum to sample inhaled water and the other tube is placed inside the osculum to sample exhaled water (Yahel et al., 2005). This water is then subjected to dissolved nutrient analysis. Two sponges found within a deep temperate

fjord were found to inhale and exhale phosphate (Yahel et al. 2007). Additionally, three temperate sponges were found to inhale phosphate with the majority of this pool then being exhaled into the surrounding water (Ribes et al. 2012).

Phosphorus is well-known to be a limiting factor within reef systems and as such must be utilized efficiently. Further, an increase of phosphorus during nutrient plumes generally leads to large algal blooms (Anderson et al. 2002) and a wide range of detrimental effects on reefs (Fabricius 2005; Silbiger et al. 2018). Most research of increased phosphate on reefs focuses on coral health. While some studies find that coral growth rate increased with marginal increases in phosphate (Koop et al. 2001; Dunn et al. 2012), others find a wide range of damaging consequences such as reduced growth rate (Fabricius 2005), decreased skeletal density (Dunn et al. 2012), and increased growth of competitive algae (Schaffelke & Klumpp 1998). It is so far unknown how increased phosphate concentrations will potentially affect marine sponges. My study is the first to show sponges taking up DIP and DOP from the water and the results indicate that marine sponges will continuously take up these forms of phosphorus in times of surplus. Further, it is important to highlight that the concentration of the ^{32}P inoculum was so low that the addition of these tracers would not affect the natural processes occurring within the tanks.

For both DIP and DOP uptake, differing initial concentrations and concentrations of the ^{32}P inoculum did not seem to play a role in uptake rates. While there were brief incidents of different P_i uptake between experimentation rounds – for example, all sponges took up different amounts of P_i within the first two hours (Figure 3.2)— no one factor such as sponge species or phosphorus concentration

seemed to be consistently linked. My approach demonstrates for the first time the utility of using ^{32}P for extremely sensitive tracing studies in marine invertebrates. Within 15 min of DIP and DOP addition, we saw sponges rapidly taking up phosphorus from the surrounding water. It could be reasonably hypothesized that the *L. chondrodes* sponge and its associated microbiome may have adaptations for more rapid uptake of phosphorus due to the fact that it lives in a more nutrient-limited habitat than *H. heliophila*. However, this was not the case and in fact the opposite was true for DOP uptake. This could be due to the fact that our individuals of *L. chondrodes* had been bred for many generations within aquaculture systems where phosphorus concentration was purposefully kept as low as possible.

3.5.2 Phosphorus intake has been confirmed, but what happens to phosphorus after uptake?

Over time, both *L. chondrodes* and *H. heliophila* showed steady rates of uptake of DIP, indicating that both sponges and their associated microbiomes were efficient at DIP uptake. This new-found characteristic of sponges raises the question: If this rapid uptake is a common trait among sponges in reefs, are sponges making dissolved phosphorus bioavailable to the surrounding ecosystems or are sponges potentially phosphorus sinks? It is quite possible that marine sponges serve as both a source and sink consistent with the way that microorganisms can act as both sinks and releasers of phosphorus in the soil biogeochemical cycle (Turner et al. 2003). Within the sponge body, it has been confirmed that some P_i may be combined with calcium to form apatite, which may lock phosphorus in a recalcitrant mineral form (Zhang et

al. 2015). Conversely, P_i was also found to be converted to poly-P by bacterial symbionts, possibly for energy and P storage (this study; Zhang et al. 2015). Poly-P may then be degraded for energy production and P_i may be released into the water. For example, rapid phosphorus turnover (relative to nitrogen) was documented in two sponges, *Aplysina* sp. and *lotrochota baculifera* (Hatcher 1994). High relative phosphorus turnover has also been recorded in a plethora of other marine invertebrates such as holothurians (Webb et al. 1977), copepods (Butler 1970), and gastropods (Hatcher 1994). Using the In-Ex method discussed previously, some sponges have been found to release P_i into the water column. P_i excretion was recorded in *Aplysina aerophoba*, *Dysedia avara,* and *Agelas oroides* (Jimenez and Ribes 2007). The same year a small, although statically significant, P_i efflux was found in the sponge *Aphrocallistes vastus* (Yahel et al. 2007). Larger and significant P_i excretion was then measured in three sponges: ca. 20%, 30% and 60% P_i excretion for *D. avara, C. reniformis* and *A. oroides* respectively (Ribes et al. 2012).

Phosphorus may also be made available by way of cell turnover and detritus production (a.k.a. the sponge loop), by which sponges would release phosphorus as POP to be consumed by detritivores, analogous to the role of sponges in carbon cycling (de Goeij et al. 2013). This potential DIP/DOP recycling by sponges would fuel higher trophic levels and contribute greatly to total reef P availability. However, all studies researching DOM production by sponges have only focused on POC and PON production (de Goeij et al. 2013; Maldonado 2014; Rix et. al. 2017; McMurray et al. 2018). Repeating sponge incubations and detritus production studies with ^{32}P-enriched DOM would provide valuable new insights into the total POM pool and the

sources of energy within oligotrophic yet thriving reefs. As this study is the first to use ^{32}P as a phosphorus tracer, it forms a foundation for expanding tracing studies to learn more about DIP, DOP, and POP.

Chapter 4: Genetic analysis and chemical extraction show sponges store phosphorus in the form of energy-rich polyphosphate (poly-P).

4.1 Abstract

In this chapter, I use genetic analysis and chemical extraction to study the phosphorus-rich energy storage compound polyphosphate (poly-P) in two marine sponges. This work builds on the dissertation research of Dr. Fan Zhang (Zhang 2015), who showed three sponges accumulate 25–40% of their phosphorus in the form of poly-P. First, the gene responsible for poly-P biosynthesis, polyphosphate kinase (*ppk*) was amplified by PCR and cloned from DNA extracted from the sponges *L. chondrodes* and *H. heliophila*. Five separate *ppk* genes were found in both sponges— two derived from bacterial symbionts of *L. chondrodes* and three from symbionts of *H. heliophila*. Poly-P was then quantified in both sponges through extraction and quantification by using a malachite green-based colorimetric assay. Results indicate that *L. chondrodes* stores 6–8% of its phosphorus as poly-P, while *H. heliophila* stores 0.55%. This difference may be because *L. chondrodes* contains a larger proportion of cyanobacterial symbionts than *H. heliophila*, as cyanobacteria have previously been shown to store poly-P (Zhang et al., 2015; Zhang, 2015). *L. chondrodes* may also store more phosphorus because this sponge lives in oligotrophic reef habitat in the south Pacific where phosphorus is at very low concentrations in the surrounding seawater—typically 0.15 ± 0.01 μM PO_4^{3-} (Fong et al. 2020)—whereas *H. heliophila* was collected from a eutrophic environment in the Maryland coastal

bays with more abundant phosphorus—0.4 – 0.6 μM PO_4^{3-} (Glibert et al. 2014) with some measurements recorded as high as 4 μM (Fang et al. 1977). The results presented here provide further evidence that marine sponges and their symbionts have a significant impact on phosphorus cycling in aquatic systems.

4.2 Introduction

4.2.1 Poly-P, a universal and central molecule

Phosphorus is an essential and central element in oceanic biogeochemical cycles, yet is often present in limiting concentrations (Karl 2014). Times of good phosphorus availability are a luxury for marine microorganisms and as such phosphorus must be utilized efficiently when available. Excess phosphate is often polymerized and stored as a long chain. This phosphate chain, known as polyphosphate (poly-P), can be a few to hundreds of phosphate molecules in length and the molecules are held together by energy-rich phosphoanhydride bonds. Poly-P is synthesized enzymatically from ATP, and bacteria are then able to re-synthesize ATP from stored poly-P during times of starvation and environmental stress (Kornberg et al. 1999). Poly-P is truly ubiquitous, having been found across all domains of life and has been hypothesized to have played a role in the origins and proliferation of the first forms of life on earth (Brown & Kornberg 2004; Björkman 2014). Due to its ubiquity and biological significance, poly-P has been utilized in various industries. For example, poly-P accumulating bacteria are used in wastewater treatment facilities to remove excess phosphate from inbound water (McMahon & Read 2013). Food-grade poly-P is used as an additive to products such as toothpaste, cheese, and processed meats (Christ et al. 2020). It can also act as an antibacterial agent in processed meat, poultry, and fish products (Lee et al. 1994).

Poly-P concentrations within organisms can vary rapidly depending on environment, availability, and metabolism. Due to the capricious nature of ambient phosphate, poly-P can be rapidly degraded and used for energy in times of

phosphorus limitation. This is one of many reasons why poly-P can be difficult to measure, although methods have been developed to visualize, extract, and quantify poly-P. For instance, 4'6-diamidino-2-phenylindole (DAPI) is used in epifluorescent microscopy as a stain that binds to nucleic acids. Because poly-P is similar in structure to the phosphate backbone of DNA and RNA, DAPI binds to polyP, shifting the peak emission wavelength from 475 nm to 525 nm under ultraviolet excitation (Tijssen et al. 1982). After cell lysis and a series of hydrolysis steps with DNase, RNase, and proteinase, DAPI can also be used to quantify poly-P by spectrofluorometry (Martin & Van Mooy 2013). Poly-P has also been quantified using a phosphate colorimetric assay. Poly-P must first be hydrolyzed with perchloric acid into phosphate molecules (Hansen & Robinson 1953). These individual phosphate molecules can then be measured with the formation of a phosphomolybdate complex and a malachite green-based colorimetric assay (Strickland & Parsons 1972; McKelvie et al. 1995; Cogan et al. 1999).

4.2.2 Polyphosphate kinase (*ppk*): using genetic analyses to locate potential poly-P production

While poly-P is found in bacteria, archaea, and eukaryotes, the enzyme responsible for its production is different in different organisms. In prokaryotes (and some microbial eukaryotes), poly-P is synthesized by the enzyme polyphosphate kinase (PPK), encoded by the polyphosphate kinase gene (*ppk*) (Zhang et al. 2007). PPK catalyzes the reversible transfer of phosphate molecules of ATP in order to augment the poly-P chain:

$$PolyP_n + ATP \leftrightarrow PolyP_{n+1} + ADP$$

This reaction is easily reversed by the enzyme responsible for cleavage of phosphate from the poly-P chain, exopolyphosphatase (PPX), encoded by the exopolyphosphatase gene (*ppx*) (Akiyama et al. 1993).

$$PolyP_n + H_2O \rightarrow PolyP_{n-1} + Pi$$

PPX can cleave poly-P in order to reform ATP. The cleaved P_i can also be used for other purposes, such as the phosphorylation of proteins (Xie & Jakob 2019). Depending on its length, one poly-P molecule can contain tens to hundreds of phosphoanhydride bonds. These high-energy bonds are the same as the bonds found within ATP. The energy released through hydrolysis can be used for crucial cell processes such as DNA replication or biosynthesis (Rao et al. 2009). PPK has been shown to be an essential enzyme for many cellular functions. Various studies using *ppk* knockouts have shown that cells lacking PPK have severely compromised cell growth, motility, quorum sensing, grazing, biofilm formation, and virulence (Rashid & Kornberg 2000; Rashid et al. 2000a, 2000b; Fraley et al. 2007; Brown & Kornberg 2008; Sanyal et al. 2013). As a result, screens for small molecules that disrupt the function of PPK have been devised as a novel approach for finding next generation antibiotics (Singh et al. 2016).

Two studies have both amplified *ppk* sequences and quantified poly-P concentrations in marine sponges. *Ppk* was successfully amplified in *M. laxissima, X. muta,* and *I. strobilina* and expression of *ppk* was successfully shown in the latter two sponges (Zhang et al. 2015). The nearest related *ppk* sequences were from nitrogen-fixing cyanobacteria, aerobic anoxygenic phototrophic bacteria, and sulfur cycle-related Proteobacteria. This metabolic diversity found within symbionts that encode for *ppk* provides further evidence that the marine sponge holobiont can use several metabolic pathways to store energy in the form of poly-P (Zhang et al. 2015). This study also determined that polyP could account for 24–40% of total phosphorus within sponge tissue (Table 4.1). In another study, half of the 30 *ppk* sequences amplified in eight sponge holobionts were highly related (above 80% similarity) to *ppk* sequences from members of the genus *Synechococcus* (Ou et al. 2020). Further, the sponges with the highest poly-P content and enrichment rates had higher abundances of cyanobacteria than the sponges with lower poly-P concentrations. Poly-P has also been found in the freshwater sponge, *Ephydatia muelleri*, with concentrations shown to change in response to adverse environmental conditions (Imsieke et al. 1996).

Table 4.1 Poly-P and Total P weight fraction in marine sponges (n = 3 for each sample). Directly from Zhang et al. 2015, provided with permission by PNAS. Zhang F., Blasiak L. C., Karolinm J. O., Powell, R. J., Geddes, C. D., Hill, R. T. (2015). Phosphorus sequestration in the form of polyphosphate by microbial symbionts in marine sponges. *Proc Natl Acad Sci*; 112: 4381–4386.

Sponge sample	PolyP ± SD, % dry weight	Total P ± SD, % dry weight	PolyP/total P, %
X. muta			
Epidermal layer	0.080 ± 0.010	0.325 ± 0.042	24.6
Mesohyl layer	0.075 ± 0.011	0.298 ± 0.034	25.2
I. strobilina	0.092 ± 0.012	0.342 ± 0.116	26.9
Whole sponge			
M. laxissima	0.126 ± 0.015	0.314 ± 0.038	40.1
Whole sponge			

4.2.3 Overview of this chapter

In this study, I extracted and quantified the percentage of the total phosphorus that was present in the form of poly-P within *H. heliophila* and *L. chondrodes.* Poly-P was quantified with a spectrophotometer after hydrolysis into P_i, formation of a phosphomolybdate complex, and staining with malachite green. Absorbance was measured at 595 nm and concentrations of poly-P were calculated from a standard curve. Five *ppk* genes in these sponges were successfully characterized by using PCR, gel purification, and cloning to detect unique *ppk* sequences in *H. heliophila* and *L. chondrodes.*

4.3 Methods

4.3.1 Polyphosphate/Total phosphorus extraction and quantification

The protocol for poly-P extraction and quantification is adapted from the methods of Canadell et al. 2016 and Cogan et al. 1999. Sponge collection and processing is described in Section 2.3.1

Polyphosphate extraction: Lyophilized sponge tissue (10 mg) was suspended in 1 ml of 5% (w/v) sodium hypochlorite and disrupted by vortexing with approx. 400 mg glass beads (size 75 µm, acid-washed, Sigma-Aldrich) at 4°C for 15 minutes. Lysate was centrifuged (18,000 g for 5 minutes) and washed once with the sodium hypochlorite solution. The pellet was resuspended in 100 µl deionized water, incubated for 5 minutes at room temperature, centrifuged as above and collected in a new 2-ml tube. The pellet was resuspended for a second time in 100 µl deionized water, incubated for 5 minutes at room temperature, and collected into the same 2-ml tube. Ice-cold ethanol (1.8 ml of 100% ethanol) was added and samples were held overnight at -20°C. Poly-P was pelleted the next day by centrifugation at 14,000 g at 4°C for 15 minutes. The supernatant was discarded and the pellet was washed once with 70% (v/v) ethanol. The ethanol was decanted and the pellet was air-dried for 30 minutes on the benchtop. The pellet was then resuspended in 40 µl deionized water and transferred to a new tube for acid hydrolysis.

Total phosphorus extraction: Lyophilized sponge tissue (10 mg) was suspended in 1 ml deionized water and disrupted by vortexing with glass beads (approx. 400 mg) at 4°C for 15 minutes. 40 µl of lysate was transferred to a new tube for acid hydrolysis.

Acid hydrolysis and quantification: A 40-µl sample containing either poly-P or total-P was converted to P_i by adding 10 µl of 1 M perchloric acid (final concentration) and incubating at 90°C for 30 minutes. Poly-P standards with an average chain length of 45 (sodium phosphate glass type 45, Sigma-Aldrich, catalog number: S4379) were used to verify the efficiency of hydrolysis by perchloric acid. P_i was then quantified by a malachite green assay in a 96-well microtiter plate containing: 10 µl sample with hydrolyzed phosphate (in 1 M perchloric acid), 90 µl phosphate assay buffer [20 mM Tris-HCl (pH 7.5), 5 mM magnesium acetate, 100 mM ammonium acetate], 86 µl of 28 mM ammonium heptamolybdate in 2.1 M H_2SO_4, and 64 µl 0.76 mM malachite green in 0.35% polyvinyl alcohol. The reaction was incubated for 20 minutes at room temperature, and absorbance was measured at 595 nm on a Spectramax M5 spectrophotometer (Molecular Devices). All absorbance peaks were corrected for the absorbance of the blank assay buffer. KH_2PO_4 standards (0 to 500 µM) were used to generate a standard curve (Figures 4.1, 4.2). The concentration of hydrolyzed orthophosphate was determined from the standard curve.

4.3.2 Cloning and sequencing of *ppk*

Sponge tissue (30 mg) was ground with a mortar and pestle, placed in a 2-ml round-bottom tube with a stainless-steel bead and 600 µl buffer (RLT plus, Qiagen), and lysed in a Tissue Lyser (Qiagen) at 30 beats/seconds for 1.5 minutes. DNA was extracted using the AllPrep DNA/RNA Minikit (Qiagen) and concentrations were confirmed using a NanoDrop spectrometer. Polymerase chain reactions were carried out in 25 µl total volume including 12.5 µl JumpStart REDTaq ReadyMix (Sigma Aldrich), 1 µl of 10 µM primer (Table 4.2), 30 ng of DNA template, and PCR-grade

water. The PCR program consisted of an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 minute, 50°C for 45 seconds, and 72°C for 2 minutes, with a final extension at 72°C for 12 minutes. DNA from *E. coli* strain K12 was used as a positive control for the presence of the *ppk* gene. PCR amplicons were isolated by size fractionation using gel electrophoresis in 1.5% TAE gels. Appropriately-sized bands were excised with a sterile scalpel and purified using the QIAquick Gel Extraction Kit (Qiagen). Gel extracts were ligated into pGEM-T-easy vectors (Promega) and subsequently transformed into 5-alpha competent *E. coli* cells (New England BioLabs). Cells were inoculated into SOC medium, shaken at 37°C for 1 hour, and plated onto LB plates with ampicillin (100 mg/ml), IPTG (10 µM), and X-Gal (2% w/v). Plates were incubated at 37°C for 18 hours. White colonies were picked and placed in 20 µl DI water for monoclonal colony PCR using M13 vector primers (Table 4.2). Appropriate amplicon size was verified by gel electrophoresis. PCR products were purified with Exo-SAPit (ThermoFisher Scientific) and sent for sequencing at the BAS Lab at IMET.

Chromatograms were trimmed and assembled with CLC Main Workbench (Qiagen). Double coverage reads allowed for confidence in base calling and accurate editing. Consensus sequences were identified using the National Center for Biotechnology Information (NCBI) BLAST (blastx & blastp) program (Altshul et al. 1990) and alignments were visualized using NCBI's Constraint-based Multiple Alignment Tool (COBALT) (Papadopoulos & Agarwala, 2007). The closest relatives to the five *ppk* sequences were downloaded from GenBank and used as references for finding an appropriate frame for alignment and tree generation. Phylogenetic trees

were created with Méthodes et algorithmes pour la bio informatique (MAB) (Dereeper et al. 2008, 2010), MUSCLE for alignment (Edgar 2004), a curation by Gblocks which eliminates poorly aligned positions and divergent regions (Castresana 2000), PhyML for phylogeny (Guindon & Gascuel 2003; Guindon et al. 2005; Anisimova 2006), and TreeDyn for tree rendering (Chevenet et al. 2006). For confidence analysis, data was resampled using 1,000 bootstrap replicates. Branches with < 50% bootstrap values were omitted from the tree. Sequences from this study were deposited in GenBank under the accession numbers MW974890–MW974893, & MW984605.

4.3.3 Technical vs. biological replicates used in poly-P extraction and quantification

Upon collection of *L. chondrodes* (supplied by aquaculture) and *H. heliophila* (collected in the field), sponge tissue was sliced into 1 cm^3 pieces with a sterile scalpel and lyophilized. From the pooled tissue, 10 mg was weighed out for poly-P/total-P extraction and quantification. Each procedure was done in triplicate and the replicates in the following data are technical replicates— as opposed to biological replicates from three different sponge individuals. *L. chondrodes* underwent two separate rounds of extraction and quantification while *H. heliophila* underwent one round. Amount of tissue was the limiting factor for *H. heliophila* as I only had ~40 mg of lyophilized tissue to work with and more sponge could not be collected in the winter months.

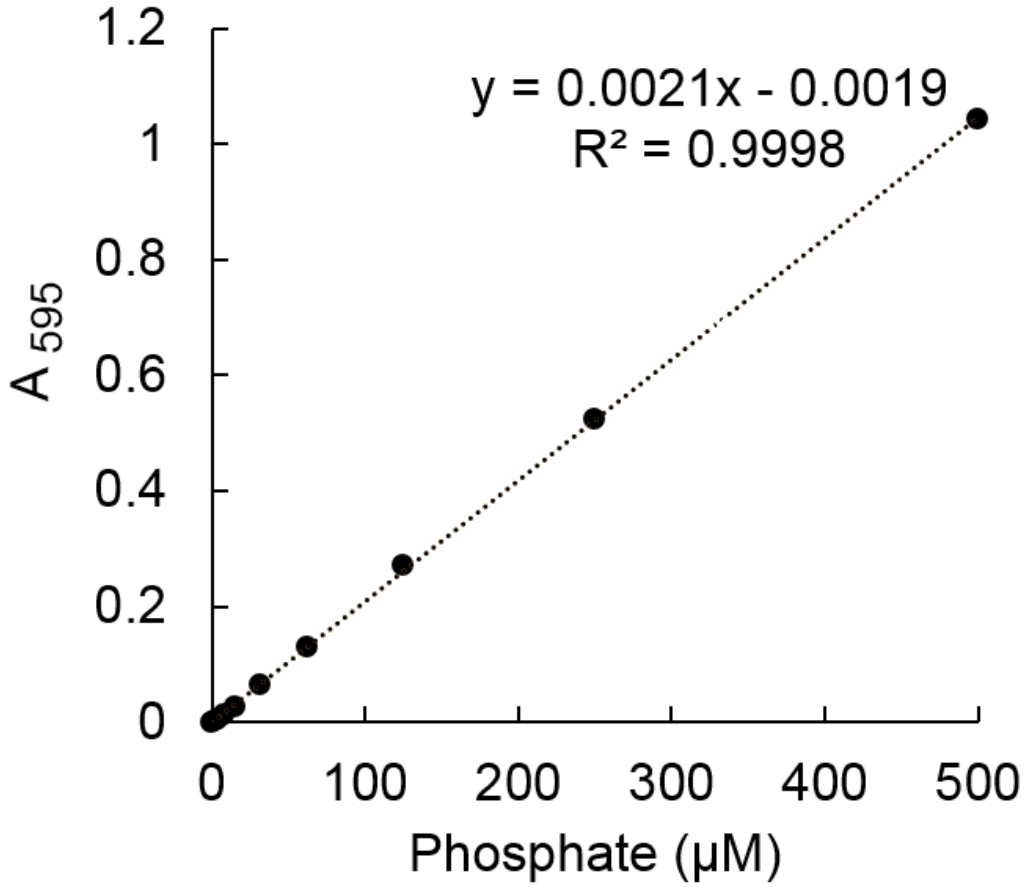


Figure 4.1 Standard curve of the absorbance at 595 nm of KH_2PO_4 standards (0 to 500 μM). Three replicate standards were measured at each concentration (Figure 4.2). All standard absorbance peaks were corrected for the absorbance of the blank assay buffer.

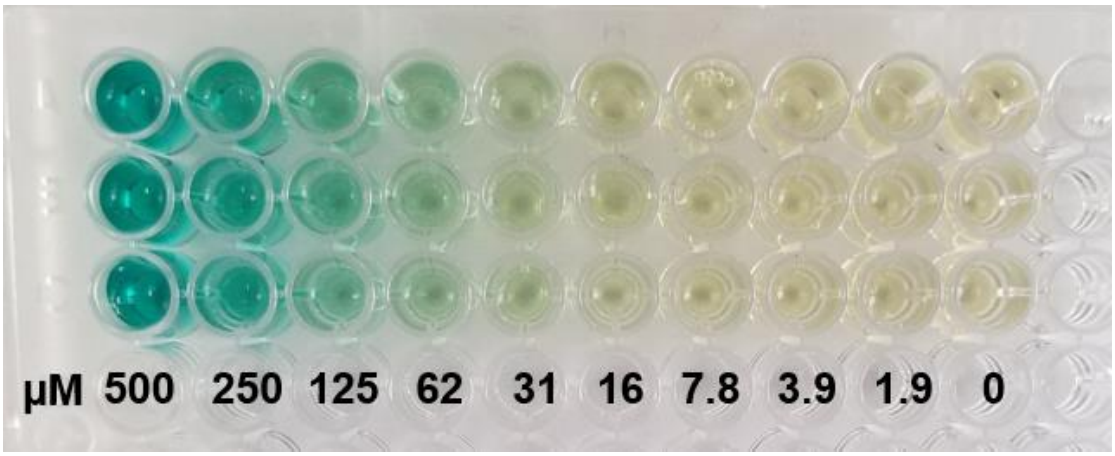


Figure 4.2 Wells used in standard curve (Figure 4.1) using a malachite green assay of KH_2PO_4 standards (0 to 500 μM). Three replicate standards were made at each concentration and were measured on a Spectramax M5 spectrophotometer (Molecular Devices).

Table 4.2 Primer sets used for the amplification of polyphosphate kinase gene, *ppk.*

Primer Name	Nucleotide Sequence	Target Region	Organism(s)	Origin
IPCRppk-1375 (forward)	**5' CAC GCC AAG ATG CTG ATG ATC G 3'**	**Polyphosphate kinase (*ppk*)**	***H. caerulea***	**McMahon et al 2002**
IPCRppk-0289 (reverse)	**5' GCG TTT CGTCGC AGA CCA GGC G 3'**	***ppk***	***H. caerulea***	**McMahon et al 2002**
ppk1_F (forward)	**5' GGS GGY CGK TAY CAY AAY TTY AAR GAC TTY ATT 3'**	***ppk***	***L. chondrodes H. heliophila***	**Ou et al 2019**
ppk1_R (reverse)	**5' ATA TTG CGC GTC ATC CAG TCS GC 3'**	***ppk***	***L. chondrodes H. heliophila***	**Ou et al 2019**
M13F (forward)	**5' GTT GTA AAA CGA CGG CCA GT 3'**	**M13 vector**		**ThermoFisher Scientific**
M13R (reverse)	**5' AGC GGA TAA CAA TTT CAC ACA GGA 3'**	**M13 vector**		**ThermoFisher Scientific**

4.4 Results

4.4.1 Quantification of Poly-P and Total-P in both sponges

L. chondrodes contained more poly-P per mg of sponge tissue and had a much higher proportion of total phosphorus stored as poly-P than *H. heliophila* (Table 4.3). From the two separate extractions, poly-P comprised ca. 6–8% of total phosphorus in *L. chondrodes* and 0.55% in *H. heliophila.* The contributing factor for lower poly-P percentage in *H. heliophila* is twofold: a lower poly-P concentration (1.4 μmol) and a larger total-P concentration (235.5 μmol). Percent poly-P profiles have been studied in three other marine sponges, and poly-P was found to make up ca. 25% of the total phosphorus in *X. muta*, ca. 27% in *I. strobilina*, and ca. 40% in *M. laxissima* (Zhang et al. 2015). Zhang et al. 2015 used a different extraction protocol and quantification by spectrofluorometry rather than by spectrophotometry as in this study. The different poly-P concentrations of *L. chondrodes* and *H. heliophila* are intriguing and in need of further verification by repeating the study with additional biological replicates and collections during different seasons.

Table 4.3 Poly-P, Total-P, and % phosphorus as poly-P in *L. chondrodes* and *H. heliophila*, n=3 (technical replicates) for each sample.

Sponge (10 mg)	Poly-P (µmol)	Total-P (µmol)	Poly-P/Total-P (%)
L. chondrodes	6.74 ± 0.40	113.3 ± 2.1	5.95
L. chondrodes	9.14 ± 0.25	114.4 ± 3.8	7.98
H. heliophila	1.41 ± 0.33	253.5 ± 9.7	0.55

4.4.2 Amplification of five novel *ppk* sequences in marine sponges

After amplification through PCR and cloning, five *ppk* sequences were identified: two from bacterial symbionts of *L. chondrodes* and three from bacterial symbionts from *H. heliophila* (Table 4.4). Sequences were confirmed to be *ppk* through the BLASTn program, which aligns nucleotides to nucleotides, BLASTx, which aligns translated nucleotides to protein sequences, and BLASTp, which aligns protein sequences to protein sequences with the NCBI GenBank database.

Table 4.4 Results from sequence alignments using NCBI BLASTx. In all cases, the primers used for amplification were the primers designated 'ppk1', listed in Table 4.2.

Sequence Name	Nearest BLASTx hit (Accession #)	Taxonomy	Identity (%)	Coverage (%)	Sponge
LC_A2	Polyphosphate kinase 1 (WP_114338655.1)	*Corallincola spongiicola*	71.36	100	*L. chondrodes*
LC_C5	Polyphosphate kinase 1 (WP_002463645.1)	*Atlantibacter hermannii*	99.46	99	*L. chondrodes*
HH_A7	Polyphosphate kinase 1 (WP_130164997.1)	*Pseudoalteromonas* sp.	72.44	99	*H. heliophila*
HH_D3	TPA Polyphosphate kinase 1 (HAB78227.1)	*Gammaproteobacteria* Isolate UBA9605	92.23	95	*H. heliophila*
HH_E5	TPA Polyphosphate kinase 1 (HAD89175.1)	*Alteromonas macleodii*	100	99	*H. heliophila*

All five of the bacteria that have the highest sequence similarity to the *ppk* sequences of this study are Gammaproteobacteria. *C. spongiicola* is a gram-negative aerobe recently isolated from a marine sponge (Choi et a. 2020) and *A. hermannii* is a gram-negative bacterium found in environmental samples as well as human wounds and sputum. *Gammaproteobacteria* Isolate UBA9605 and *A. macleodii* are common marine heterotrophic Gammaproteobacteria. Interestingly, *Pseudoalteromonas* sp. is a genus of Gammaproteobacteria that contains many species which have been found to synthesize biologically active compounds (Holmström and Kjelleberg 1999). Species of *Pseudoalteromonas* are generally found in association with marine eukaryotes, including marine sponges (Ivanova et al. 1998) and often display antibacterial, agarolytic and algicidal activities. However, the sequence that aligned with the *Pseudoalteromonas* sp. sequence, HH_A7, does have the lowest percent identity of the five *ppk* sequences found.

The low percent identity of LC_A2, HH_A7, and HH_D3 suggest that the bacteria from which these sequences originate may not be closely related to the exact species listed in Table 4.3, but perhaps to other species within the genus or in the case of HH_A7 (72.44% identity) perhaps another genus altogether. Phylogenetic relationships between the five *ppk* sequences found in this study and other related sequences was visualized through a phylogenetic tree using maximum likelihood methods (Figure 4.3). The outgroup seen at the top of the tree was kept due to the fact that a few species relevant to this study clustered together. For example, *ppk* sequences have been found in *Cyanobium* sp. (WP_106500455.1) and *Pseudanabaena* sp. (GBO54392.1). Both species were cultured from *L. chondrodes* (Table 2.3), but *ppk* was not amplified from these

bacteria in this study. *Geobacter sulfurreducens* was the closest BLAST hit to one of the *ppk* sequences found by Zhang et al. 2015.

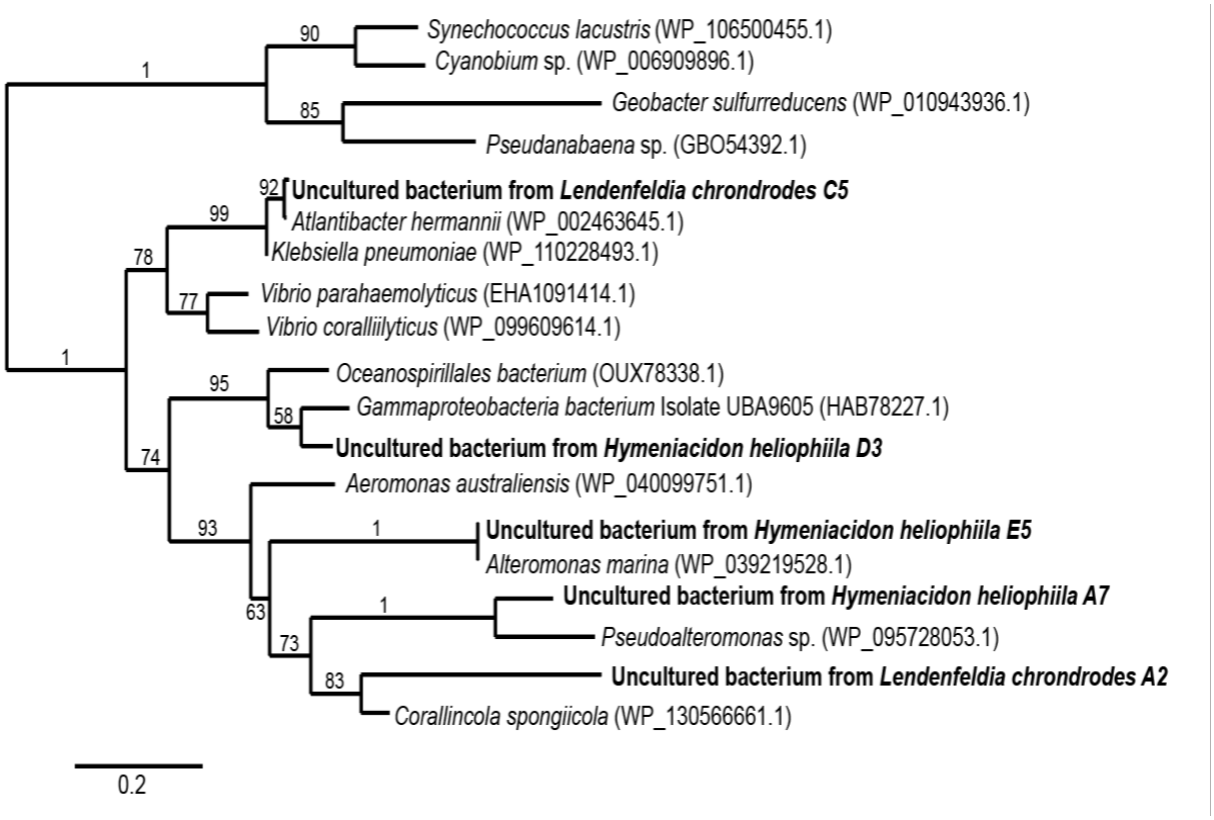


Figure 4.3 Phylogenetic relationships between this study's partial *ppk* sequences (amino acid) and others downloaded and trimmed from GenBank. Sequences from this study are in bold. The steps for tree construction are explained in Figure 4.4. The tree was constructed using the maximum likelihood method and boot strap confidence values (expressed as percentages of 1000 replications) are shown at the branch nodes. Branches with bootstrap values less than 50% were eliminated.

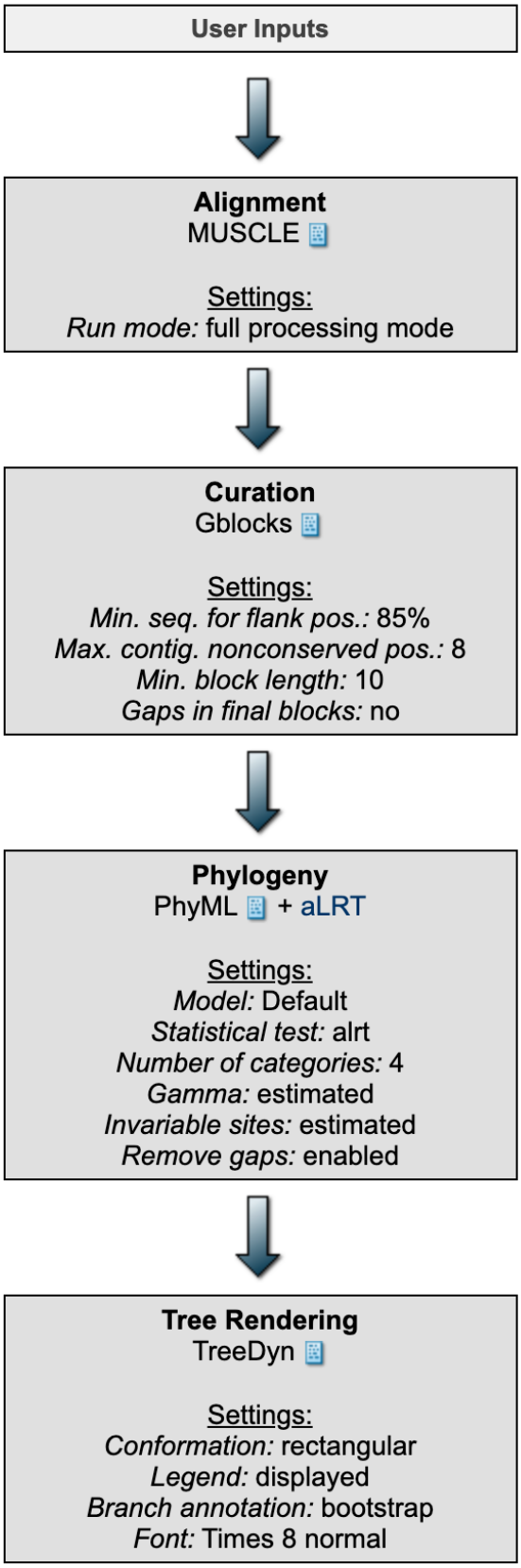


Figure 4.4 Overview of bioinformatic pipeline used in tree construction by Méthodes et algorithmes pour la bio informatique (Dereeper et al. 2008). Default settings were used in this study uses the "one-click mode" including a curation by the Gblocks program. Text of tree was then exported and edited within Adobe Illustrator.

4.5 Discussion

4.5.1 The sponges *L. chondrodes* and *H. heliophila* store different amounts of poly-P and this may be related to their distinct habitats or microbial assemblages.

Chemical extraction showed that both sponges store phosphorus in the form of energy-rich poly-P. *L. chondrodes* stores more phosphorus in this form (ca. 6–8%) compared to *H. heliophila* (0.55%). It can be hypothesized that sponges from nutrient-limited environments may store more phosphorus in the form of poly-P since phosphorus is considered a luxury in oligotrophic habitats. This was found to be the case by Ou et al. (2020) who reported poly-P enrichment rates being higher in sponges found in waters with lower DIP concentrations (Ou et al. 2020). The higher poly-P enrichment rates were also correlated with the sponges' bacterial communities, specifically with the presence of the cyanobacterial genus, *Synechococcus. L. chondrodes* was found in this study and others (Ridley et al. 2005a; Freeman & Easson 2016; Vargas et al. 2020) to harbor abundant cyanobacterial symbionts (Figure 2.4). Zhang et al (2015) reported accumulation of poly-P granules within a cyanobacterial symbiont, suggesting a key role for cyanobacteria in poly-P accumulation in sponges, although other bacterial genera may also be implicated. Thus, I hypothesize that the abundant *Synechococcus spongarium* in *L. chondrodes* could play a key role in accumulating poly-P. However, neither of the two *ppk* sequences amplified from DNA extracted from the *L. chondrodes* holobiont had amino acid similarity to *ppk* sequences found within *Synechococcus* or other cyanobacteria. Because of the dense microbial assemblages present in some sponges, cloning may need to be repeated numerous times to fully elucidate the various bacterially-derived *ppk* sequences present in this sponge. Repeated cloning or careful

primer design using the sequences from this study, Zhang et al. 2015, and Ou et al. 2020 would most likely reveal more *ppk* sequences, perhaps from cyanobacteria. In addition to genetic analysis, there is direct evidence of cyanobacteria playing a large role in phosphorus accumulation. *Leptolyngbya*, a filamentous and diazotrophic cyanobacteria enriched from the sponge *I. strobilina*, was directly shown through microscopy to contain numerous poly-P granules (Zhang et al. 2015). I was able to culture another filamentous cyanobacterium, *Pseudanabaena* sp. from *L. chondrodes*, which has already been shown to encode for *ppk* (Figure 4.3). Further, *Limnothrix/Pseudanabaena* sp. have been reported to accumulate large amounts of poly-P granules (Kitazaki et al. 2013). This specific group of symbionts could be responsible for the higher poly-P percentages we see in this sponge. No cyanobacteria were able to be cultured from *H. heliophila* and cyanobacteria do not make up a large proportion of its microbiome (Figure 2.4).

4.5.2 Sponge symbionts store poly-P and this could impact phosphorus cycling in their surrounding environment.

Poly-P has been shown to a principal component in a wide range of survival mechanisms used by bacteria, including but not limited to motility, DNA replication, and cell division (Rao et al. 2009). By contributing to symbiont health and persistence, higher concentrations of poly-P may help sponges be more competitive. For example, *Pseudoalteromonas* symbionts that contain large amounts of poly-P could use this phosphorus reserve to produce a wide range of metabolites that prevent the settlement of common fouling organisms on their sponge hosts (Holmström & Kjelleberg 1999). Poly-P could have a functional reach far beyond that of the sponge holobiont. Poly-P is

speculated to have contributed to the origin of life as a prebiotic phosphate-containing compound that made up the ribose-phosphate backbone of life's genetic material (Keefe & Miller 1995). There is no conclusive verification to support this hypothesis, however many pieces of isolated evidence are being assembled by evolutionary biologists and geologists. For example, poly-P is abundant in volcanic exudates and deep-sea steam vents (Yamataga et al. 1991) and can be synthesized by heating phosphate minerals under geological conditions with the use of high-energy bonds from cyanimide or hydrogen cyanide (Schwartz et al. 1975). However, even under extreme scenarios in which this type of poly-P synthesis were to occur, the poly-P concentration in the ocean would still have been insignificant (Keefe & Miller 1995). The origin of water-soluble polyphosphates required for prebiotic evolution has therefore been up for debate (Gulick 1957; Miller & Urey 1959; Miller & Paris 1964). No abiotic forms of poly-P have been found and most of the phosphorus on the early Earth would have been in the form of apatite, an insoluble phosphate mineral (Keefe & Miller 1995). Within biological systems, however, the importance of poly-P cannot be overstated as it contributes directly to phosphorus storage and energy needed to produce all vital biological molecules (Achbergerová & Nahálka 2011). Combining the fact that poly-P is found within every living cell (Kulaev & Vagabov 1983) and fact that sponges are among the first metazoans to appear on earth (Taylor et al. 2007b), it can be deduced that poly-P has played a role in the evolution of all subsequent metazoans and their microbial symbionts. It could be of great interest to the field of sponge microbiology and evolutionary biology to conduct more experiments with poly-P and marine sponges to better comprehend the role of poly-P as a potential prebiotic reagent.

4.5.3 Potential drivers of phosphorus acquisition in marine sponges: vacillating ambient phosphate concentrations or host phylogeny?

Phosphorus is generally supplied to marine systems via rivers and terrestrial runoff (Paytan & McLaughlin 2007). Unlike nitrogen, phosphorus cannot be fixed from the atmosphere and thus drives microorganisms to deplete dissolved phosphorus prior to dissolved nitrogen (Jackson & Williams 1985). The temporal variability of phosphorus, combined with the fact that it is essential for growth, drives its rapid turnover within aquatic systems (Benitez-Nelson 2000). Phosphate availability may also be the driving factor behind uptake by the sponge holobiont. This was found to be the case for the twelve sponges studied by Ou et al. in 2020; the sponges living under oligotrophic conditions were found to have higher poly-P enrichment rates compared to their eutrophic counterparts (Ou et al. 2020). These high enrichment rates and subsequent poly-P storage could be an important piece in understanding how marine sponges thrive within environments with low ambient nutrients. This certainly is the case for planktonic microorganisms living in the extremely oligotrophic Sargasso Sea, as the phytoplankton found there are highly enriched in poly-P that may be contributed to the surrounding environment and enhance primary production (Martin et al. 2014). More research on the relationship between ambient phosphate concentrations and marine sponge poly-P accumulation would not only help in characterizing local phosphorus cycling, but also would further our understanding on the high rates of primary production found within reef systems.

A second driving factor of poly-P accumulation by marine sponge is microbial phylogeny. A canonical correspondence analysis revealed a strong correlation between the polyP enrichment rate and the sponge microbial community structures studied by Ou et al. 2020. Further, the genera *Synechococcus*, *Blastopirellula*, *Rhodopirellula*, and *Rubripirellula*, had the strongest correlation with the polyP enrichment rate (Ou et al. 2020). Cyanobacteria have historically been found to contribute greatly to their host through a wide range of metabolic functions. Cyanobacterial symbionts, most commonly *Synechococcus*, are major contributors of carbon and nitrogen to several different sponges in a range of geographic locations (Zhang et al. 2019). It is known that free-living cyanobacteria can accumulate poly-P from water (Mukherjee et al. 2015; Aguilar-May & del Pilar Sánchez-Saavedra 2009) and filamentous cyanobacterial symbionts of sponges may be the driving taxa of poly-P accumulation (Zhang et al. 2019). In this study, *L. chondrodes* has a microbiome dominated by cyanobacteria and also stores a higher proportion of its phosphorus as poly-P compared to *H. heliophila*. However, *L. chondrodes* stores a smaller proportion of poly-P than the three Caribbean sponges studied by Zhang et al. 2015. Those sponges may have had a more dominant assemblage of cyanobacteria or have developed other mechanisms for efficient poly-P storage. There may be other bacterial taxa within the microbiome of these sponges that contribute to higher poly-P enrichment. For example, one study found that Actinomycetes are able to solubilize phosphate compounds, potentially providing a bioavailable form of phosphorus for the holobiont (Sabarathnam et al. 2010). Further, Planctomycetes, Proteobacteria, and Acidobacteria are hypothesized to be involved in phosphorus sequestration (Ou et al. 2020).

More studies on phosphorus cycling within the sponge holobiont are needed to better understand what factors—nutrient availability, phylogeny, etc.—determine whether sponge store large amounts of phosphorus as poly-P. Considering the oligotrophic environments in which most sponges dominate, any amount of phosphorus stored within sponges is likely to play a major role in phosphorus biogeochemistry on reefs.

Chapter 5: Conclusions and Future Directions

5.1 Sponges' involvement in the phosphorus cycle

Sponges often exist in ecosystems with low concentrations of phosphorus, for example the South Pacific (Vacelet 2007; Freeman & Easson 2016), Mediterranean (Friedrich et al. 2001; Lazzari et al. 2016), and Caribbean (Diaz & Rützler 2001). Due to their pumping capacity, sponges come into contact with large amounts of water—in some cases, 24,000 l day^{-1}(Vogel 1977)— and as such, come into direct contact with more phosphorus than the majority of organisms on reefs. My results demonstrate that sponges have the capacity to take up phosphorus in both its inorganic and organic forms and store it as poly-P. To date, this is the first study to use ^{32}P to trace phosphorus uptake by sponges. Both *L. chondrodes* and *H. heliophila* took up DIP and DOP from the surrounding water and both were found to store this phosphorus as poly-P. These results indicate that both of these sponge species impact the phosphorus biogeochemistry of their respective habitats. Considering their worldwide occurrence, high abundance, ability to filter large volumes of water, and expanded metabolism due to associated microorganisms, marine sponges have the potential to be central organisms in benthic-pelagic coupling and nutrient biogeochemistry in coral reef ecosystems.

The sponge holobiont is responsible for various biogeochemical transformations that influence nutrient availability on reefs (Figure 5.1A). It has been well-characterized that pelagic nutrient cycling is ruled by planktonic photoautotrophic and heterotrophic prokaryotes (Azam et al. 1983; Ferrier-Pagés & Gattuso, 1998; Partensky et al., 1999). Through their active suspension feeding, sponges provide a link between the pelagic zone and the benthos, a process known as benthic-pelagic coupling (Lesser 2006; Coppari et a.

2016). Not only do sponges connect the reef with the overlying water column, some sponges also facilitate the transformation of DON and DOC into particulate detritus that is returned to higher trophic levels (de Goeij et al. 2013). This sponge loop hypothesis is continually being tested at various depths (Lesser et al. 2019) and within various types of sponges (McMurray et al. 2016). Discovering the mechanisms for the transformation of DOM to bioavailable particulate forms is fundamental to understanding the major flow of energy and nutrients within coral reef ecosystems, and research is needed to extend the sponge loop hypothesis to include phosphorus. Discovering the mechanisms that drive various nutrient cycles within the sponge holobiont has long been a focus for the field of sponge microbiology. For a deeper look into the various nutrient cycles carried out by the sponge holobiont, one can read the publication, "Microbially mediated nutrient cycles in marine sponges" that I co-wrote with Dr. Fan Zhang during the first year of my master's degree (Zhang et al. 2019).

This thesis puts forth new insights into marine sponges' role in phosphorus cycling within reefs (Figure 5.1B). Through six rounds of experimentation using ^{32}P-tracing, I discovered two marine sponges, *L. chondrodes* and *H. heliophila*, rapidly taking up both inorganic and organic forms of phosphorus. The sponges took up DOP at a higher rate than DIP, but uptake of DIP was more consistent over the 78-hour times series. Further, *H. heliophila* took up DOP at a higher rate than *L. chondrodes.* Rates of uptake could be driven by pumping rates, host phylogeny, microbial assemblage, or ambient phosphorus availability. Carbon fixation and nitrogen transformations have been previously reported in *H. heliophila* (Hammen & Osborne 1959; Weigel & Erwin 2017). The DIP and DOP uptake presented by this thesis now enables *H. heliophila,* alongside *L.*

chondrodes, to be considered in the context of phosphorus cycling as well. This thesis did not determine whether sponges take up particulate organic phosphorus (POP) to determine if phosphorus is supplied by the sponges' bacterial diet. There are many potential outputs of phosphorus by sponges. Some sponges have been found to release P_i into the water column (Hatcher 1994; Jimenez & Ribes 2007; Yahel et al. 2007; Ribes et al. 2012). Further, poly-P may be degraded for energy production, releasing P_i. Given sponge abundance across the ecosystem, this P_i output could fuel primary production and greatly affect the overall energy dynamics of the surrounding phosphorus-starved microorganisms. Conversely, poly-P could be combined with calcium to produce insoluble apatite, which may lock phosphorus in a recalcitrant mineral form (Hirschler et al. 1990; Zhang et al. 2015). The fate of phosphorus within the sponge body remains to be fully characterized.

Whether it is degraded or stored, poly-P accumulation by sponges has the potential to affect the energy dynamics of reef microcosms. Similar to that of a wastewater treatment plant, sponge tissue provides an adequate redox environment for poly-P accumulation (Zhang et al. 2015). In addition to a suitable redox environment, there are other potential drivers of poly-P storage. Poly-P storage could be driven by limitation, as bacteria have mechanisms in place to accumulate poly-P when under nutritional stress (Rao et al. 1998). The phosphate regulon (Pho regulon) is a response regulator that is activated by the depletion of ambient P_i (Torriani-Gorini 1994; Wanner 1994, 1995, 1996). Thus, oligotrophy could be one of the drivers for energy storage in the form of poly-P within sponge symbionts. Another driver could be the microbial assemblage; this study found that *L. chondrodes* stored more phosphorus as poly-P and

also had a higher abundance of cyanobacterial symbionts. Not only did cyanobacteria make up 68% of the *L. chondrodes* bacterial community, but two cyanobacterial isolates (*Cyanobium* sp. and *Pseudanabaena* sp.) were previously found to encode for *ppk*. Cyanobacterial abundance could be proportional to poly-P accumulation, and considering their widespread abundance as sponge symbionts (Usher 2008), an abundant cyanobacterial assemblage could be a key characteristic when looking for sponges that are central to phosphorus cycling.

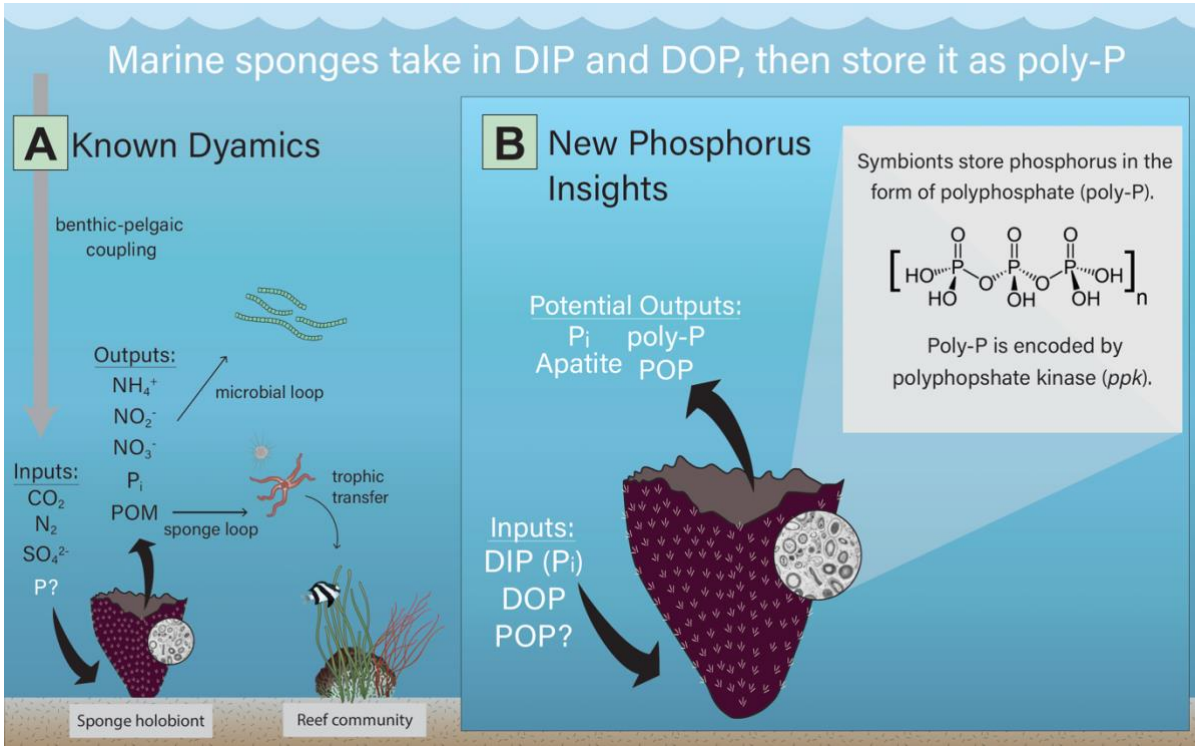


Figure 5.1. Microbial symbionts of marine sponges have diverse metabolic strategies that provide sponges access to nutrients that would be otherwise unattainable. **(A)** Known microbially mediated nutrient cycles of marine sponges (as reviewed by Zhang, Jonas et al. 2019). Abbreviations: carbon dioxide (CO_2); atmospheric nitrogen (N_2); ammonium ($NH4^+$) nitrite ($NO2^-$); nitrate ($NO3^-$); phosphate (PO_4^{3-}); particulate organic matter (POM). **(B)** Insights into phosphorus cycling provided by this thesis. Through ^{32}P tracing, sponges were found to take in both orthophosphate (P_i) and dissolved organic phosphorus (DOP) in the form of ATP. It has yet to be determined whether sponges take up phosphorus as particulate organic phosphorus (POP), either as detrital-POP or living-POP from their bacterial diet. Sponges have been shown to release P_i (Ribes et al. 2012) and are potential sources of POP through cell shedding (de Goeij et al. 2013) or potential sinks by mineralizing phosphate into recalcitrant apatite (Zhang et al. 2015). Further determining in

which forms phosphorus is released by sponges will be crucial to further our understanding of sponges and their impact on phosphorus cycling.

5.2 Limitations and Future Directions

5.2.1 Expanding on the community of *H. heliophila* and *L. chondrodes*.

The universal bacterial primer set 27F/1492R does not adequately amplify archaea, which have been characterized in *H. heliophila* (Turque et al. 2010; Weigel & Erwin 2016; Coelho et al. 2018). To expand the metagenomic analysis of both *H. heliophila* and *L. chondrodes*, one could add an analysis using the universal bacterial/archaeal forward primer 515F and reverse primer 806R (Caporaso et al. 2011) or do a separate analysis specifically of the archaeal community, using primers such as SSU1ArF, SSU520R, 340F, SSU666ArR and SSU1000ArR (combinations discussed in Bahram et al. 2018). Understanding the archaeal assemblage within these sponges could be important, especially because ammonia oxidizing archaea that have been hypothesized to increase *H. heliophila* fitness within polluted environments (Turque et al. 2010; Weigel & Erwin 2016). Another way to deepen my analysis on the sponges' microbiomes would have been to increase culturing efforts. In the future, culturing efforts should be started when sponges are first collected, and perhaps with more subculturing or longer incubation times, more symbionts could be isolated. To understand the functioning of the sponge holobiont, especially in the context of biogeochemistry, future studies must pair the genetic analysis of microbial communities within any other experimentation. Sponge microbial communities are a dynamic collection of diverse bacteria, archaea, and microalgae; metagenomics and metatranscriptomic analysis will be instrumental in understanding putative functioning of the holobiont.

5.2.2 More phosphorus tracing studies are needed to better understand coral reef functioning and the impact of the marine sponges.

Working with radioisotopes presented some limitations on the types of experiments I was able to conduct. For example, I had originally planned to separate bacterial symbionts from sponge tissue by gradient density fractionation and assay them separately to determine the proportion of phosphorus that is incorporated into bacterial cells vs. sponge tissue. ^{32}P is a strong beta emitter, therefore, this isotope poses an external (skin) and internal hazard to persons handling it (University of Maryland Baltimore Environmental Health and Safety). Consequently, I wanted to limit my exposure as much as possible and decided to only count radiation found within sponge tissue. If safety hazards were not of concern, it would have been ideal to pair my tracing experiments with my poly-P assays. I could extract poly-P from ^{32}P-labelled sponge tissue over time to determine the proportion of phosphorus that is incorporated into polyP. However, this extraction process involves various types of equipment that were not designated to be used with radioactive material. Further, the assay's multiple steps take two days to complete and I was not comfortable with that level of prolonged exposure.

If I were to repeat my ^{32}P tracing study, I would have used the same molarity of P_i and ATP in each round. Because of my various tank sizes, I was unable to maintain the same concentration between rounds. For example, I used the 80 µl of $^{32}P_i$ in my first round in my 27.5 l tank, which made the final concentration of the inoculum, 3.58 pM (Table 3.1). To make the following round have the same final concentration using my larger tank of 61 l, I would have had to have added 200 µl of $^{32}P_i$. This amount was too

expensive to be repeated over the course of my experiment. If repeated, I would have started my first round with a smaller final concentration in order to maintain that molarity of both P_i and ATP between rounds. I did, however, adjust my concentrations when tracing uptake in *H. heliophila* so that my finals concentrations of the P_i and ATP inoculum were 1.36 pM (Table 3.1)

When handled carefully, ^{32}P is a safe and powerful tool for tracing phosphorus. Sponge microbiology in particular would benefit from more tracing studies using either ^{32}P or ^{33}P with more sponge species. It would be beneficial for future studies to extend the time series beyond 78 hours, taking sponge and water samples 1x per day for several weeks to better understand if sponges will take in phosphorus uninterrupted or if they reach a certain uptake threshold. Additionally, studies using a range of DIP or DOP concentrations could reveal how uptake rates correlate to substrate concentrations in order to apply a Michaelis-Menten kinetics model.

Although this study confirms uptake of DIP and DOP by sponges, it is still unknown whether sponges acquire phosphorus from POP from heterotrophic feeding or symbiont acquisition. By radiolabeling bacterial cultures and subsequently inoculating tanks with this source of POP, further insights can be made into the source of phosphorus in the sponge diet. Secondly, we stress the importance of extending the knowledge of the sponge loop beyond the scope of carbon and nitrogen. Using ^{32}P within cell shedding and detritus production studies would add an essential element to the narrative of the transformations of DOM to POM by marine sponges and their symbionts. Lastly, there is a large research gap regarding the outputs of phosphorus by sponges. More studies that measure phosphorus efflux will be useful in understanding if various sponge species are

sources of bioavailable P_i. Further, one could attempt to determine if apatite is found in the sand beneath sponges to see if sponges may be sinks with coral reefs.

5.2.3 Moving forward with more genetic and chemical analysis

The limiting factor for my poly-P analysis was the amount of sponge tissue available. I collected my sponges before knowing how much material I would need for my extraction protocol. In order to do the assay with replicates, I was only able to complete one round with *H. heliophila*. These replicates were technical replicates as I pooled and lyophilized all sponge tissue together after collection. I have decided to pursue this experiment further in the spring of 2021, once weather permits *H. heliophila* to be collected again by kayak and snorkel. The extraction protocol will be repeated with both sponges and biological replicates will be used to strengthen the data. I will also collect water samples for nutrient analysis as I am particularly interested in the amount of phosphate in the water.

Within the past few decades, extensive genetic insights into the marine sponge microbiome have been made (reviewed by Hentschel et al. 2012). However, compared with other relevant gene markers involving nutrient cycling (such as *nifH* and *amoA*), *ppk* and *ppx* are understudied. It could of great interest to amplify *ppk* and *ppx* genes from a wide range of sponges including those found near hydrothermal vent systems, those containing high and low assemblages of cyanobacteria, and sponges found within oligotrophic and eutrophic habitats. In addition to these genetic analyses, further insights into poly-P concentrations within sponges is needed. Along with Zhang et al. 2015 and Ou et al. 2020, this study sets the stage for understanding sponges' role in phosphorus

accumulation. We now propose the following questions for future studies: (1) Which sponges in coral reefs store the most phosphorus in the form of poly-P? (2) Do these sponges release a substantial amount of P_i while pumping or do they combine phosphate molecules with calcium to form recalcitrant apatite? Measuring poly-P accumulation, P_i efflux, and apatite formation within more sponges would be a crucial step in understanding whether sponges are a source of sink of phosphorus.

A dual hypothesis that has come out of my thesis research is that phosphorus uptake, as well as poly-P storage, could be driven by two factors: cyanobacterial abundance and nutrient availability. In the case of the two sponges studied here, *L. chondrodes* stored more phosphorus as poly-P. Additionally, this sponge is found in oligotrophic habitats in the South Pacific (0.15 ± 0.01 μM PO_4^{3-}, Fong et al. 2020) and has a large assemblage of cyanobacteria (68% of its total bacterial community). I am not able to conclude which factor is the most important in predicting a high concentration of poly-P within sponges. However, these correlations that I found are consistent with the other studies on marine sponges and phosphorus acquisition. First, Ou et al. reported that poly-P enrichment rates were higher in sponges found in waters with low nutrient availability (Ou et al. 2020). This group also reported higher poly-P enrichment rates were correlated with the cyanobacterial genus *Synechococcus* (Ou et al. 2020). Second, Zhang et al. reported accumulation of poly-P granules within an enrichment culture of a cyanobacterial symbiont, suggesting a key role for cyanobacteria in poly-P accumulation in the sponge host. Designing studies that would aim to determine if cyanobacterial abundance or oligotrophy are driving factors of phosphorus storage would be valuable in developing this story further. It would be particularly interesting to examine sponges with

significantly different cyanobacterial abundances to further determine if these taxa are a driving factor in phosphorus cycling. One way of approaching this study design would be to look at a sponge species under different conditions of light availability. For example, Burgsdorf et al. (2014) sampled two morphotypes of *Petrosia ficiformis*, one inside caves with low cyanobacterial abundance, and one living in lighted areas and thus hosting a higher abundance of cyanobacteria (Burgsdorf et al. 2014). Studying the individuals of the same species but with dissimilar cyanobacterial abundances would provide an opportunity to examine if these taxa are key in poly-P storage.

5.2.4 Final thoughts

Because of their prevalence within phosphorus-limited ecosystems and their ability to pump large amounts of seawater, sponges are likely to be significant players within the phosphorus biogeochemical cycles of reefs. Further, availability of phosphorus may control atmospheric oxygen and carbon dioxide levels over geologic timescales due to the involvement of phosphorus in primary production (Van Cappellen & Ingall 1996). This thesis shows that two marine sponges with dissimilar microbial assemblages and geographic habitats take up DIP and DOP from the surrounding water. I elucidated that *L. chondrodes*, a sponge dominated by cyanobacteria, stores 6–8% of its phosphorus as poly-P, while *H. heliophila,* a sponge dominated by Proteobacteria, stores 0.55%. Further, both sponges were found to host symbionts that encode for *ppk*. A more complete understanding of how marine sponges control phosphorus availability requires more attention, including additional tracing studies, genetic analyses, and the characterization of potential sponge effluxes such as poly-P and apatite.

Appendix to Chapter 2

SN 15 Medium
(marine autotrophic isolation)

Mix 750 ml filtered seawater and 250 ml distilled H_2O (final salinity 15 ppt). Autoclave in a teflon lined bottle, then add the following compounds aseptically:

$NaNO_3$ (300.0 g/L dH_2O)	2.5 ml
K_2HPO_4 (anhydrous) (6.1 g/L dH_2O)	2.6 ml
$Na_2EDTA \cdot 2\ H_2O$ (1.0 g/L dH_2O)	5.6 ml
Na_2CO_3 (4.0 g/L dH_2O)	2.6 ml
Vitamin B_{12} (1.0 mg/L dH_2O)	1.0 ml
Cyano Trace Metal Solution	1.0 ml

Cyano Trace Metal Solution:

Citric Acid • H_2O	6.25 g
Ferric Ammonium Citrate	6.0 g
$MnCl_2 \cdot 4\ H_2O$	1.4 g
$Na_2MoO_4 \cdot 2\ H_2O$	0.39 g
$Co(NO_3)_2 \cdot 6\ H_2O$	0.025 g
$ZnSO_4 \cdot 7\ H_2O$	0.222 g

Dissolve each metal compound individually in 100 ml dH_2O. Combine and mix the six solutions, then bring volume up to 1.0 L with dH_2O.

Appendix to Chapter 4

Table A4.1 Primers used to amplify *ppk* gene from the sponge *Haliclona caerulea*.

Primer Name	Nucleotide Sequence	Target Region	Organism	Original Reference
IPCRppk-1375 (forward)	5' CAC GCC AAG ATG CTG ATG ATC G 3'	Polyphosphate kinase (*ppk*)	*Haliclona caerulea*	McMahon et al. 2002
IPCRppk-0289 (reverse)	5' GCG TTT CGTCGC AGA CCA GGC G 3'	*ppk*	*Haliclona caerulea*	McMahon et al 2002

Table A4.2 Results from sequence alignment using NCBI BLASTx.

Sequence Name	Nearest BLASTx hit (Accession #)	Taxonomy	Identity (%)	Coverage (%)	Sponge	Primer
HC_9B	polyphosphate kinase 1 (WP_025769808)	*Thioalkalivibrio* sp.	100	82	*Haliclona caerulea*	IPCR

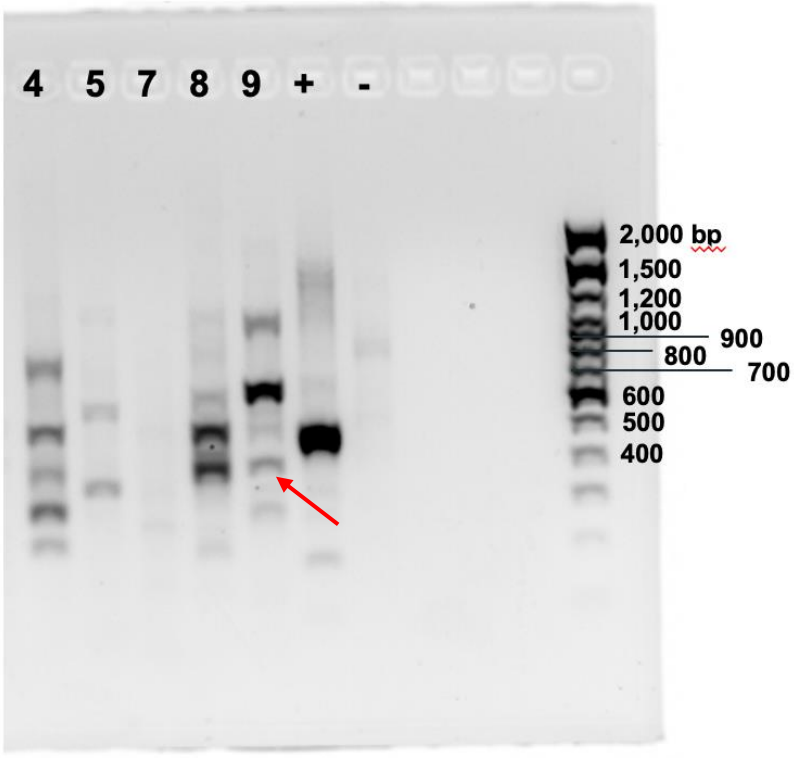


Figure A4.1 Results of gel electrophoresis after amplification of *ppk*. Bands in lane '9' are DNA from the sponge, *Haliclona caerulea.* Red arrow indicates the band that was found to be *ppk*. Band produced a sequence that aligned to *ppk*1 from *Thioalkalivibrio* sp. (Table A4.2). All other lanes and bands were unable to be sequenced due to low quality. The *ppk* gene was detected in the sponge *Haliclona caerulea*, collected from Kaneohe Bay until 2018. Table A4.1 shows the primer pair used the PCR reaction to amplify *ppk*.

Bibliography

Achbergerová, L., & Nahálka, J. (2011). Polyphosphate - an ancient energy source and active metabolic regulator. *Microbial Cell Factories* 10: 63. https://doi.org/10.1186/1475-2859-10-63

Acinas, S. G., Haverkamp, T. H. A., Huisman, J., & Stal, L. J. (2009). Phenotypic and genetic diversification of *Pseudanabaena* spp. (cyanobacteria). *ISME Journal* 3: 31-46. https://doi.org/10.1038/ismej.2008.78

Aguilar-May, B., & del Pilar Sánchez-Saavedra, M. (2009). Growth and removal of nitrogen and phosphorus by free-living and chitosan-immobilized cells of the marine cyanobacterium *Synechococcus elongatus*. *Journal of Applied Phycology* 21: 353-360. https://doi.org/10.1007/s10811-008-9376-7

Akiyama, M., Crooke, E., & Kornberg, A. (1993). An exopolyphosphatase of *Escherichia coli*. The enzyme and its *ppx* gene in a polyphosphate operon. *The Journal of Biological Chemistry* 268: 633-639. https://doi.org/10.1016/S0021-9258(18)54198-3

Albrecht, M., Pröschold, T., & Schumann, R. (2017). Identification of Cyanobacteria in a eutrophic coastal lagoon on the Southern Baltic Coast. *Frontiers in Microbiology* 8: 923. https://doi.org/10.3389/fmicb.2017.00923

Alex, A., Vasconcelos, V., Tamagnini, P., Santos, A., & Antunes, A. (2012). Unusual symbiotic cyanobacteria association in the genetically diverse intertidal marine sponge *Hymeniacidon perlevis* (Demospongiae, Halichondrida). *PLoS ONE* 7: e51834. https://doi.org/10.1371/journal.pone.0051834

Alex, A., Silva, V., Vasconcelos, V., & Antunes, A. (2013). Evidence of unique and generalist microbes in distantly related sympatric intertidal marine sponges (Porifera: Demospongiae). *PLoS ONE* 8: e80653. https://doi.org/10.1371/journal.pone.0080653

Allgaier, M., Uphoff, H., Felske, A., & Wagner-Döbler, I. (2003). Aerobic anoxygenic photosynthesis in *Roseobacter* clade bacteria from diverse marine habitats. *Applied and Environmental Microbiology* 69: 5051-5059. https://doi.org/10.1128/AEM.69.9.5051-5059.2003

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology* 215: 403-410. https://doi.org/10.1016/S0022-2836(05)80360-2

Anderson, D. M., Glibert, P. M., & Burkholder J. M. (2002). Harmful algal blooms and eutrophication: Nutrient sources, composition, and consequences. *Estuaries* 25: 704-726. https://doi.org/10.1007/BF02804901

Anisimova, M., & Gascuel, O. (2006). Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. *Systematic Biology* 55: 539-552. https://doi.org/10.1080/10635150600755453

Atkinson, M. J. (1987a). Rates of phosphate uptake by coral reef flat communities. *Limnology and Oceanography* 32: 426-435. https://doi.org/10.4319/lo.1987.32.2.0426

Atkinson M. J. (1987b). Alkaline phosphatase activity of coral reef benthos. *Coral Reefs* 6: 59-62. https://doi.org/10.1007/BF00301374

Atkinson, M. J., & Smith, D. F. (1987). Slow uptake of ^{32}P over a barrier reef flat. *Limnology and Oceanography* 32: 436-441. https://doi.org/10.4319/lo.1987.32.2.0436

Azam, F., Fenchel, T., Field, J., Gray, J., Meyer-Reil, L., & Thingstad, F. (1983). The ecological role of water-column microbes in the sea. *Marine Ecology Progress Series* 10: 257-263. https://doi.org/10.3354/meps010257

Bahram, M., Anslan, S., Hildebrand, F., Bork, P., & Tedersoo, L. (2019). Newly designed 16S rRNA metabarcoding primers amplify diverse and novel archaeal taxa from the environment. *Environmental Microbiology Reports* 11: 487-494. https://doi.org/10.1111/1758-2229.12684

Batista, D., Muricy, G., Rocha, R. C., & Miekeley, N. F. (2014). Marine sponges with contrasting life histories can be complementary biomonitors of heavy metal pollution in coastal ecosystems. *Environmental Science and Pollution Research* 21: 5785-5794. https://doi.org/10.1007/s11356-014-2530-7

Batista, D., Costa, R., Carvalho, A. P., Batista, W. R., Rua, C. P. J., de Oliveira, L., Leomil, L., Fróes, A. M., Thompson, F. L., Coutinho, R., & Dobretsov, S. (2018). Environmental conditions affect activity and associated microorganisms of marine sponges. *Marine Environmental Research* 142: 59-68. https://doi.org/10.1016/j.marenvres.2018.09.020

Becerro, M. A., Thacker, R. W., Turon, X., Uriz, M. J., & Paul, V. J. (2003). Biogeography of sponge chemical ecology: Comparisons of tropical and temperate defenses. *Oecologia* 135: 91-101. https://doi.org/10.1007/s00442-002-1138-7

Belda, C. A., Lucas, J. S., & Yellowlees, D. (1993). Nutrient limitation in the giant clam-zooxanthellae symbiosis: effects of nutrient supplements on growth of the symbiotic partners. *Marine Biology* 117: 655-664. https://doi.org/10.1007/BF00349778

Bell, J. J., Rovellini, A., Davy, S. K., Taylor, M. W., Fulton, E. A., Dunn, M. R., Bennett, H. M., Kandler, N. M., Luter, H. M., & Webster, N. S. (2018). Climate change alterations to ecosystem dominance: how might sponge-dominated reefs function? *Ecology* 99: 1920-1931. https://doi.org/10.1002/ecy.2446

Bell, D. W., Pellechia, P. J., Ingall, E. D., & Benitez-Nelson, C. R. (2020). Resolving marine dissolved organic phosphorus (DOP) composition in a coastal estuary. *Limnology and Oceanography* 65: 2787-2799. https://doi.org/10.1002/lno.11552

Benitez-Nelson, C. R., & Buesseler, K. O. (1998). Measurement of cosmogenic ^{32}P and ^{33}P activities in rainwater and seawater. *Analytical Chemistry* 70: 64-72. https://doi.org/10.1021/ac9707500

Benitez-Nelson, C. R., & Buesseler, K. O. (1999). Variability of inorganic and organic phosphorus turnover rates in the coastal ocean. *Nature* 398: 502-505. https://doi.org/10.1038/19061

Benitez-Nelson, C. R. (2000). The biogeochemical cycling of phosphorus in marine systems. *Earth Science Reviews* 51: 109-135. https://doi.org/10.1016/S0012-8252(00)00018-0

Berthold, M., & Schumann, R. (2020). Phosphorus dynamics in a eutrophic lagoon: uptake and utilization of nutrient pulses by phytoplankton. *Frontiers in Marine Science* 7: 281. https://doi.org/10.3389/fmars.2020.00281

Björkman, K. M. (2014). Polyphosphate goes from pedestrian to prominent in the marine P-cycle. *Proceedings of the National Academy of Sciences of the United States of America* 111: 7890-7891. https://doi.org/10.1073/pnas.1407195111

Björkman, K. M, & Karl, D. M. (2003). Bioavailability of dissolved organic phosphorus in the euphotic zone at Station ALOHA, North Pacific Subtropical Gyre. *Limnology and Oceanography* 48: 1049-1057. https://doi.org/10.4319/lo.2003.48.3.1049.

Blanchard, Z. (2014). Saltwater fish and reef tanks: From beginner to expert. Zechariah Blanchard; Second Edition.

Bond, C., & Harris, A. K. (1988). Locomotion of sponges and its physical mechanism. *Journal of Experimental Zoology* 246: 271-284. https://doi.org/10.1002/jez.1402460307

Bordenstein, S. R., & Theis, K. R. (2015). Host biology in light of the microbiome: Ten principles of holobionts and hologenomes. *PLoS Biology* 13: e1002226. https://doi.org/10.1371/journal.pbio.1002226

Bovio, E., Fauchon, M., Toueix, Y., Mehiri, M., Varese, G. C., & Hellio, C. (2019). The sponge-associated fungus *Eurotium chevalieri* mut 2316 and its bioactive

molecules: potential applications in the field of antifouling. *Marine Biotechnology* 21: 743-752. https://doi.org/10.1007/s10126-019-09920-y

Broecker, W. S., & Peng, T. H. (1983). Tracers in the Sea. https://doi.org/10.1016/0016-7037(83)90075-3

Brown, M. R. W., & Kornberg, A. (2004). Inorganic polyphosphate in the origin and survival of species. *Proceedings of the National Academy of Sciences of the United States of America* 101: 16085-16087. https://doi.org/10.1073/pnas.0406909101

Brown, M. R. W., & Kornberg, A. (2008). The long and short of it - polyphosphate, PPK and bacterial survival. *Trends in Biochemical Sciences* 33: 284-290. https://doi.org/10.1016/j.tibs.2008.04.005.

Brümmer, F., & Nickel, M. (2003). Sustainable use of marine resources: cultivation of sponges. *Progress in Molecular and Subcellular Biology* 37:143-162. https://doi.org/10.1007/978-3-642-55519-0_6

Bryan, P. G. (1973). Growth rate, toxicity, and distribution of the encrusting sponge *Terpios* sp. (Hadromerida: Suberitidae) in Guam, Mariana Islands. *Micronesica*.

Burgsdorf, I., Erwin, P. M., López-Legentil, S., Cerrano, C., Haber, M., Frenk, S., & Steindler, L. (2014). Biogeography rather than association with cyanobacteria structures symbiotic microbial communities in the marine sponge *Petrosia ficiformis*. *Frontiers in Microbiology* 5: 529. https://doi.org/10.3389/fmicb.2014.00529

Burgsdorf, I., Slaby, B. M., Handley, K. M., Haber, M., Blom, J., Marshall, C. W., Gilbert, J. A., Hentschel, U., & Steindler, L. (2015). Lifestyle evolution in cyanobacterial symbionts of sponges. *MBio* 6: e00391-15. https://doi.org/10.1128/mBio.00391-15

Butler, E. I., Corner, E. D. S., & Marshall, S. M. (1970). On the nutrition and metabolism of zooplankton 7. Seasonal survey of nitrogen and phosphorus excretion by *Calanus* in the Clyde Sea-area. *Journal of the Marine Biological Association of the United Kingdom* 50: 525-560. https://doi.org/10.1017/S0025315400004707

Chanas, B., Pawlik, J. R., Lindel, T., & Fenical, W. (1997). Chemical defense of the Caribbean sponge *Agelas clathrodes* (Schmidt). *Journal of Experimental Marine Biology and Ecology* 208: 185-196. https://doi.org/10.1016/S0022-0981(96)02653-6

Candelas, G. C., & Candelas, G. A. (1963). Notes on the seasonal distribution of the sponge *Hymeniacidon heliophila* at Beaufort, North Carolina. *Ecology* 44: 595-597. https://doi.org/10.2307/1932546

Candelas, G. C., & Candelas, G. A. (1964). A criterion for tolerance investigations in marine sponges. *Nature* 201: 101-102. https://doi.org/10.1038/201101a0

Cantera, J. J. L., Kawasaki, H., & Seki, T. (2004). The nitrogen-fixing gene (*nifH*) of *Rhodopseudomonas palustris*: A case of lateral gene transfer? *Microbiology* 150: 2237-2246. https://doi.org/10.1099/mic.0.26940-0

Charpy L. (2001). Phosphorus supply for atoll biological productivity. *Coral Reefs* 20: 357-360. https://doi.org/10.1007/s00338-001-0182-9

Canadell, D., Bru, S., Clotet, J., & Ariño, J. (2016). Extraction and quantification of polyphosphate in the budding yeast *Saccharomyces cerevisiae*. *Bio-Protocol* 6: e1874. https://doi.org/10.21769/BioProtoc.1874

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N., & Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the National Academy of Sciences of the United States of America* 108: 4516-4522. https://doi.org/10.1073/pnas.1000080107

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* 17: 540-552. https://doi.org/10.1093/oxfordjournals.molbev.a026334

Chevenet, F., Brun, C., Bañuls, A. L., Jacq, B., & Christen, R. (2006). TreeDyn: Towards dynamic graphics and annotations for analyses of trees. *BMC Bioinformatics* 7: 439. https://doi.org/10.1186/1471-2105-7-439

Choi, H., Im, W. T., & Park, J. S. (2020). *Corallincola spongiicola* sp. nov., isolated from sponge. 113: 643-650. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology.* https://doi.org/10.1007/s10482-019-01371-8.

Chombard, C., Boury-Esnault, N., & Tillier, S. (1998). Reassessment of homology of morphological characters in Tetractinellid sponges based on molecular data. *Systematic Biology* 47: 351-366. https://doi.org/10.1080/106351598260761

Christ, J. J., Smith, S. A., Willbold, S., Morrissey, J. H., & Blank, L. M. (2020). Biotechnological synthesis of water-soluble food-grade polyphosphate with *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering* 117: 2089-2099. https://doi.org/10.1002/bit.27337

Coelho, F. J. R. C., Cleary, D. F. R., Gomes, N. C. M., Pólonia, A. R. M., Huang, Y. M., Liu, L. L., & de Voogd, N. J. (2018). Sponge prokaryote communities in Taiwanese coral reef and shallow hydrothermal vent ecosystems. *Microbial Ecology* 75: 239-254. https://doi.org/10.1007/s00248-017-1023-x

Cogan, E. B., Birrell, G. B., & Griffith, O. H. (1999). A robotics-based automated assay for inorganic and organic phosphates. *Analytical Biochemistry* 271: 29-35. https://doi.org/10.1006/abio.1999.4100.

Colman, A. S. (2015). Sponge symbionts and the marine P cycle. *Proceedings of the National Academy of Sciences* 112: 4191-4192. https://doi.org/10.1073/pnas.1502763112

Cook, P. A., Stewart, B. A., & Achituv, Y. (1991). The symbiotic relationship between the hydrocoral *Millepora dichotoma* and the barnacle *Savignium milleporum*. *Hydrobiologia* 216: 285-290. https://doi.org/10.1007/BF00026476

Corredor, J. E., Wilkinson, C. R., Vicente, V. P., Morell, J. M., & Otero, E. (1988). Nitrate release by Caribbean reef sponges. *Limnology and Oceanography* 33: 114-120. https://doi.org/10.4319/lo.1988.33.1.0114

Costa, M. L., de Andrade Rosa, I., Andrade, L., Mermelstein, C., & C. Coutinho, C. (2020). Distinct interactions between epithelial and mesenchymal cells control cell morphology and collective migration during sponge epithelial to mesenchymal transition. *Journal of Morphology* 281: 183-195. https://doi.org/10.1002/jmor.21090

Cox, G. C., Hiller, R. G., & Larkum, A. W. D. (1985). An unusual cyanophyte, containing phycourobilin and symbiotic with ascidians and sponges. *Marine Biology* 89: 149-163. https://doi.org/10.1007/BF00392886

Custódio, M. R., Hajdu, E., & Muricy, G. (2004). Cellular dynamics of in vitro allogeneic reactions of *Hymeniacidon heliophila* (Demospongiae: Halichondrida). *Marine Biology* 144: 999-1010. https://doi.org/10.1007/s00227-003-1265-7

Coutinho, C. C., De Andrade Rosa, I., De Oliveira Teixeira, J. D., Andrade, L. R., Costa, M. L., & Mermelstein, C. (2017). Cellular migration, transition and interaction during regeneration of the sponge *Hymeniacidon heliophila*. *PLoS ONE* 12: e0178350. https://doi.org/10.1371/journal.pone.0178350

D'Angelo, C., & Wiedenmann, J. (2014). Impacts of nutrient enrichment on coral reefs: New perspectives and implications for coastal management and reef survival. *Current Opinion in Environmental Sustainability* 7: 82-93. https://doi.org/10.1016/j.cosust.2013.11.029

Davis, K. E. R., Joseph, S. J., & Janssen, P. H. (2005). Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. *Applied and Environmental Microbiology* 71: 826-834. https://doi.org/10.1128/AEM.71.2.826-834.2005

Deane, E. M., & O'Brien, R. W. (1981). Uptake of phosphate by symbiotic and free-living dinoflagellates. *Archives of Microbiology* 128: 307-310. https://doi.org/10.1007/BF00422536

D'Elia, C. F. (1977). The uptake and release of dissolved phosphorus by reef corals. *Limnology and Oceanography* 22: 301-315. https://doi.org/10.4319/lo.1977.22.2.0301

de Goeij, J. M., van Oevelen, D., Vermeij, M. J. a, Osinga, R., Middelburg, J. J., de Goeij, A. F. P. M., & Admiraal, W. (2013). Surviving in a marine desert : The sponge loop retains resources within coral reefs. *Science* 342: 108-110. https://doi.org/10.1126/science.1241981

de Goeij, J. M., Moodley, L., Houtekamer, M., Carballeira, N. M., & Van Duyl, F. C. (2008). Tracing 13C-enriched dissolved and particulate organic carbon in the bacteria-containing coral reef sponge *Halisarca caerulea*: Evidence for DOM feeding. *Limnology and Oceanography* 53: 1376-1358. https://doi.org/10.4319/lo.2008.53.4.1376

den Haan, J., Huisman, J., Brocke, H. J., Goehlich, H., Latijnhouwers, K. R. W., van Heeringen, S., et al. (2016). Nitrogen and phosphorus uptake rates of different species from a coral reef community after a nutrient pulse. *Scientific Reports* 6: 28821. https://doi.org/10.1038/srep28821

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., et al. (2008). Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Research* 36: W465-9. https://doi.org/10.1093/nar/gkn180.

Dereeper, A., Audic, S., Claverie, J. M., & Blanc, G. (2010). BLAST-EXPLORER helps you building datasets for phylogenetic analysis. *BMC Evolutionary Biology* 10: 8. https://doi.org/10.1186/1471-2148-10-8.

Diaz, J., Ingall, E., Benitez-Nelson, C., Paterson, D., De Jonge, M. D., McNulty, I., & Brandes, J. A. (2008). Marine polyphosphate: A key player in geologic phosphorus sequestration. *Science* 320: 652-655. https://doi.org/10.1126/science.1151751

Diaz, M. C., & Ward, B. B. (1997). Sponge-mediated nitrification in tropical benthic communities. *Marine Ecology Progress Series* 156: 97-107. https://doi.org/10.3354/meps156097

Diaz, M. C., & Rützler, K. (2001). Sponges: An essential component of Caribbean coral reefs. *Bulletin of Marine Science* 69: 535-546.

Dinn, C., & Leys, S. P. (2018). Field guide to sponges of the Eastern Canadian Arctic. A field and laboratory identification guide Ver. 1.0 *Department of Biological Sciences University of Alberta Edmonton*

Dinn, C., Zhang, X., Edinger, E., & Leys, S. P. (2020). Sponge communities in the eastern Canadian Arctic: species richness, diversity and density determined using targeted benthic sampling and underwater video analysis. *Polar Biology* 43: 1287-1305. https://doi.org/10.1007/s00300-020-02709-z

Dittami, S. M., Arboleda, E., Auguet, J. C., Bigalke, A., Briand, E., Cárdenas, P., Cardini, U., Decelle, J., Engelen, A., Eveillard, D., Gachon, C. M. M., Griffiths, S., Harder, T., Kayal, E., Kazamia, E., Lallier, F. H., Medina, M., Marzinelli, E. M., Morganti, T., et al. (2019). A community perspective on the concept of marine holobionts: current status, challenges, and future directions. *PeerJ* 9: e10911. http://doi.org/10.7717/peerj.10911

Dyhrman, S. T., Ammerman, J. W., & Van Mooy, B. A. S. (2007). Microbes and the marine phosphorus cycle. *Oceanography* 20: 110-116. https://doi.org/10.5670/oceanog.2007.54

Easson, C. G., & Thacker, R. W. (2014). Phylogenetic signal in the community structure of host-specific microbiomes of tropical marine sponges. *Frontiers in Microbiology* 5: 532. https://doi.org/10.3389/fmicb.2014.00532

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32: 1792- 1797. https://doi.org/10.1093/nar/gkh340

Enticknap, J. J., Kelly, M., Peraud, O., & Hill, R. T. (2006). Characterization of a culturable alphaproteobacterial symbiont common to many marine sponges and evidence for vertical transmission via sponge larvae. *Applied and Environmental Microbiology* 72: 3724-3732. https://doi.org/10.1128/AEM.72.5.3724-3732.2006

Erpenbeck, D., Sutcliffe, P., Cook, S. de C., Dietzel, A., Maldonado, M., van Soest, R. W. M., Hooper, J. N. A., & Wörheide, G. (2012). Horny sponges and their affairs: On the phylogenetic relationships of keratose sponges. *Molecular Phylogenetics and Evolution* 63: 809-816. https://doi.org/10.1016/j.ympev.2012.02.024

Erwin, P. M., & Thacker, R. W. (2007). Incidence and identity of photosynthetic symbionts in Caribbean coral reef sponge assemblages. *Journal of the Marine Biological Association of the United Kingdom* 87: 1683-1692. https://doi.org/10.1017/S0025315407058213

Erwin, P. M., & Thacker, R. W. (2008a). Phototrophic nutrition and symbiont diversity of two Caribbean sponge-cyanobacteria symbioses. *Marine Ecology Progress Series*. https://doi.org/10.3354/meps07464

Erwin, P. M., & Thacker, R. W. (2008b). Cryptic diversity of the symbiotic cyanobacterium *Synechococcus spongiarum* among sponge hosts. *Molecular Ecology* 362: 139-147. https://doi.org/10.1111/j.1365-294X.2008.03808.x

Erwin, P. M., López-Legentil, S., & Turon, X. (2012). Ultrastructure, molecular phylogenetics, and chlorophyll a content of novel cyanobacterial symbionts in temperate sponges. *Microbial Ecology* 64: 771-783. https://doi.org/10.1007/s00248-012-0047-5

Fabricius, K. E. (2005). Effects of terrestrial runoff on the ecology of corals and coral reefs: Review and synthesis. *Marine Pollution Bulletin* 50: 125-146. https://doi.org/10.1016/j.marpolbul.2004.11.028

Fan, L., Reynolds, D., Liu, M., Stark, M., Kjelleberg, S., Webster, N. S., & Thomas, T. (2012). Functional equivalence and evolutionary convergence in complex communities of microbial sponge symbionts. *Proceedings of the National Academy of Sciences* 109: E1878-87. https://doi.org/10.1073/pnas.1203287109

Faulkner, D. J., Harper, M. K., Haygood, M. G., Salomon, C. E., & Schmidt, E. W. (2004). Symbiotic bacteria in sponges: Sources of bioactive substances. *In* Fusetani, N. (ed), Drugs from the Sea. https://doi.org/10.1159/000062486

Ferrier-Pagès, C., & Gattuso, J. P. (1998). Biomass, production and grazing rates of pico- and nanoplankton in coral reef waters (Miyako Island, Japan). *Microbial Ecology* 35: 46-57. https://doi.org/10.1007/s002489900059

Ferrier-Pagès C., Gattuso, J. P., Dallot, S., Jaubert, J. (2000). Effect of nutrient enrichment on growth and photosynthesis of the zooxanthellate coral *Stylophora pistillata Coral Reefs* 19: 103-113. https://doi.org/10.1007/s003380000078

Fiore, C. L., Jarett, J. K., Olson, N. D., & Lesser, M. P. (2010). Nitrogen fixation and nitrogen transformations in marine symbioses. *Trends in Microbiology* 18: 455-63. https://doi.org/10.1016/j.tim.2010.07.001

Fiore, C. L., Labrie, M., Jarett, J. K., & Lesser, M. P. (2015). Transcriptional activity of the giant barrel sponge, *Xestospongia muta* holobiont: Molecular evidence for metabolic interchange. *Frontiers in Microbiology* 6: 364. https://doi.org/10.3389/fmicb.2015.00364

Flórez, L. V., Biedermann, P. H. W., Engl, T., & Kaltenpoth, M. (2015). Defensive symbioses of animals with prokaryotic and eukaryotic microorganisms. *Natural Product Reports* 32: 904-936. https://doi.org/10.1039/c5np00010f

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3: 294-9. https://doi.org/10.1071/ZO9660275

Föllmi, K. B. (1996). The phosphorus cycle, phosphogenesis and marine phosphate-rich deposits. *Earth-Science Reviews* 4); 55-124. https://doi.org/10.1016/0012-

8252(95)00049-6

Fong, C. R., Gaynus, C. J., & Carpenter, R. C. (2020). Extreme rainfall events pulse substantial nutrients and sediments from terrestrial to nearshore coastal communities: a case study from French Polynesia. *Scientific Reports* 10: 2955. https://doi.org/10.1038/s41598-020-59807-5

Fieseler, L., Horn, M., Wagner, M., & Hentschel, U. (2004). Discovery of the novel candidate phylum "Poribacteria" in marine sponges. *Applied and Environmental Microbiology* 70: 3724-3732. https://doi.org/10.1128/AEM.70.6.3724-3732.2004

Fraley, C. D., Rashid, P. H., Lee, S. S. K., Gottschalk, R., Harrison, J., Wood, P. J., Brown, M. R. W., & Kornberg A. (2007). A polyphosphate kinase 1 (*ppk1*) mutant of *Pseudomonas aeruginosa* exhibits multiple ultrastructural and functional defects. *Proceedings of the National Academy of Sciences of the United States of America* 104: 3526-31. https://doi.org/10.1073/pnas.0609733104

Freeman, C. J., & Thacker, R. W. (2011). Complex interactions between marine sponges and their symbiotic microbial communities. *Limnology and Oceanography* 56: 1577-1586. https://doi.org/10.4319/lo.2011.56.5.1577

Freeman, C. J., Thacker, R. W., Baker, D. M., & Fogel, M. L. (2013). Quality or quantity: Is nutrient transfer driven more by symbiont identity and productivity than by symbiont abundance? *ISME Journal* 7: 1116-1125. https://doi.org/10.1038/ismej.2013.7

Fricke, H., Giere, O., Stetter, K., Alfredsson, G. A., Kristjansson, J. K., Stoffers, P., & Svavarsson, J. (1989). Hydrothermal vent communities at the shallow subpolar Mid-Atlantic ridge. *Marine Biology* 102: 425-429. https://doi.org/10.1007/BF00428495

Friedrich, A. B., Fischer, I., Proksch, P., Hacker, J., & Hentschel, U. (2001). Temporal variation of the microbial community associated with the Mediterranean sponge *Aplysina aerophoba*. *FEMS Microbiology Ecology* 38: 105-113. https://doi.org/10.1016/S0168-6496(01)00185-4

Galitz, A., Cook, S. D. E. C., Ekins, M., Hooper, J. N. A., Naumann, P. T., de Voogd, N. J., Wahab, M. A., Wörheide, G., & Erpenbeck, D. (2018). Identification of an aquaculture poriferan "Pest with Potential" and its phylogenetic implications. *PeerJ* 6: e5586. https://doi.org/10.7717/peerj.5586

Gao, Z. M., Wang, Y., Tian, R. M., Wong, Y. H., Batang, Z. B., Al-Suwailem, A. M., Bajic, V. B., & Qian, P. Y. (2014). Symbiotic adaptation drives genome streamlining of the cyanobacterial sponge symbiont "*Candidatus* Synechococcus spongiarum". *MBio* 5: e00079-14. https://doi.org/10.1128/mBio.00079-14

Giguere, L. A. (1981). Food assimilation efficiency as a function of temperature and meal size in larvae of *Chaoborus trivittatus* (Diptera: Chaoboridae). *The Journal of Animal Ecology* 50: 103-109. https://doi.org/10.2307/4034

Gili, J. M., & Coma, R. (1998). Benthic suspension feeders: Their paramount role in littoral marine food webs. *Trends in Ecology and Evolution* 13: 316-321. https://doi.org/10.1016/S0169-5347(98)01365-2

Ginn, B. K., Logan, A., & Thomas, M. L. H. (2000). Sponge ecology on sublittoral hard substrates in a high current velocity area. *Estuarine, Coastal and Shelf Science* 50: 403-413. https://doi.org/10.1006/ecss.1999.0563

Glibert, P. M., Hinkle, D. C., Sturgis, B., & Jesien, R. V. (2014). Eutrophication of a Maryland/Virginia coastal lagoon: a tipping point, ecosystem changes, and potential causes. *Estuaries and Coasts* 37: 128-146. https://doi.org/10.1007/s12237-013-9630-3

Gloeckner, V., Wehrl, M., Moitinho-Silva, L., Gernert, C., Hentschel, U., Schupp, P., Pawlik, J. R., Lindquist, N. L., Erpenbeck, D., & Wörheide, G. (2014). The HMA-LMA dichotomy revisited: An electron microscopical survey of 56 sponge species. *Biological Bulletin* 227: 78-88. https://doi.org/10.1086/BBLv227n1p78

Godinot, C., Ferrier-Pagès, C., & Grover, R. (2009). Control of phosphate uptake by zooxanthellae and host cells in the scleractinian coral *Stylophora pistillata*. *Limnology and Oceanography.* https://doi.org/10.4319/lo.2009.54.5.1627

Godinot, C., Ferrier-Pagès, C., Sikorski, S., & Grover, R. (2013). Alkaline phosphatase activity of reef-building corals. *Limnology and Oceanography* 54: 1627-1633. https://doi.org/10.4319/lo.2013.58.1.0227

Granato, A. C., Berlinck, R. G. S., Magalhães, A., Schefer, A. B., Ferreira, A. G., De Sanctis, B., De Freitas, J. C., Hajdu, E., & Migotto, A. E. (2000). Produtos naturais das esponjas marinhas *Aaptos* sp., *Hymeniacidon* aff. *heliophila*, e do nudibrânquio *Doris* aff. *verrucosa*. *Quimica Nova* 23: 594-599. https://doi.org/10.1590/s0100-40422000000500005

Grant, R. E. (1825). Observations on the structure and function of the sponge. *Edinburgh Philosophical Journal.* Edinburgh: Printed for A. Constable.

Grant, S. R., Church, M. J., Ferrón, S., Laws, E. A., & Rappé, M. S. (2019). Elemental composition, phosphorous uptake, and characteristics of growth of a SAR11 strain in batch and continuous culture. *mSystems* 4: e00218-18. https://doi.org/10.1128/msystems.00218-18

Guindon, S., & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* 52: 696-704. https://doi.org/10.1080/10635150390235520

Guindon, S., Lethiec, F., Duroux, P., & Gascuel, O. (2005). PHYML Online - A web server for fast maximum likelihood-based phylogenetic inference. *Nucleic Acids Research* 33: W557-9. https://doi.org/10.1093/nar/gki352

Gulick, A. (1957). Phosphorus and the origin of life. *Annals of the New York Academy of Sciences* 69: 309-313. https://doi.org/10.1111/j.1749-6632.1957.tb49666.x

Hadas, E., Shpigel, M., & Ilan, M. (2009). Particulate organic matter as a food source for a coral reef sponge. *Journal of Experimental Biology* 212: 3643-50. https://doi.org/10.1242/jeb.027953

Hadas, E., Marie, D., Shpigel, M., & Ilan, M. (2006). Virus predation by sponges is a new nutrient-flow pathway in coral reef food webs. *Limnology and Oceanography* 51: 1548-1550. https://doi.org/10.4319/lo.2006.51.3.1548

Hallam, S. J., Mincer, T. J., Schleper, C., Preston, C. M., Roberts, K., Richardson, P. M., & DeLong, E. F. (2006). Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analyses of marine Crenarchaeota. *PLoS Biology* 4: e437. https://doi.org/10.1371/journal.pbio.0040095

Hammen, C. S., & Osborne, P. J. (1959). Carbon dioxide fixation in marine invertebrates: A survey of major phyla. *Science* 130: 1409-1410. https://doi.org/10.1126/science.130.3386.1409

Hansen, A. L., Robinson, R. J. (1953). The determination of organic phosphorus in seawater using perchloric acid oxidation. *The Journal of Marine Research* 12: 31-42

Hanson, C. E., McLaughlin, M. J., Hyndes, G. A., & Strzelecki, J. (2009). Selective uptake of prokaryotic picoplankton by a marine sponge (*Callyspongia* sp.) within an oligotrophic coastal system. *Estuarine, Coastal and Shelf Science* 84: 289-297. https://doi.org/10.1016/j.ecss.2009.05.019

Hatcher, A. (1994). Nitrogen and phosphorus turnover in some benthic marine invertebrates: implications for the use of C:N ratios to assess food quality. *Marine Biology* 121: 161-166. https://doi.org/10.1007/BF00349485

Henrikson, A. A., & Pawlik, J. R. (1995). A new antifouling assay method: Results from field experiments using extracts of four marine organisms. *Journal of Experimental Marine Biology and Ecology* 194: 157-165. https://doi.org/10.1016/00220981(95)00088-7

Hentschel, U., Fieseler, L., Wehrl, M., Gernert, C., Steinert, M., Hacker, J., & Horn, M. (2003). Microbial diversity of marine sponges. *Progress in Molecular and Subcellular Biology* 37: 59-88. https://doi.org/10.1007/978-3-642-55519-0_3

Hentschel, U, Hopke, J., Horn, M., Friedrich, A. B., Wagner, M., Hacker, J., & Moore, B. S. (2002). Molecular evidence for a uniform microbial community in sponges from different oceans. *Applied and Environmental Microbiology* 68: 4431-4440. https://doi.org/10.1128/AEM.68.9.4431-4440.2002

Hentschel, U., Piel, J., Degnan, S. M., & Taylor, M. W. (2012). Genomic insights into the marine sponge microbiome. *Nature Reviews Microbiology* 10: 641-654. https://doi.org/10.1038/nrmicro2839

Hernandez-Marine, M., Turon, X., & Catalan, J. (1990). A marine Synechocystis (Chroococcales, Cyanophyta) epizoic on didemnid ascidians from the Mediterranean Sea. *Phycologia*. https://doi.org/10.2216/i0031-8884-29-3-275.1

Hirschler, A., Lucas, J., & Hubert, J. C. (1990). Bacterial involvement in apatite genesis. *FEMS Microbiology Letters* 3: 275-284. https://doi.org/10.1016/0378-1097(90)90732-6

Hoffmann, F., Larsen, O., Thiel, V., Rapp, H. T., Pape, T., Michaelis, W., & Reitner, J. (2005). An anaerobic world in sponges. *Geomicrobiology Journal* 22: 1-10. https://doi.org/10.1080/01490450590922505

Hoffmann, F., Rapp, H. T., & Reitner, J. (2006). Monitoring microbial community composition by fluorescence *in situ* hybridization during cultivation of the marine cold-water sponge *Geodia barretti*. *Marine Biotechnology* 8: 373-379. https://doi.org/10.1007/s10126-006-5152-3

Holmström, C., Kjelleberg, S. (1999). Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. *FEMS Microbiology Ecol*ogy 30:285-293. https://doi.org/10.1111/j.1574-6941.1999.tb00656.x

Imhoff, J. F., & Trüper, H. G. (1976). Marine sponges as habitats of anaerobic phototrophic bacteria. *Microbial Ecology* 3: 1-9. https://doi.org/10.1007/BF02011449

Imsiecke, G., Münkner, J., Lorenz, B., Müller, W. E. G., Schroder, H. C., & Bachinski, N. (1996). Inorganic polyphosphates in the developing freshwater sponge *Ephydatia muelleri*: Effect of stress by polluted waters. *Environmental Toxicology and Chemistry* 15: 1329-1334. https://doi.org/10.1002/etc.5620150811

Itskovich, V. B., Kaluzhnaya, O. V., Veynberg, E., & Erpenbeck, D. (2015). Endemic Lake Baikal sponges from deep water.1: Potential cryptic speciation and discovery

of living species known only from fossils. *Zootaxa* 3990: 123. https://doi.org/10.11646/zootaxa.3990.1.7

Ivanova, E. P., Kiprianova, E. A., Mikhailov, V. V., Levanova, G. F., Garagulya, A. D., Gorshkova, N. M., et al. (1998). Phenotypic diversity of *Pseudoalteromonas citrea* from different marine habitats and emendation of the description. *International Journal of Systematic Bacteriology* 48: 247-256. https://doi.org/10.1099/00207713-48-1-247

Jackson, A. E., & Yellowlees, D. (1990). Phosphate uptake by zooxanthellae isolated from corals. *Proceedings of the Royal Society B: Biological Sciences* 242: 201-204. https://doi.org/10.1098/rspb.1990.0125

Jackson, G. A., Williams, P. M. (1985). Importance of dissolved organic nitrogen and phosphorus to biological nutrient cycling. *Deep Sea Research Part A. Oceanographic Research Papers* 32: 223-235. *https*://doi.org/10.1016/0198-0149(85)90030-5

Jackson, J. B. C., & Winston, J. E. (1982). Ecology of cryptic coral reef communities. I. Distribution and abundance of major groups of encrusting organisms. *Journal of Experimental Marine Biology and Ecology* 57: 135-147. https://doi.org/10.1016/0022-0981(82)901885

Jiménez, E., & Ribes, M. (2007). Sponges as a source of dissolved inorganic nitrogen: Nitrification mediated by temperate sponges. *Limnology and Oceanography* 52: 948-958. https://doi.org/10.4319/lo.2007.52.3.0948

Kamke, J., Sczyrba, A., Ivanova, N., Schwientek, P., Rinke, C., Mavromatis, K., Woyke, T., & Hentschel, U. (2013). Single-cell genomics reveals complex carbohydrate degradation patterns in poribacterial symbionts of marine sponges. *ISME Journal* 7: 2287-2300. https://doi.org/10.1038/ismej.2013.111

Karimi, E., Slaby, B. M., Soares, A. R., Blom, J., Hentschel, U., & Costa, R. (2018). Metagenomic binning reveals versatile nutrient cycling and distinct adaptive features in alphaproteobacterial symbionts of marine sponges. *FEMS Microbiology Ecology* 94: fiy074. https://doi.org/10.1093/femsec/fiy074

Karl, D. M., Letelier, R., Hebel, D. V., Bird, D. F., & Winn, C. D. (1992). *Trichodesmium* blooms and new nitrogen in the North Pacific gyre. *Marine Pelagic Cyanobacteria* 362: 219-237. https://doi.org/10.1007/978-94-015-7977-314

Karl, D. M., & Björkman, K. M. (2002). Dynamics of DOP. *In* Hansell. D.A., & Carlson, C. A. (eds). Biogeochemistry of Marine Dissolved Organic Matter 233-334. https://doi.org/10.1016/b978-012323841-2/50008-7.

Karl, D. M. (2014). Microbially mediated transformations of phosphorus in the sea: new views of an old cycle. *Annual Review of Marine Science* 6: 279-337. https://doi.org/10.1146/annurev-marine-010213-135046

Karl, D. M., & Tien, G. (1997). Temporal variability in dissolved phosphorus concentrations in the subtropical North Pacific Ocean. *Marine Chemistry* 56: 77-96. https://doi.org/10.1016/S0304-4203(96)00081-3

Keefe, A. D., Miller, S. L. (1995). Are polyphosphates or phosphate esters prebiotic reagents? *Journal of Molecular Evolution* 41: 693-702. https://doi.org/10.1007/BF00173147

Keesing, J. K., Usher, K. M., & Fromont, J. (2012). First record of photosynthetic cyanobacterial symbionts from mesophotic temperate sponges. *Marine and Freshwater Research* 63: 403-408. https://doi.org/10.1071/MF11216

Kersken, D., Göcke, C., Brandt, A., Lejzerowicz, F., Schwabe, E., Anna Seefeldt, M., Veit-Köhler, G., & Janussen, D. (2014). The infauna of three widely distributed sponge species (Hexactinellida and Demospongiae) from the deep Ekström Shelf in the Weddell Sea, Antarctica. *Deep-Sea Research Part II: Topical Studies in Oceanography* 108: 102-112. https://doi.org/10.1016/j.dsr2.2014.06.005

Kitazaki, C., Numano, S., Takanezawa, A., Nishizawa, T., Shirai, M., & Asayama, M. (2013). Characterization of lysis of the multicellular cyanobacterium *Limnothrix/Pseudanabaena* sp. strain ABRG5-3. *Bioscience, Biotechnology and Biochemistry* 77: 2239-2347. https://doi.org/10.1271/bbb.130409

Kling, H. J., Dail Laughinghouse, I., Šmarda, J., Komárek, J., Acreman, J., Bruun, K., Watson, S. B., & Chen, F. (2012). A new red colonial *Pseudanabaena* (Cyanoprokaryota, Oscillatoriales) from North American large lakes. *Fottea* 12: 327-339. https://doi.org/10.5507/fot.2012.023

Kobayashi, J., & Ishibashi, M. (1993). Bioactive metabolites of symbiotic marine microorganisms. *Chemical Reviews* 93: 1753-1769. https://doi.org/10.1021/cr00021a005

Kobayashi, M. (2004). Search for biologically active substances from marine sponges. *In* Fusetani, N. (ed): Drugs from the Sea. 46-45. https://doi.org/10.1159/000062481

Kolowith, L. C., Ingall, E. D., & Benner, R. (2001). Composition and cycling of marine organic phosphorus. *Limnology and Oceanography* 46: 309-320. https://doi.org/10.4319/lo.2001.46.2.0309

Koop, K., Booth, D., Broadbent, A., Brodie, J., Bucher, D., Capone, D., et al. (2001). ENCORE: The effect of nutrient enrichment on coral reefs. Synthesis of results and

conclusions. *Marine Pollution Bulletin* 42: 91-120. https://doi.org/10.1016/S0025-326X(00)00181-8

Kornberg, A., Rao, N. N., & Ault-Riché, D. (1999). Inorganic polyphosphate: A molecule of many functions. *Annual Review of Biochemistry* 68: 89-125. https://doi.org/10.1146/annurev.biochem.68.1.89

Kulaev I. S., & Vagabov, V. M. (1983). Polyphosphate metabolism in microorganisms. *Advances in Microbial Physiology* 24: 83-171. https://doi.org/10.1016/S0065-2911(08)60385-9

Lafi, F. F., Fuerst, J. A., Fieseler, L., Engels, C., Goh, W. W. L., & Hentschel, U. (2009). Widespread distribution of Poribacteria in demospongiae. *Applied and Environmental Microbiology* 75: 5695-5699. https://doi.org/10.1128/AEM.00035-0

Lal, D., & Lee, T. (1988). Cosmogenic ^{32}P and ^{33}P used as tracers to study phosphorus recycling in the upper ocean. *Nature* 333: 752-754. https://doi.org/10.1038/333752a0

Lane, D. J. (1991). 16S/23S rRNA Sequencing. *In* Stackebrandt, E., & Goodfellow, M. (eds). Nucleic acid techniques in bacterial systematics 115-175.

Lazzari, P., Solidoro, C., Salon, S., & Bolzon, G. (2016). Spatial variability of phosphate and nitrate in the Mediterranean Sea: A modeling approach. *Deep-Sea Research Part I: Oceanographic Research Papers* 108: 39-52. https://doi.org/10.1016/j.dsr.2015.12.006

Lee, J. J., McEnery, M. E., & Garrison, J. R. (1980). Experimental studies of larger foraminifera and their symbionts from the Gulf of Elat on the Red Sea. *Journal of Foraminiferal Research* 10: 31-47. https://doi.org/10.2113/gsjfr.10.1.31

Lee, R. M., Hartman, P. A., Olson, D. G., & Williams, F. D. (1994). Bactericidal and bacteriolytic effects of selected food-grade phosphates, using *Staphylococcus aureus* as a model system. *Journal of Food Production* 57: 276-283. https://doi.org/10.4315/0362-028X-57.4.276

Lee, Y. K., Lee, J. H., & Lee, H. K. (2001). Microbial symbiosis in marine sponges. *Journal of Microbiology* 39: 254-264

Leigh, B. A. (2019). Cooperation among conflict: prophages protect bacteria from phagocytosis. *Cell Host and Microbe* 26: 450-452. https://doi.org/10.1016/j.chom.2019.09.003

Lerner, C., Mothes, B., & Carraro, J. L. (2005). Novos registros e ampliação de limites meridionais de distribuição de poríferos (Porifera, Demospongiae) no Atlântico

sudoeste. *Revista Brasileira de Zoologia* 22: 596-612. https://doi.org/10.1590/s0101-81752005000300012

Lerouge, I., & Vanderleyden, J. (2002). O-antigen structural variation: Mechanisms and possible roles in animal/plant-microbe interactions. *FEMS Microbiology Reviews* 26: 17-47. https://doi.org/10.1016/S0168-6445(01)00070-5

Lesser, M. P. (2006). Benthic-pelagic coupling on coral reefs: Feeding and growth of Caribbean sponges. *Journal of Experimental Marine Biology and Ecology* 328: 277-288. https://doi.org/10.1016/j.jembe.2005.07.010

Lesser, M. P., Fiore, C., Slattery, M., & Zaneveld, J. (2016). Climate change stressors destabilize the microbiome of the Caribbean barrel sponge, *Xestospongia muta*. *Journal of Experimental Marine Biology and Ecology* 475: 11-18. https://doi.org/10.1016/j.jembe.2015.11.004

Lesser, M. P., Mueller, B., Pankey, M. S., Macartney, K. J., Slattery, M., & de Goeij, J. M. (2020). Depth-dependent detritus production in the sponge, *Halisarca caerulea*. *Limnology and Oceanography* 65: 1200-1216. https://doi.org/10.1002/lno.11384

Leys, S. P., Yahel, G., Reidenbach, M. A., Tunnicliffe, V., Shavit, U., & Reiswig, H. M. (2011). The sponge pump: The role of current induced flow in the design of the sponge body plan. *PLoS ONE* 6: e27787. https://doi.org/10.1371/journal.pone.0027787

Liu, J., & Vyverman, W. (2015). Differences in nutrient uptake capacity of the benthic filamentous algae *Cladophora* sp., *Klebsormidium* sp. and *Pseudanabaena* sp. under varying N/P conditions. *Bioresource Technology* 179: 234-242. https://doi.org/10.1016/j.biortech.2014.12.028

Liu, M., Fan, L., Zhong, L., Kjelleberg, S., & Thomas, T. (2012). Metaproteogenomic analysis of a community of sponge symbionts. *ISME Journal* 6: 1515-1525. https://doi.org/10.1038/ismej.2012.1

Liu, M. Y., Kjelleberg, S., & Thomas, T. (2011). Functional genomic analysis of an uncultured δ-proteobacterium in the sponge Cymbastela concentrica. *ISME Journal* 5: 427-435. https://doi.org/10.1038/ismej.2010.139

Liu, W. T., Nielsen, A. T., Wu, J. H., Tsai, C. S., Matsuo, Y., & Molin, S. (2001). *In situ* identification of polyphosphate- and polyhydroxyalkanoate-accumulating traits for microbial populations in a biological phosphorus removal process. *Environmental Microbiology* 3: 110-22. https://doi.org/10.1046/j.1462-2920.2001.00164.x

Lôbo-Hajdu, G., Guimarães, A. C. R., Salgado, A., Lamarão, F. R. M., Vieiralves, T., Mansure, J. J., & Albano, R. M. (2004). Intragenomic, intra- and interspecific variation in the rDNA ITS of Porifera revealed by PCR-Single-Strand Conformation

Polymorphism (PCR-SSCP). *Bollettino dei musei e degli istituti biologici dell'Universita di Genova* 68: 413-423.

Lopez, J. V., McCarthy, P. J., Janda, K. E., Willoughby, R., & Pomponi, S. A. (1999). Molecular techniques reveal wide phyletic diversity of heterotrophic microbes associated with *Discodermia* spp. (Porifera: Demospongiae). *Memoirs of the Queensland Museum* 44: 329-341.

Lysek, N., Kinscherf, R., Claus, R., & Lindel, T. (2003). L-5-hydroxytryptophan: Antioxidant and anti-apoptotic principle of the intertidal sponge *Hymeniacidon heliophila*. *Zeitschrift Fur Naturforschung - Section C Journal of Biosciences* 58: 568-72. https://doi.org/10.1515/znc-2003-7-821

Maldonado, M., Ribes, M., & van Duyl, F. C. (2012). Nutrient fluxes through sponges. Biology, budgets, and ecological implications. *Advances in Marine Biology* 62: 113-182. https://doi.org/10.1016/B978-0-12-394283-8.00003-5

Maldonado, M., Zhang, X., Cao, X., Xue, L., Cao, H., & Zhang, W. (2010). Selective feeding by sponges on pathogenic microbes: A reassessment of potential for abatement of microbial pollution. *Marine Ecology Progress Series* 403: 75-89. https://doi.org/10.3354/meps08411

Manz, W., Arp, G., Schumann-Kindel, G., Szewzyk, U., & Reitner, J. (2000). Widefield deconvolution epifluorescence microscopy combined with fluorescence in situ hybridization reveals the spatial arrangement of bacteria in sponge tissue. *Journal of Microbiological Methods* 40: 125-134. https://doi.org/10.1016/S0167-7012(99)00103-7

Martin, P., Dyhrman, S. T., Lomas, M. W., Poulton, N. J., & Van Mooy, B. A. S. (2014). Accumulation and enhanced cycling of polyphosphate by Sargasso Sea plankton in response to low phosphorus. *Proceedings of the National Academy of Sciences* 111: 8089-8094. https://doi.org/10.1073/pnas.1321719111

McClintock, J. B., Amsler, C. D., Baker, B. J., & van Soest, R. W. M. (2005). Ecology of antarctic marine sponges: An overview. *Integrative and Comparative Biology* 45: 359-368. https://doi.org/10.1093/icb/45.2.359

McFall-Ngai, M., Hadfield, M. G., Bosch, T. C. G., Carey, H. V., Domazet-Lošo, T., Douglas, A. E., et al. (2013). Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences of the United States of America* 110: 3229-3236. https://doi.org/10.1073/pnas.1218525110

McKelvie, I. D., Peat, D. M. W., & Worsfold, P. J. (1995). Analytical perspective. Techniques for the quantification and speciation of phosphorus in natural waters. *Analytic Proceedings Including Analytical Communications* 32: 437-445. https://doi.org/10.1039/AI9953200437

McMahon, K. D., Dojka, M. A., Pace, N. R., Jenkins, D., & Keasling, J. D. (2002). Polyphosphate kinase from activated sludge performing enhanced biological phosphorus removal. *Applied and Environmental Microbiology* 68: 4971-4978. https://doi.org/10.1128/AEM.68.10.4971-4978.2002

McMahon, K. D., & Read, E. K. (2013). Microbial contributions to phosphorus cycling in eutrophic lakes and wastewater. *Annual Review of Microbiology* 67: 199-219. https://doi.org/10.1146/annurev-micro-092412-155713

McMurray, S. E., Johnson, Z. I., Hunt, D. E., Pawlik, J. R., & Finelli, C. M. (2016). Selective feeding by the giant barrel sponge enhances foraging efficiency. *Limnology and Oceanography* 61: 1271-1286. https://doi.org/10.1002/lno.10287

McMurray, S. E., Stubler, A. D., Erwin, P. M., Finelli, C. M., & Pawlik, J. R. (2018). A test of the sponge-loop hypothesis for emergent Caribbean reef sponges. *Marine Ecology Progress Series* 588: 1-14. https://doi.org/10.3354/meps12466

Meyer, B., & Kuever, J. (2008). Phylogenetic diversity and spatial distribution of the microbial community associated with the Caribbean deep-water sponge *Polymastia* cf. *corticata* by 16S rRNA, aprA, and *amoA* gene analysis. *Microbial Ecology* 56: 306-21. https://doi.org/10.1007/s00248-007-9348-5

Meylan, A. (1988). Spongivory in hawksbill turtles: A diet of glass. *Science* 239: 393-395. https://doi.org/10.1126/science.239.4838.393

Miller, S. L., & Parris, M. (1964). Synthesis of pyrophosphate under primitive earth conditions. *Nature* 204: 1248-1250. https://doi.org/10.1038/2041248a0

Miller, S. L., & Urey, H. C. (1959). Organic compound synthesis on the primitive earth. *Science* 130: 245-251.https://doi.org/10.1126/science.130.3370.245

Mohamed, N. M., Rao, V., Hamann, M. T., Kelly, M., & Hill, R. T. (2008a). Monitoring bacterial diversity of the marine sponge *Ircinia strobilina* upon transfer into aquaculture. *Applied and Environmental Microbiology* 74: 4133-4143. https://doi.org/10.1128/AEM.00454-08

Mohamed, N. M., Enticknap, J. J., Lohr, J. E., McIntosh, S. M., & Hill, R. T. (2008b). Changes in bacterial communities of the marine sponge *Mycale laxissima* on transfer into aquaculture. *Applied and Environmental Microbiology* 74: 1209-1222. https://doi.org/10.1128/AEM.02047-07

Mohamed, N. M., Colman, A. S., Tal, Y., & Hill, R. T. (2008c). Diversity and expression of nitrogen fixation genes in bacterial symbionts of marine sponges. *Environmental Microbiology* 10: 2910-2921. https://doi.org/10.1111/j.14622920.2008.01704.x

Montalvo, N. F., & Hill, R. T. (2011). Sponge-associated bacteria are strictly maintained in two closely related but geographically distant sponge hosts. *Applied and Environmental Microbiology* 20: 7207-7216. https://doi.org/10.1128/AEM.05285-11

Monteiro, L. C., & Muricy, G. (2004). Patterns of sponge distribution in Cagarras Archipelago, Rio de Janeiro, Brazil. *Journal of the Marine Biological Association of the United Kingdom* 84: 681-687. https://doi.org/10.1017/S0025315404009750h

Morrow, K. M., Bourne, D. G., Humphrey, C., Botté, E. S., Laffy, P., Zaneveld, J., Uthicke, S., Fabricius, K. E., & Webster, N. S. (2015). Natural volcanic CO_2 seeps reveal future trajectories for host-microbial associations in corals and sponges. *ISME Journal* 9: 894-908. https://doi.org/10.1038/ismej.2014.188

Mukherjee, C., Chowdhury, R., & Ray, K. (2015). Phosphorus recycling from an unexplored source by polyphosphate accumulating microalgae and cyanobacteria-a step to phosphorus security in agriculture. *Frontiers in Microbiology* 6: 1421. https://doi.org/10.3389/fmicb.2015.01421

Muller-Parker, G., Cook, C., & D'Elia, C. (1990). Feeding affects phosphate fluxes in the symbiotic sea anemone *Aiptasia pallida*. *Marine Ecology Progress Series* 60: 283-29. https://doi.org/ 10.3354/meps060283.

Muricy, G., & Hajdu, E. (2006). Porifera brasilis: guia de identificação das esponjas mais comuns do Sudeste. *Série livros Museu Nacional, Rio de Janeiro.* Print.

Nguyen, M. T. H. D., Liu, M., & Thomas, T. (2014). Ankyrin-repeat proteins from sponge symbionts modulate amoebal phagocytosis. *Molecular Ecology* 23: 1635-1645. https://doi.org/10.1111/mec.12384

Odorico, D. M., & Miller, D. J. (1997). Internal and external relationships of the Cnidaria: Implications of primary and predicted secondary structure of the 5'-end of the 23S-like rDNA. *Proceedings of the Royal Society B: Biological Sciences* 264: 77-82. https://doi.org/10.1098/rspb.1997.0011

Okino, T., Yoshimura, E., Hirota, H., & Fusetani, N. (1995). Antifouling kalihinenes from the marine sponge Acanthella cavernosa. *Tetrahedron Letters* 36: 8637-8640. https://doi.org/10.1016/0040-4039(95)01861-B

O'Neil, J. M., & Capone, D. G. (2008). Nitrogen cycling in coral reef environments. *In* Capone, D. G., Bronk, D. A., Mullholland ,M. R., & Carpenter, E. J. (eds). Nitrogen in the Marine Environment. https://doi.org/10.1016/B978-0-12-372522-6.00021-9

Orchard, E. D., Ammerman, J. W., Lomas, M. W., & Dyhrman, S. T. (2010). Dissolved inorganic and organic phosphorus uptake in *Trichodesmium* and the microbial

community: The importance of phosphorus ester in the Sargasso Sea. *Limnology and Oceanography* 55: 1390-1399. https://doi.org/10.4319/lo.2010.55.3.1390

Oren, M., Steindler, L., & Ilan, M. (2005). Transmission, plasticity and the molecular identification of cyanobacterial symbionts in the Red Sea sponge *Diacarnus erythraenus*. *Marine Biology* 148: 35-41. https://doi.org/10.1007/s00227-005-0064-8

Ou, H., Li, M., Wu, S., Jia, L., Hill, R. T., & Zhao, J. (2020). Characteristic microbiomes correlate with polyphosphate accumulation of marine sponges in South China sea areas. *Microorganisms* 8: 63. https://doi.org/10.3390/microorganisms8010063

Partensky, F., Hess, W. R., & Vaulot, D. (1999). *Prochlorococcus*, a marine photosynthetic prokaryote of global significance. *Microbiology and Molecular Biology Reviews* 63: 106-27. https://doi.org/10.1128/mmbr.63.1.106-127.1999

Papadopoulos, J. S., & Agarwala, R. (2007). COBALT: Constraint-based alignment tool for multiple protein sequences. *Bioinformatics* 23: 1073-9. https://doi.org/10.1093/bioinformatics/btm076

Pawlik, J. R., Chanas, B., Toonen, R. J., & Fenical, W. (1995). Defenses of Caribbean sponges against predatory reef fish. I. Chemical deterrency. *Marine Ecology Progress Series* 127: 183-194. https://doi.org/10.3354/meps127183

Pawlik, Joseph R. (2011). The chemical ecology of sponges on caribbean reefs: Natural products shape natural systems. *BioScience* 61: 888-898. https://doi.org/10.1525/bio.2011.61.11.8

Paytan, A., & McLaughlin, K. (2007). The oceanic phosphorus cycle. *Chemical Reviews* 107: 563- 576. https://doi.org/10.1021/cr0503613

Pedrete, T. A., Coutinho, C. ., Batista, D., Hauser-Davis, R. A., Nudi, A. H., & Wagener, A. L. R. (2017). Toxicity evaluation of PAHs in the sponge *Hymeniacidon heliophila*: field assessment and laboratory assays - a preliminary study. *Ecotoxicology and Environmental Contamination* 12: 113-131. https://doi.org/10.5132/eec.2017.01.14

Phillips, D. H., & Arlt, V. M. (2007). The ^{32}P-postlabeling assay for DNA adducts. *Nature Protocols* 2: 2772-81. https://doi.org/10.1038/nprot.2007.394

Piel, J. (2005). Bacterial symbionts: Prospects for the sustainable production of invertebrate-derived pharmaceuticals. *Current Medicinal Chemistry* 13: 39-50. https://doi.org/10.2174/092986706775197944

Piel, Jörn, Hui, D., Wen, G., Butzke, D., Platzer, M., Fusetani, N., & Matsunaga, S. (2004). Antitumor polyketide biosynthesis by an uncultivated bacterial symbiont of

the marine sponge *Theonella swinhoei*. *Proceedings of the National Academy of Sciences of the United States of America* 101: 16222-16227. https://doi.org/10.1073/pnas.0405976101

Pile, A. J., Patterson, M. R., Savarese, M., Chernykh, V. I., & Fialkov, V. A. (1997). Trophic effects of sponge feeding within Lake Baikal's littoral zone. 2. Sponge abundance, diet, feeding efficiency, and carbon flux. *Limnology and Oceanography* 42: 178-184. https://doi.org/10.4319/lo.1997.42.1.0178

Pile, A. J., Patterson, M. R., & Witman, J. D. (1996). In situ grazing on plankton. *Marine Ecology Progress Series* 141: 95-102.

Pita, L., Rix, L., Slaby, B. M., Franke, A., & Hentschel, U. (2018). The sponge holobiont in a changing ocean: from microbes to ecosystems. *Microbiome* 6: 46. https://doi.org/10.1186/s40168-018-0428-1

Pomeroy, L. R., Kuenzler, E. J. (1969). Phosphorus turnover by coral reef animals. *In* Nelson, D.J., & Evans, F. C. (eds). Symposium on Radioecology 473-482.

Raheb, J., Naghdi, S., & Flint, K. P. (2006). Alkaline phosphatase activity of *Flexibacter chinensis* under starvation stress in water microcosms. *Enzyme and Microbial Technology* 40: 13-16. https://doi.org/10.1016/j.enzmictec.2005.12.024

Randall, J. E., & Hartman, W. D. (1968). Sponge-feeding fishes of the West Indies. *Marine Biology* 1: 216-225. https://doi.org/10.1007/BF00347115

Rao, N. N., Liu, S., & Kornberg, A. (1998). Inorganic polyphosphate in *Escherichia coli*: The phosphate regulon and the stringent response. *Journal of Bacteriology* 18: 2186-2193. https://doi.org/10.1128/jb.180.8.2186-2193.1998

Rao, N. N., Gómez-García, M. R., & Kornberg, A. (2009). Inorganic polyphosphate: Essential for growth and survival. *Annual Review of Biochemistry* 78: 605-647. https://doi.org/10.1146/annurev.biochem.77.083007.093039

Rashid, M. H., & Kornberg, A. (2000). Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the United States of America* 97: 4885-4890. https://doi.org/10.1073/pnas.060030097

Rashid, M. H., Rao, N. N., & Kornberg, A. (2000a). Inorganic polyphosphate is required for motility of bacterial pathogens. *Journal of Bacteriology* 182: 225-227. https://doi.org/10.1128/jb.182.1.225-227.2000.

Rashid, M. H., Rumbaugh, K., Passador, L., Davies, D. G., Hamood, A. N., Iglewski, B. H., et al. (2000b). Polyphosphate kinase is essential for biofilm development, quorum sensing, and virulence of Pseudomonas aeruginosa. *Proceedings of the*

National Academy of Sciences of the United States of America 97: 9636-41. https://doi.org/10.1073/pnas.170283397

Redfield, A. C. (1960). The biological control of chemical factors in the environment. *Science Progress* 46: 205-221. https://doi.org/10.2307/27828530

Reichert, W. L., French, B. L., & Stein, J. E. (1999). Exposure of marine mammals to genotoxic environmental contaminants: Application of the ^{32}P-postlabeling assay for measuring DNA-xenobiotic adducts. *Environmental Monitoring and Assessment* 56: 225–239. https://doi.org/10.1023/A:1005991116993

Reinfelder, J. R., & Fisher, N. S. (1991). The assimilation of elements ingested by marine copepods. *Science* 251: 794-796. https://doi.org/10.1126/science.251.4995.794

Reiswig, H. M. (1971). In situ pumping activities of tropical Demospongiae. *Marine Biology* 9: 38-50. https://doi.org/10.1007/BF00348816

Reiswig, H. M. (1971). Particle feeding in natural populations of three marine demosponges. *The Biological Bulletin* 141: 568-591. https://doi.org/10.2307/1540270

Reiswig, H. M. (1974). Water transport, respiration and energetics of three tropical marine sponges. *Journal of Experimental Marine Biology and Ecology* 14: 231-249. https://doi.org/10.1016/0022-0981(74)90005-7

Ribeiro, S. M., Bianco, É. M., Rogers, R., Teixeira, V. L., & Pereira, R. C. (2010). Chemical defense of *Hymeniacidon heliophila* (Porifera: Halichondrida) against tropical predators. *Brazilian Journal of Oceanography* 58: 315-321. https://doi.org/10.1590/S1679-87592010000400006

Ribes, M., Dziallas, C., Coma, R., & Riemann, L. (2015). Microbial diversity and putative diazotrophy in high- and low- microbial-abundance Mediterranean sponges. *Applied and Environmental Microbiology* 81: 5683-5693. https://doi.org/10.1128/AEM.01320-15

Ridley, C. P., Faulkner, D. J., and Haygood, M. G. (2005a). Investigation of *Oscillatoria spongeliae*-dominated bacterial communities in four Dictyoceratid sponges. *Applied and Environmental Microbiology* 71: 7366-75. doi:10.1128/AEM.71.11.7366-7375.2005

Ridley, C. P., Bergquist, P. R., Harper, M. K., Faulkner, D. J., Hooper, J. N. A., & Haygood, M. G. (2005b). Speciation and biosynthetic variation in four dictyoceratid sponges and their cyanobacterial symbiont, *Oscillatoria spongeliae*. *Chemistry and Biology* 12: 397-406. https://doi.org/10.1016/j.chembiol.2005.02.003

Ribes, M., Jiménez, E., Yahel, G., López-Sendino, P., Diez, B., Massana, R., Sharp, J.H.,

& Coma, R. (2012). Functional convergence of microbes associated with temperate marine sponges. *Environmental Microbiology* 14: 1224-1239. https://doi.org/10.1111/j.1462-2920.2012.02701.x

Ribes, M., Coma, R., & Gili, J. M. (1999). Natural diet and grazing rate of the temperate sponge *Dysidea avara* (Demospongiae, Dendroceratida) throughout an annual cycle. *Marine Ecology Progress Series* 176: 179-190. https://doi.org/10.3354/meps176179

Ribes, M., Calvo, E., Movilla, J., Logares, R., Coma, R., & Pelejero, C. (2016). Restructuring of the sponge microbiome favors tolerance to ocean acidification. *Environmental Microbiology Reports* 8: 536-544. https://doi.org/10.1111/1758-2229.12430

Richter, C., & Wunsch, M. (1999). Cavity-dwelling suspension feeders in coral reefs - A new link in reef trophodynamics. *Marine Ecology Progress Series* 188: 105-116. https://doi.org/10.3354/meps188105

Richter, C., Wunsch, M., Rasheed, M., Kötter, I., & Badran, M. I. (2001). Endoscopic exploration of Red Sea coral reefs reveals dense populations of cavity-dwelling sponges. *Nature* 413: 726-730. https://doi.org/10.1038/35099547

Rix, L., de Goeij, J. M., van Oevelen, D., Struck, U., Al-Horani, F. A., Wild, C., & Naumann, M. S. (2017). Differential recycling of coral and algal dissolved organic matter via the sponge loop. *Functional Ecology* 31: 778-789. https://doi.org/10.1111/1365-2435.12758

Rohde, S., Nietzer, S., & Schupp, P. J. (2015). Prevalence and mechanisms of dynamic chemical defenses in tropical sponges. *PLoS ONE* 10: e0132236. https://doi.org/10.1371/journal.pone.0132236

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

Rozas, E. E., Albano, R. M., Lôbo-Hajdu, G., Müller, W. E. G., Schröder, H. C., & Custödio, M. R. (2011). Isolation and cultivation of fungal strains from in vitro cell cultures of two marine sponges (Porifera: Halichondrida and Haplosclerida). *Brazilian Journal of Microbiology* 243: 1-10. https://doi.org/10.1590/S1517-83822011000400043

Rozas, E. E., Mendes, M. A., Nascimento, C. A. O., Espinosa, D. C. R., Oliveira, R., Oliveira, G., & Custodio, M. R. (2017). Bioleaching of electronic waste using bacteria isolated from the marine sponge *Hymeniacidon heliophila* (Porifera). *Journal of Hazardous Materials* 329: 120-130. https://doi.org/10.1016/j.jhazmat.2017.01.037

Rützler, K. (1990). Associations between Caribbean Sponges and photosynthetic organisms. *In* Rützler, K. (ed). New Perspectives in Sponge Biology 455-466.

Sabarathnam, B., Manilal, A., Sujith, S., Kiran, G. S., Selvin, J., Thomas, A., & Ravji, R. (2010). Role of sponge associated actinomycetes in the marine phosphorous biogeochemical cycles. *American-Eurasian Journal of Agricultural & Environmental Sciences* 8: 253–256. https://doi.org/UMI : 3257958

Sanyal, S., Banerjee, S. K., Banerjee, R., Mukhopadhyay, J., & Kundu, M. (2013). Polyphosphate kinase 1, a central node in the stress response network of *Mycobacterium tuberculosis*, connects the two-component systems MprAB and SenX3-RegX3 and the extracytoplasmic function sigma factor, sigma E. *Microbiology* 159: 2074-2086. https://doi.org/10.1099/mic.0.068452-0

Sarà, M., Balduzzi, A., Barbieri, M., Bavestrello, G., & Burlando, B. (1992). Biogeographic traits and checklist of Antarctic demosponges. *Polar Biology* 12: 559-585. https://doi.org/10.1007/BF00236980

Schaffelke, B., & Klumpp, D.W. (1998). Nutrient-limited growth of the coral reef macroalga *Sargassum baccularia* and experimental growth enhancement by nutrient addition in continuous flow culture. *Marine Ecology Progress Series* 164: 199-211. https://doi.org/ 10.3354/meps164199

Schaffelke, B., Carleton, J., Skuza, M., Zagorskis, I., &Furnas, M. J. (2012). Water quality in the inshore Great Barrier Reef lagoon: Implications for long-term monitoring and management. *Marine Pollution Bulletin* 65: 249-260. https://doi.org/10.1016/j.marpolbul.2011.10.031

Schander, C., Rapp, H. T., Kongsrud, J. A., Bakken, T., Berge, J., Cochrane, S., et al. (2010). The fauna of hydrothermal vents on the Mohn Ridge (North Atlantic). *Marine Biology Research* 6: 155-171.https://doi.org/10.1080/17451000903147450

Schmitt, S., Angermeier, H., Schiller, R., Lindquist, N., & Hentschel, U. (2008). Molecular microbial diversity survey of sponge reproductive stages and mechanistic insights into vertical transmission of microbial symbionts. *Applied and Environmental Microbiology* 74: 7624-708. https://doi.org/10.1128/AEM.00878-08

Schmitt, S., Tsai, P., Bell, J., Fromont, J., Ilan, M., Lindquist, N., Perez, T., Rodrigo, A., Schupp, P. J., Vacelet, J., Webster, N., Hentschel, U., & Taylor, M. W. (2012). Assessing the complex sponge microbiota: Core, variable and species-specific bacterial communities in marine sponges. *ISME Journal* 6: 564-576. https://doi.org/10.1038/ismej.2011.116

Schumannkindel, G., Bergbauer, M., Manz, W., Szewzyk, U., & Reitner, J. (1997). Aerobic and anaerobic microorganisms in modern sponges - A possible relationship to fossilization-processes. *Facies* 36: 268–272.

https://doi.org/10.1023/A:1012717200362

Schwartz, A. W., van der Veen, M., Bisseling, T., & Chittenden, G. J. F. (1975). Prebiotic nucleotide synthesis-demonstration of a geologically plausible pathway. *Origins of Life and Evolution of Biospheres* 6: 163-168. https://doi.org/10.1007/BF01372401

Schwintner, C., Sabaty, M., Berna, B., Cahors, S., & Richaud, P. (1998). Plasmid content and localization of the genes encoding the denitrification enzymes in two strains of *Rhodobacter sphaeroides*. *FEMS Microbiology Letters* 165: 313-321. https://doi.org/10.1016/S0378-1097(98)00294-

Sears, M. A., Gerhart, D. J., & Rittschof, D. (1990). Antifouling agents from marine sponge *Lissodendoryx isodictyalis* carter. *Journal of Chemical Ecology* 16: 791-799. https://doi.org/10.1007/BF01016489

Sennet, S. H., Wright, A. E., Pomponi, S. A., Armstrong, J. E., Willoughby, R., &Bingham, B.L. (1990). Cellular localization and ecological role of secondary metabolites from the sponge *Hymeniacidon heliophila*. *International Society of Chemical Ecology Annual Meeting* 1990:8–15

Sera, Y., Adachi, K., & Shizuri, Y. (1999). A new epidioxy sterol as an antifouling substance from a Palauan marine aponge, *Lendenfeldia chondrodes*. *Journal of Natural Products* 62: 152-154. https://doi.org/10.1021/np980263v

Shieh W. Y., & Lin Y. M. (1994). Association of heterotrophic nitrogen-fixing bacteria with a marine sponge of *Halichondria* sp. *Bulletin of Marine Science* 54: 557-564.

Siegl, A., Kamke, J., Hochmuth, T., Piel, J., Richter, M., Liang, C., Dandekar, T., & Hentschel, U. (2011). Single-cell genomics reveals the lifestyle of Poribacteria, a candidate phylum symbiotically associated with marine sponges. *ISME Journal* 5: 61-70. https://doi.org/10.1038/ismej.2010.95

Silbiger, N. J., Nelson, C. E., Remple, K., Sevilla, J. K., Quinlan, Z. A., Putnam, H. M., Fox, M. D., & Donahue, M. J. (2018). Nutrient pollution disrupts key ecosystem functions on coral reefs. *Proceedings of the Royal Society B: Biological Sciences* 285: 20172718. https://doi.org/10.1098/rspb.2017.2718

Simister, R., Taylor, M. W., Tsai, P., Fan, L., Bruxner, T. J., Crowe, M. L., & Webster, N. (2012). Thermal stress responses in the bacterial biosphere of the great barrier reef sponge, *Rhopaloeides odorabile*. *Environmental Microbiology*. 14: 3232-46. https://doi.org/10.1111/1462-2920.12010

Singh, M., Tiwari, P., Arora, G., Agarwal, S., Kidwai, S., & Singh, R. (2016). Establishing virulence associated polyphosphate kinase 2 as a drug target for

Mycobacterium tuberculosis. *Scientific Reports* 6: 26900. https://doi.org/10.1038/srep26900

Sipkema, D., Holmes, B., Nichols, S. A., & Blanch, H. W. (2009). Biological characterisation of *Haliclona* (?gellius) sp.: Sponge and associated microorganisms. *Microbial Ecology* 58: 903-920. https://doi.org/10.1007/s00248-009-9534-8

Sipkema, D., Schippers, K., Maalcke, W. J., Yang, Y., Salim, S., & Blanch, H. W. (2011). Multiple approaches to enhance the cultivability of bacteria associated with the marine sponge *Haliclona* (gellius) sp. *Applied and Environmental Microbiology* 77: 2130-2140. https://doi.org/10.1128/AEM.01203-10

Slaby, B. M., & Hentschel, U. (2017). Draft genome sequences of "*Candidatus* Synechococcus spongiarum," cyanobacterial symbionts of the Mediterranean sponge *Aplysina aerophoba*. *Genome Announcements* 5: e0026817. https://doi.org/10.1128/genomeA.00268-17

Smith, J. T., Bowes, M. J., & Cailes, C. R. (2011). A review and model assessment of ^{32}P and ^{33}P uptake to biota in freshwater systems. *Journal of Environmental Radioactivity* 102: 317-325. https://doi.org/10.1016/j.jenvrad.2010.12.006

Sohrabi, M., Nair, R. G., Samaranayake, L. P., Zhang, L., Zulfiker, A. H. M., Ahmetagic, A., Good, D., & Wei, M. Q. (2016). The yield and quality of cellular and bacterial DNA extracts from human oral rinse samples are variably affected by the cell lysis methodology. *Journal of Microbiological Methods* 122: 64-72. https://doi.org/10.1016/j.mimet.2016.01.013

Sorokin, Y. I. (1992). Phosphorus metabolism in coral reef communities: exchange between the water column and bottom biotopes. *Hydrobiologia* 242: 105-114. https://doi.org/10.1007/BF00018066

Southwell, M. W., Popp, B. N., & Martens, C. S. (2008). Nitrification controls on fluxes and isotopic composition of nitrate from Florida Keys sponges. *Marine Chemistry* 108: 96-108. https://doi.org/10.1016/j.marchem.2007.10.005

Southwell, M. W., Weisz, J. B., Martens, C. S., & Lindquist, N. (2008). In situ fluxes of dissolved inorganic nitrogen from the sponge community on Conch Reef, Key Largo, Florida. *Limnology and Oceanography* 53: 986-996. https://doi.org/10.4319/lo.2008.53.3.0986

Stachowicz, J. J., & Hay, M. E. (2000). Geographic variation in camouflage specialization by a decorator crab. *American Naturalist* 156: 59-71. https://doi.org/10.1086/303366

Stackebrandt, E., & Liesack, W. (1993). Nucleic acids and classification. *In* Goodfellow, M., & O'Donnell, A. G. (eds). Handbook of new bacterial systematics 152–189.

Stein, J. E., Reichert, W. L., & Varanasi, U. (1994). Molecular epizootiology: Assessment of exposure to genotoxic compounds in teleosts. *Environmental Health Perspectives* 102: 19-23. https://doi.org/10.1289/ehp.94102s1219

Steindler, L., Huchon, D., Avni, A., & Ilan, M. (2005). 16S rRNA phylogeny of sponge-associated cyanobacteria. *Applied and Environmental Microbiology* 71: 4127-4131. https://doi.org/10.1128/AEM.71.7.4127-4131.2005

Steinert, G., Taylor, M. W., Deines, P., Simister, R. L., De Voogd, N. J., Hoggard, M., & Schupp, P. J. (2016). In four shallow and mesophotic tropical reef sponges from Guam the microbial community largely depends on host identity. *PeerJ* 4: e1936. https://doi.org/10.7717/peerj.1936

Strickland, J. D. H., & Parsons, T. R. (1972). Determination of reactive silicate. *In* Stevenson, J. C., Watson, J., Reinhart, J. M., & Camp, J. (eds). A Practical Handbook of Seawater Analysis 65-71.

Taylor, M. W., Radax, R., Steger, D., & Wagner, M. (2007a). Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiology and Molecular Biology Reviews* 71: 295-347. https://doi.org/10.1128/mmbr.00040-06

Taylor, M. W., Hill, R. T., Piel, J., Thacker, R. W., Hentschel, U. (2007b). Soaking it up: The complex lives of marine sponges and their microbial associates. *ISME Journal* 1: 187-190. https://doi.org/10.1038/ismej.2007.32

Taylor, M. W., Tsai, P., Simister, R. L., Deines, P., Botte, E., Ericson, G., Schmidt S., & Webster, N. S. (2013). Sponge-specific bacteria are widespread (but rare) in diverse marine environments. *ISME Journal* 7: 438-443. https://doi.org/10.1038/ismej.2012.111

Temperton, B., Gilbert, J. A., Quinn, J. P., & McGrath, J. W. (2011). Novel analysis of oceanic surface water metagenomes suggests importance of polyphosphate metabolism in oligotrophic environments. *PLoS ONE* 6: e16499. https://doi.org/10.1371/journal.pone.0016499

Thacker, R. W., Becfrro, M. A., Lumbang, W. A., & Paul, V. J. (1998). Allelopathic interactions between sponges on a tropical reef. *Ecology* 79: 1740-1750. https://doi.org/10.1890/0012-9658(1998)079[1740:AIBSOA]2.0.CO;2

Thacker, R. W., & Freeman, C. J. (2012). Sponge-microbe symbioses. Recent advances and new directions. *Advances in Marine Biology* 62: 57-111. https://doi.org/10.1016/B978-0-12-394283-8.00002-3

Thakur, N. L., Hentschel, U., Krasko, A., Pabel, C. T., Anil, A. C., & Müller, W. E. G.

(2003). Antibacterial activity of the sponge *Suberites domuncula* and its primmorphs: Potential basis for epibacterial chemical defense. *Aquatic Microbial Ecology* 31:77-83. https://doi.org/10.3354/ame031077

Thomas, T., Rusch, D., DeMaere, M. Z., Yung, P. Y., Lewis, M., Halpern, A., Heidelberg, K. B., Egan, S., Steinberg, P. D., & Kjelleberg, S. (2010). Functional genomic signatures of sponge bacteria reveal unique and shared features of symbiosis. *ISME Journal* 4: 1557-1567. https://doi.org/10.1038/ismej.2010.74

Thomson-Bulldis, A., & Karl, D. (1998). Application of a novel method for phosphorus determinations in the oligotrophic North Pacific Ocean. *Limnology and Oceanography* 43: 1565-1577. https://doi.org/10.4319/lo.1998.43.7.1565

Thomson, W. (1869). On Holtenia, A genus of vitreous sponges. *Proceedings of the Royal Society of London* 18: 32-35. https://doi.org/10.1111/j.1365-2818.1869.tb01857.x

Tijssen, J. P. F., Beekes, H. W., & Van Steveninck, J. (1982). Localization of polyphosphates in *Saccharomyces fragilis*, as revealed by 4′,6-diamidino-2-phenylindole fluorescence. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 721: 394-398. https://doi.org/10.1016/0167-4889(82)90094-5

Timmers, M. A., Vicente, J., Webb, M., Jury, C. P., & Toonen, R. J. (2020). Sponging up diversity: Evaluating metabarcoding performance for a taxonomically challenging phylum within a complex cryptobenthic community. *Environmental DNA* 00: 1-15. https://doi.org/10.1002/edn3.163

Todd, B. D., Thornhill, D. J., Fitt, W. K. (2006). Patterns of inorganic phosphate uptake in *Cassiopea xamachana*: A bioindicator species. *Marine Pollution Bulletin* 52: 515-521. https://doi.org/ 10.1016/j.marpolbul.2005.09.044

Toggweiler, J. R. (1999). An ultimate limiting nutrient. *Nature* 400: 511-512. https://doi.org/10.1038/22892

Torriani-Gorini, A. (1994). Introduction: the pho regulon of *Escherichia coli*. *In* A. Torriani-Gorini, A., Yagil, E. & Silver, S. (eds). Phosphate in microorganisms: cellular and molecular biology 1-4.

Turner, B. L., Cade-Menun, B. J., & Westermann, D. T. (2003). Organic phosphorus composition and potential bioavailability in semi-arid arable soils of the Western United States. *Soil Science Society of America Journal* 67: 1168-1179. https://doi.org/10.2136/sssaj2003.1168

Turner, T. L. (2020). The marine sponge *Hymeniacidon perlevis* is a globally-distributed invasive species. *bioRxiv* 1-23. https://doi.org/10.1101/2020.02.13.948000

Turon, X., Becerro, M. A., Uriz, M. J., & Llopis, J. (1996). Small-scale association measures in epibenthic communities as a clue for allelochemical interactions. *Oecologia* 108: 351-360. https://doi.org/10.1007/BF00334661

Turon, X., Galera, J., & Uriz, M. J. (1997). Clearance rates and aquiferous systems in two sponges with contrasting life-history strategies. *Journal of Experimental Zoology* 278: 22-36. https://doi.org/10.1002/(SICI)1097-010X(19970501)278:1<22::AID-JEZ3>3.0.CO;2-8

Turque, A. S., Batista, D., Silveira, C. B., Cardoso, A. M., Vieira, R. P., Moraes, F. C., Clementino, M. M., Albano, R. M., Paranhos, R., Martins, O. B., & Muricy, G. (2010). Environmental shaping of sponge associated archaeal communities. *PLoS ONE* 5: e15774. https://doi.org/10.1371/journal.pone.0015774

Turque, A. S., Cardoso, A. M., Silveira, C. B., Vieira, R. P., Freitas, F. A. D., Albano, R. M., Gonzalez, A. M., Paranhos, R., Muricy, G., & Martins, O. B. (2008). Bacterial communities of the marine sponges *Hymeniacidon heliophila* and *Polymastia janeirensis* and their environment in Rio de Janeiro, Brazil. *Marine Biology* 155: 135-146. https://doi.org/10.1007/s00227-008-1008-x

Tyrrell, T. (1999). The relative influences of nitrogen and phosphorus on oceanic primary production. *Nature* 400: 525-531. https://doi.org/10.1038/22941

University of Maryland Baltimore Environmental Health and Safety. (2021). Radionuclides Commonly Used in Research. https://www.umaryland.edu/ehs/research-safety/radiation-safety/radionuclides/

Usher, K. M., Kuo, J., Fromont, J., Sutton, D.C. (2001). Vertical transmission of cyanobacterial symbionts in the marine sponge *Chondrilla australiensis* (Demospongiae). *Hydrobiologia* 461: 9-13. doi:10.1023/A:1012748727678

Usher, K. M., Fromont, J., Sutton, D. C., & Toze, S. (2004). The biogeography and phylogeny of unicellular cyanobacterial symbionts in sponges from Australia and the Mediterranean. *Microbial Ecology* 48: 167-177. https://doi.org/10.1007/s00248-003-1062-3

Usher, K. M., Sutton, D. C., Toze, S., Kuo, J., & Fromont, J. (2005). Intergenerational transmission of microbial symbionts in the marine sponge *Chondrilla australiensis* (Demospongiae). *Marine and Freshwater Research* 56: 125-131. https://doi.org/10.1071/MF04304

Usher, K. M. (2008). The ecology and phylogeny of cyanobacterial symbionts in sponges. *Marine Ecology* 29: 178-192. https://doi.org/10.1111/j.1439-0485.2008.00245.x

Vacelet, J. (2007). Diversity and evolution of deep-sea carnivorous sponges. Porifera

research: Biodiversity, innovation and sustainability. *Série Livros* 28: 107–115.

Valiela, I., McClelland, J., Hauxwell, J., Behr, P. J., Hersh, D., & Foreman, K. (1997). Macroalgal blooms in shallow estuaries: Controls and ecophysiological and ecosystem consequences. *Limnology and Oceanography* 42: 1105-1118. https://doi.org/10.4319/lo.1997.42.5_part_2.1105

Van Cappellen, P., & Ingall, E. D. (1994). Benthic phosphorus regeneration, net primary production, and ocean anoxia: A model of the coupled marine biogeochemical cycles of carbon and phosphorus. *Paleoceanography* 9: 677-692. https://doi.org/10.1029/94PA01455

van Duyl, F. C., Hegeman, J., Hoogstraten, A., & Maier, C. (2008). Dissolved carbon fixation by sponge-microbe consortia of deep water coral mounds in the northeastern Atlantic Ocean. *Marine Ecology Progress Series* 358: 137-150. https://doi.org/10.3354/meps07370

Van Mooy, B. A. S., Rocap, G., Fredricks, H. F., Evans, C. T., & Devol, A. H. (2006). Sulfolipids dramatically decrease phosphorus demand by picocyanobacteria in oligotrophic marine environments. *Proceedings of the National Academy of Sciences of the United States of America* 103: 8607-8612. https://doi.org/10.1073/pnas.0600540103

van Soest, R. W. M., Boury-Esnault, N., Hooper, J. N. A., Rützler, K., de Voogd, N. J., Alvarez, B., Hajdu, E., Pisera, A.B., Manconi, R., Schönberg, C., Klautau, M., Kelly, M., Vacelet, J., Dohrmann, M., Díaz, M. C., Cárdenas, P., Carballo, J.L. , Ríos, P., Downey, R., & Morrow, C. C. (2020) *Hymeniacidon heliophila* (Wilson, 1911). *World Porifera Database*. www.marinespecies.org/porifera/porifera.php?p=taxdetails&id=132652

Vargas, S., Leiva, L., & Wörheide, G. (2020). Short-term exposure to high-water temperature causes a shift in the microbiome of the common aquarium sponge *Lendenfeldia chondrodes*. *Microbial Ecology* 81: 213-222. https://doi.org/10.1101/2020.01.10.901363

Vernon, E. L., Smith, J. T., & Jha, A. N. (2018). Relative comparison of tissue specific bioaccumulation and radiation dose estimation in marine and freshwater bivalve molluscs following exposure to phosphorus-32. *Journal of Environmental Radioactivity* 192: 312-320. https://doi.org/10.1016/j.jenvrad.2018.07.005

Versluis, D., McPherson, K., van Passel, M. W. J., Smidt, H., & Sipkema, D. (2017). Recovery of previously uncultured bacterial genera from three Mediterranean sponges. *Marine Biotechnology* 19: 454-468. https://doi.org/10.1007/s10126-017-9766-4

Vogel, S. (1974). Current-induced flow through the sponge, *Halichondria*. *The*

Biological Bulletin 147: 443-456. https://doi.org/10.2307/1540461

Vogel, S. (2006). Current-induced flow through living sponges in nature. *Proceedings of the National Academy of Sciences* 74: 2069-71. https://doi.org/10.1073/pnas.74.5.2069

Wang, J. T., Chen, Y. Y., Meng, P. J., Sune, Y. H., Hsu, C. M., Wei, K. Y., & Chen, C. A. (2012). Diverse interactions between corals and the coral-killing sponge, *Terpios hoshinota* (Suberitidae: Hadromerida). *Zoological Studies* 51: 150-159.

Wang, J. T., Hsu, C. M., Kuo, C. Y., Meng, P. J., Kao, S. J., & Chen, C. A. (2015). Physiological outperformance at the morphologically-transformed edge of the cyanobacteriosponge *Terpios hoshinota* (Suberitidae: Hadromerida) when confronting opponent corals. *PLoS ONE* 10: e0131509. https://doi.org/10.1371/journal.pone.0131509

Wanner, B. L. (1994). Multiple controls of the *Escherichia coli* Pho regulon by the Pi sensor PhoR, the catabolite regulatory sensor CreC, and acetyl phosphate. *In* Torriani-Gorini, A., Yagil, E., Silver, S. (eds). Phosphate in microorganisms: cellular and molecular biology.

Wanner, B. L. (1995). Signal transduction and cross regulation in the *Escherichia coli* phosphate regulon by PhoR, CreC, and acetyl phosphate. *In* Hoch, J. A., & Silhavy T. J. (eds). Two-Component Signal Transduction. https://doi.org/10.1128/9781555818319.ch12

Wanner, B. L. (1996). Phosphorus assimilation and control of the phosphate regulon. *In* Neidhardt, F. C. (ed). Escherichia coli and Salmonella: Cellular and Molecular Biology.

Wazniak, C., & Hall, M. (2004). Chapter 1.2. The Maryland Coastal Bays ecosystem. In: Maryalnd's Coastal Bays: Ecosystem Health Assessment 2004. https://dnr.maryland.gov/waters/coastalbays/Documents/Ch1.2.pdf

Webb, K., DuPaul, W., & D'Elia, C. (1977). Biomass and nutrient flux measurements on *Holothuria atra* populations on windward reef flats at Enewetak, Marshall Islands. *Proceedings of The Third International Coral Reef Symposium* 409-416. https://doi.org/10.1023/A:1004121818691.

Webster, N. S., & Hill, R. T. (2001). The culturable microbial community of the great barrier reef sponge *Rhopaloeides odorabile* is dominated by an α-Proteobacterium. *Marine Biology* 138: 843-851. https://doi.org/10.1007/s002270000503

Webster, N. S., Wilson, K. J., Blackall, L. L., & Hill, R. T. (2001). Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. *Applied and Environmental Microbiology* 67: 434-444.

https://doi.org/10.1128/AEM.67.1.434-444.2001

Webster, N. S., Taylor, M. W., Behnam, F., Lücker, S., Rattei, T., Whalan, S., Horn, M., & Wagner, M. (2010). Deep sequencing reveals exceptional diversity and modes of transmission for bacterial sponge symbionts. *Environmental Microbiology* 12: 2070-2082. https://doi.org/10.1111/j.1462-2920.2009.02065.x

Webster, N. S., & Taylor, M. W. (2012). Marine sponges and their microbial symbionts: Love and other relationships. *Environmental Microbiology* 14: 335-346. https://doi.org/10.1111/j.1462-2920.2011.02460.x

Webster, N. S., & Thomas, T. (2016). The sponge hologenome. *MBio* 7: e00135-16. https://doi.org/10.1128/mBio.00135-16

Weisz, J. B., Hentschel, U., Lindquist, N., & Martens, C. S. (2007). Linking abundance and diversity of sponge-associated microbial communities to metabolic differences in host sponges. *Marine Biology* 152: 475-483. https://doi.org/10.1007/s00227-007-0708-y

Weisz, J. B., Massaro, A. J., Ramsby, B. D., & Hill, M. S. (2010). Zooxanthellar symbionts shape host sponge trophic status through translocation of carbon. *Biological Bulletin* 219: 189-197. https://doi.org/10.1086/BBLv219n3p189

Wendt, P. H., Van Dolah, R. F., & O'Rourke, C. B. (1985). A comparative study of the invertebrate macrofauna associated with seven sponge and coral species collected from the South Atlantic bight. *Journal of the Elisha Mitchell Scientific Society* 101: 187-203.

Wiedenmayer, F. (1977). Shallow-water sponges of the western Bahamas. doi:10.1007/978-3-0348-5797-0

Wilkinson C. R. (1979). Nutrient translocation from symbiotic cyanobacteria to coral reef sponges. *Biologie des Spongiarires* 291: 373-378.

Wilkinson, C. R., & Fay, P. (1979). Nitrogen fixation in coral reef sponges with symbiotic cyanobacteria. *Nature* 279: 527-529. https://doi.org/10.1038/279527a0

Wilkinson, C., & Garrone, R. (1980). Nutrition of marine sponges. Involvement of symbiotic bacteria in the uptake of dissolved carbon. In Smith, D. C., & Tiffon, Y. (eds). Nutrition in the Lower Metazoa 157-161. https://doi.org/10.1016/b978-0-08-025904-8.50016-x

Wilkinson, C. R. (1983). Net primary productivity in coral reef sponges. *Science* 219: 410-412. https://doi.org/10.1126/science.219.4583.410

Wilkinson, C. R., Garrone, R., & Vacelet, J. (1984). Marine sponges discriminate

between food bacteria and bacterial symbionts: Electron microscope radioautography and in situ evidence. *Proceedings of the Royal Society of London - Biological Sciences* 220: 519-528. https://doi.org/10.1098/rspb.1984.0018

Wilkinson C. R. (1992). Symbiotic interactions between marine sponges and algae. *In* Reisser, W. (ed). Algae and Symbiosis, Plants, Animals, Fungi, Viruses. Interactions Explored 111-151.

Wilkinson, C. R., Summons, R. E., & Evans, E. (1999). Nitrogen fixation in symbiotic marine sponges: Ecological significance and difficulties in detection. *Memoirs of the Queensland Museum* 44: 667-673.

Wilson, W. H. (1979). Community structure and species diversity of the sedimentary reefs constructed by *Petaloproctus socialis* (Polychaeta: Maldanidae). *Journal of Marine Research* 37: 623-641.

Winpenny, K., Knowles, J. F., & Smith, D. L. (1998). The uptake of radioactive phosphorus by brown trout (*Salmo trutta* L.) from water and food. *Journal of Environmental Radioactivity* 38: 211-221. https://doi.org/10.1016/S0265-931X(97)00030-1

Wörheide, G. (1998). The reef cave dwelling ultraconservative coralline demosponge *Astrosclera willeyana* Lister 1900 from the Indo-Pacific. *Facies* 38: 1-88. https://doi.org/10.1007/BF02537358

Wu J., Sunda W., Boyle E. A., & Karl D. M. (2000). Phosphate depletion in the Western North Atlantic Ocean. *Science* 289: 759-762. https://doi.org/10.1126/science.289.5480.759

Wulff, J. L., & Buss, L. W. (1979). Do sponges help hold coral reefs together? *Nature* 281: 474-475. https://doi.org/10.1038/281474a0

Xie, L., & Jakob, U. (2019). Inorganic polyphosphate, a multifunctional polyanionic protein scaffold. *Journal of Biological Chemistry* 294: 2180-2190. https://doi.org/10.1074/jbc.REV118.002808

Yahel, G., Sharp, J. H., Marie, D., Häse, C., & Genin, A. (2003). In situ feeding and element removal in the symbiont-bearing sponge *Theonella swinhoei*: Bulk DOC is the major source for carbon. *Limnology and Oceanography* 48: 141-149. https://doi.org/10.4319/lo.2003.48.1.0141

Yahel, G., Marie, D., & Genin, A. (2005). InEx-a direct in situ method to measure filtration rates, nutrition, and metabolism of active suspension feeders. *Limnology and Oceanography: Methods* 3: 46-53. https://doi.org/10.4319/lom.2005.3.46

Yahel, G., Eerkes-Medrano, D. I., & Leys, S. P. (2006). Size independent selective filtration of ultraplankton by hexactinellid glass sponges. *Aquatic Microbial Ecology* 45: 181-194. https://doi.org/10.3354/ame045181

Yahel, G., Whitney, F., Reiswig, H. M., Eerkes-Medrano, D. I., & Leys, S. P. (2007). In situ feeding and metabolism of glass sponges (Hexactinellida, Porifera) studied in a deep temperate fjord with a remotely operated submersible. *Limnology and Oceanography* 52: 428-440. https://doi.org/10.4319/lo.2007.52.1.0428

Yamagata Y., Watanabe, H., Saitoh, M., & Namba, T. (1991). Volcanic production of polyphosphates and its relevance to prebiotic evolution. *Nature* 352: 516-519. https://doi.org/10.1038/352516a0

Yang, Q., Franco, C. M. M., & Zhang, W. (2019). Uncovering the hidden marine sponge microbiome by applying a multi-primer approach. *Scientific Reports* 9: 1-13. https://doi.org/10.1038/s41598-019-42694-w

Yuan, S., Cohen, D. B., Ravel, J., Abdo, Z., & Forney, L. J. (2012). Evaluation of methods for the extraction and purification of DNA from the human microbiome. *PLoS ONE* 7: e33865. https://doi.org/10.1371/journal.pone.0033865

Zakhia, F., & De Lajudie, P. (2001). Taxonomy of rhizobia. *Agronomie* 21: 569-576. https://doi.org/10.1051/agro:2001146

Zhang, F., Blasiak, L. C., Karolin, J. O., Powell, R. J., Geddes, C. D., & Hill, R. T. (2015). Phosphorus sequestration in the form of polyphosphate by microbial symbionts in marine sponges. *Proceedings of the National Academy of Sciences* 112: 4361-4386. https://doi.org/10.1073/pnas.1423768112

Zhang, F., Jonas, L., Lin, H., & Hill, R. T. (2019). Microbially mediated nutrient cycles in marine sponges. *FEMS Microbiology Ecology* 95: 1-13. https://doi.org/10.1093/femsec/fiz155

Zhang, F., Vicente, J., & Hill, R. T. (2014). Temporal changes in the diazotrophic bacterial communities associated with Caribbean sponges *Ircinia stroblina* and *Mycale laxissima. Frontiers in Microbiology* 5: 1-9. https://doi.org/10.3389/fmicb.2014.00561

Zhang, F. (2015). Roles of the symbiotic microbial communities associated with sponge hosts in the nitrogen and phosphorus cycles. Ph.D. dissertation. University of Maryland College Park

Zhang, H., Gómez-García, M. R., Shi, X., Rao, N. N., & Kornberg, A. (2007). Polyphosphate kinase 1, a conserved bacterial enzyme, in a eukaryote, *Dictyostelium discoideum*, with a role in cytokinesis. *Proceedings of the National*

Academy of Sciences of the United States of America 104: 16486-16491. https://doi.org/10.1073/pnas.0706847104

Zilber-Rosenberg, I., & Rosenberg, E. (2008). Role of microorganisms in the evolution of animals and plants: The hologenome theory of evolution. *FEMS Microbiology Reviews* 32: 723-735. https://doi.org/10.1111/j.1574-6976.2008.00123.x