

ABSTRACT

Title of Dissertation:

**ROLES OF THE SYMBIOTIC MICROBIAL
COMMUNITIES ASSOCIATED WITH
SPONGE HOSTS IN THE NITROGEN AND
PHOSPHORUS CYCLES**

Fan Zhang, Doctor of Philosophy, 2015

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Marine sponges are habitat-forming organisms in coral reefs. Many sponge species host highly abundant microorganisms inside their bodies, forming symbiotic relationships. Efficient nutrient cycling between the symbiotic microbial communities and their hosts is considered to be a vital mechanism to retain limited resources inside the holobiont, providing a competitive edge in an environment where ambient nutrient availability is extremely low. In this dissertation, I describe microbially mediated nitrogen fixation, ammonia oxidation and phosphorus accumulation in keystone sponge species, combining culture dependent and independent methods to characterize these functional pathways. Firstly, I characterized the symbiotic diazotrophic communities using nitrogenase gene marker

nifH and by culturing representative diazotrophs. I found that various groups of cyanobacteria and heterotrophic bacteria actively express *nifH* genes during the entire day-night cycle, an indication that the nitrogen fixation potential was fully exploited by different N-fixing bacterial groups associated with their hosts. Archaea associated with marine sponges can actively affect the fate of fixed nitrogen in the holobiont. In order to elucidate the relative importance of host specificity and biogeographic background in shaping the symbiotic archaeal communities, I investigated these communities in sympatric sponges from the Mediterranean and the Caribbean. Based on 16S rRNA and *amoA* genes, the community structure in *M. laxissima* differed from that in *Ircinia spp.*, including the sympatric sponge *I. strobilina*, indicating that host-specific processes control the sponge-archaeal communities. Compared with the nitrogen cycle, the phosphorus cycle has been little studied in sponge microbiology. I found significant accumulation of polyphosphate (polyP) granules in three common sponge species from Caribbean coral reefs. The identity of the polyP granules was confirmed by energy-dispersive spectroscopy and by fluorescence properties of the granules. Microscopic images revealed that a large proportion of microbial cells associated with sponge hosts contained intracellular polyP granules. Based on these findings, I propose a potentially important phosphorus sequestration pathway through symbiotic microorganisms of marine sponges. Considering the widespread sponge population and abundant microbial cells associated with them, these pathways are likely to have a significant impact on the nitrogen and phosphorus cycle in benthic coral reef ecosystems.

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Dedication

I dedicate this dissertation to my wife Zheng Liu, and my parents Yiwen Fang and Jianmin Zhang for their unconditional love and support.

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I am very grateful to a number of highly talented scientists with whom I have had the honor to collaborate throughout my Ph.D. study. First and foremost, I am very fortunate to have Dr. Russell Hill as my mentor, and I have learnt a great deal from him, scientifically and personally. His high expectation and trust are my biggest motivations to push the project forward. I benefit greatly from working in a loving laboratory environment created by Russell's care for others and his management style. I also extend sincere thanks to my other committee members for their guidance and expertise: Dr. Feng Chen for the assistance in cyanobacteria isolation and personal help; Dr. Hal Schreier for helping me set up anaerobic enrichment culture experiment and forging a productive collaboration; Drs. Judy O'Neil and Frank Robb for scientific discussions that helped to steer my project.

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Finally, I am forever in debt to my parents who raised me up and supported my career choice to study abroad. I will always remember their encouragement: “you only feel fulfilled when you are swimming upward”. And to my wife, who cheers me up every day and sacrificed her career back home to be by my side.

Statement of contribution

Dr. Lucia Pita, from the Department of Animal Biology, University of Barcelona, cloned archaeal 16S rRNA and *amoA* genes from the microbial communities associated with the Mediterranean sponges *I. fasciculata* and *I. oros*, and performed statistical analyses to generate Figures 3.2, 3.3 and 3.4 and Table 3.1 and 3.2.

Ryan McDonald and Dr. Harold Schreier, from the Department of Marine Biotechnology, IMET, University of Maryland Baltimore County, provided assistance and helpful advice in the anaerobic enrichment culture experiment and acetylene reduction assay.

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Chapter 1: Introduction

1.1 Coral reef ecosystems, a paradox of low nutrients and high production

1.1.1 High diversity and primary production in an oligotrophic environment

In an ecosystem, sources and sinks of nutrients constantly seek long-term equilibrium; energy supply fuels the strength of each nutrient transformation flux. Living organisms in the ecosystem carry out the anabolic and metabolic processes connecting all trophic levels. Every ecosystem is unique in its structure and biological community. Coral reef ecosystems are an intriguing example. Coral reefs are built upon the foundation of carbonate skeletons from coral polyps. Regarded as the “rainforest of the sea”, coral reef environments cover just under 0.1% of the world ocean surface, but are home to about 25% of marine living organisms species and rank among the top primary production ecosystems on Earth [1]. These ecosystems are found mostly in the euphotic zone of temperate and tropical regions with very high seawater light transparency [2], and very low nutrient availability in the water column, with nutrient concentrations comparable to those of the vast open oligotrophic ocean surface. For a long time, researchers have been puzzled by an ecological conundrum in the coral reef environment, commonly referred to as “Darwin’s Paradox” [3]: how can the low nutrient concentrations in the water surrounding coral reefs support one of the most productive and bio-diverse ecosystems?

1.1.2 Efficient recycling of existing nutrient pool

Coral reef ecosystems feature high gross primary production with an estimation of carbon (C) fixation of 700 tg C/year, but low net primary production at the scale of 20 tg C/year, less than 3% of the gross production [4]. This scale of net production is similar in number to the integrated water column production in the

euphotic zone of the oligotrophic marine environment. It is believed that in this euphotic zone system, fueled by the high photosynthetic rate as a strong energy engine, limited nutrients are efficiently recycled at a high turnover rate among organisms in the whole food web, resulting in high local biomass and a low standing stock of nutrients in the ambient water column. In coral reefs, nutrient sources can be supplied from physical processes like atmospheric deposition, riverine input, sediment release and deep seawater upwelling into this system. Nutrients also seep from the cracks and cavities that spread through the reef framework [5]. The pore water in the sediment contains ten-fold higher nutrient concentrations than the water column, therefore sediment can function as a nutrient rich reservoir which can either feed back to the water column or form minerals that result in long term nutrient sequestration [6]. Another important source of nutrients comes from internal biological processes like in situ photosynthesis and benthic nitrogen (N) fixation, providing additional resources for biomass growth [7-9]. On the other hand, nutrients can be taken out of the ecosystem by ocean current flow, sediment sequestration and human activities such as fishing. Microbial processes like denitrification and anammox in the sediment can also contribute to the removal of bioavailable N from the system [7]. Highly variable rates for these processes have been detected in the few field studies that have been done, making it difficult to estimate their significance to nutrient fluxes in the ecosystem. The variability in availability of organic matter substrate for denitrifying bacteria might be the explanation for these variations [10].

1.2 Marine sponges in the coral reef benthic community

1.2.1 Role of marine sponges in the community

The organisms living on the seafloor comprise a large proportion of the coral reef biomass and play a central role in transferring nutrients from the water column to the sediment and vice versa. The limited nutrient availability in this environment may favor close microbial symbioses with the invertebrates over evolutionary time scales. Coral is a well-studied example of this collaboration between organisms. Coral polyps form a close symbiotic relationship with zooxanthellae. The photosynthetic products fixed by this dinoflagellate can fulfill up to 90% of total host nutrient demand [11]. The expression of nitrogenase by cyanobacteria that are symbionts of corals [12], suggests that these cyanobacteria may provide an important N source to the corals and could balance the C input from zooxanthellae. Other than corals, diverse photosynthetic organisms in the benthic community like seagrasses, macroalgae and cyanobacteria (often present as cyanobacterial mats) can also provide carbohydrate resources from C fixation by photosynthesis, alleviating nutrient constraints in the ecosystem.

Among these benthic organisms, marine sponges are important habitat forming organisms in the benthic community. Their filter-feeding trophic strategy can remove large amounts of organic particles in the size range between 0.2 to 10 μm , mainly bacterioplankton and phytoplankton [13], from the water column in the local environment (Fig. 1.1). These plankton are consumed as part of the sponge diet. The proposed concept of the “sponge loop” [14] describes this process as accelerating the remineralization of dissolved organic matter (DOM) into the inorganic form, contributing to enhanced elemental turnover and low net export (Fig. 1.2).

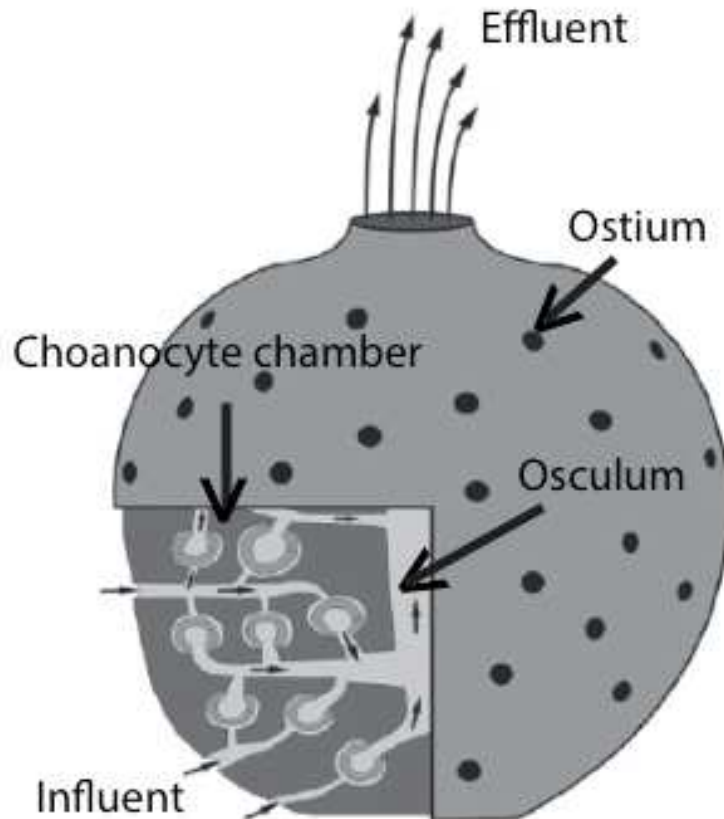


Figure 1.1. A schematic representation of a sponge with a single osculum. Seawater flows through the sponge aquiferous system along the following trajectory. Influent water enters the small inhalant openings (ostia) in the outer pinacodermal tissue, through the inhalant canal propelled by the beating of flagellated choanocyte cells lining the inside of the sponge. The choanocytes, which are specialized cells that form choanocyte chambers, serve to capture food particles in the seawater. After passage through choanocyte chambers, near-sterile seawater enters the effluent canals, and is then discharged from the osculum. Adapted from Mendola [15] with permission.

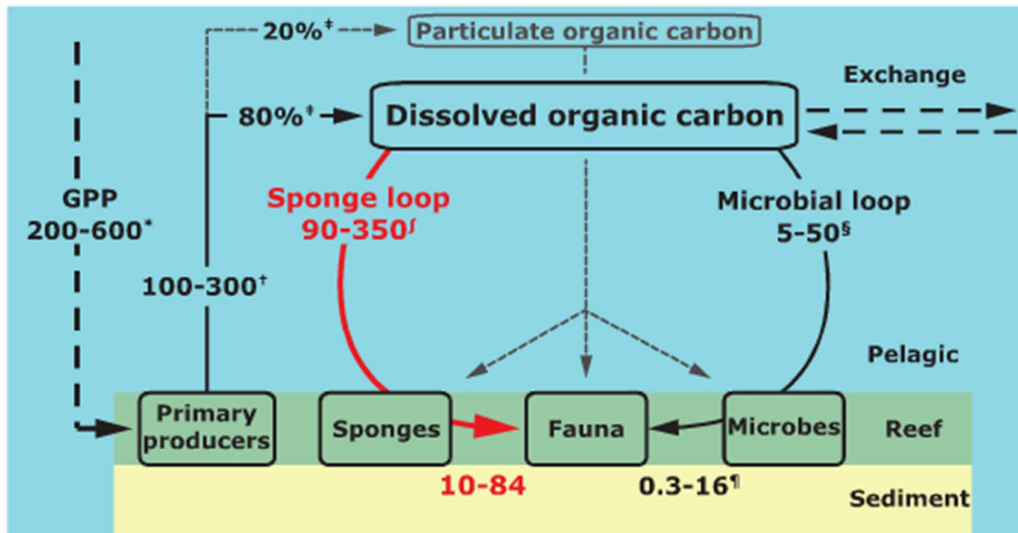


Figure 1.2. A simplified scheme of the DOM-sponge-detritus-fauna feedback loop. Three major ecosystem compartments: pelagic (blue), benthic reef (green), and sediment (yellow) are connected by organic C transfer pathways indicated by arrows and corresponding fluxes are estimated (unit: millimoles of C $\text{m}^{-2} \text{day}^{-1}$) on coral reefs as presented in de Goeij et al. [14] with permission.

Sponges also serve as hosts to many microbes that live as symbionts within the sponge mesohyl, the gelatinous matrix within a sponge between the outer pinacoderm layer and the inner choanoderm (Fig. 1.3). The terms “symbiont” and “symbiosis” are used here consistent with the usage by Taylor et al. [16], according to the original definition by de Bary to refer to two or more organisms found living together for a long period and does not imply that the organisms benefit or harm each other. The density of microbial symbionts in sponges can reach a billion cells per ml volume, approximately three orders of magnitude higher than in the surrounding seawater [16]. Considering these high densities, symbionts are likely to play important roles for the hosts and the local environment.

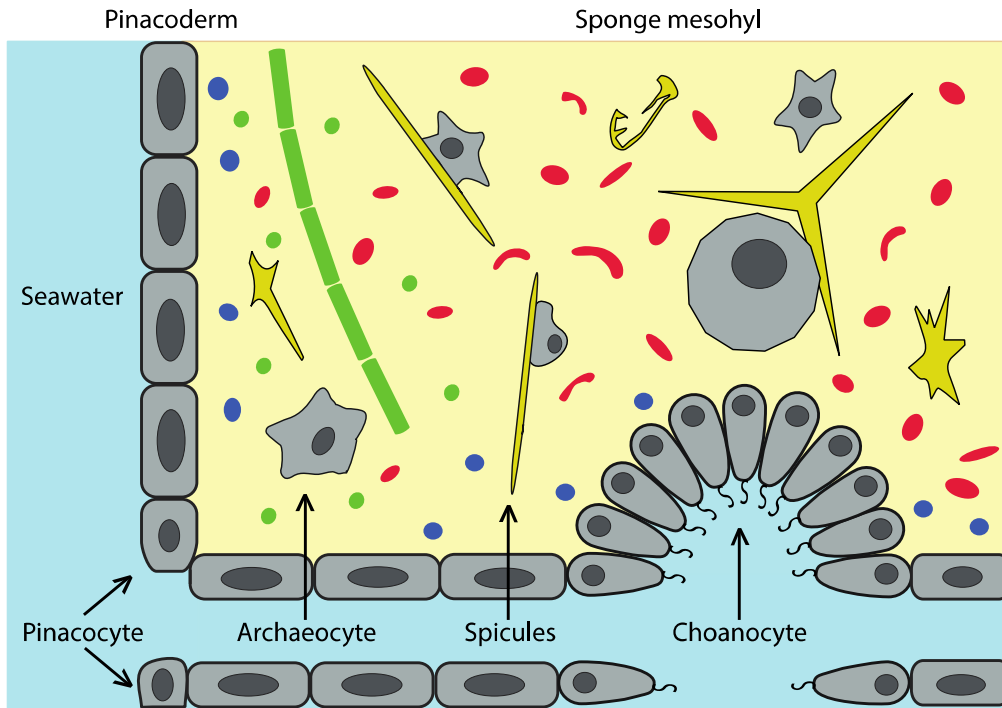


Figure 1.3. An illustration of the internal structure of a typical demersponge and the microbial symbionts. Major sponge cell (grey) types include pinacocytes, building blocks of the pinacodermal layer; archaeocytes, amoeba-like cells responsible for feeding and cell differentiation; and choanocytes, which form choanocyte chambers to harvest particles in the flow through seawater. 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 demersponges. Representative microbial symbionts are cyanobacteria (green), archaea (blue) and heterotrophic bacteria (red). Siliceous spicules (yellow) act as structural skeleton to support to the morphology of demersponges.

1.2.2 The abundance of sponges is increasing in the coral reef environment

As a consequence of rising anthropogenic CO₂ concentration in the atmosphere and subsequently dissolved in seawater, long term ocean acidification is considered to be detrimental to corals because of the negative impact on their calcium carbonate skeleton [17] but could impose less stress on sponges as their silica (Si)-based skeletons might be less affected by the acidification. Recently, many studies have reported an increasing prevalence of sponges in many coral reef ecosystems worldwide, accompanied by a declining number of corals in these areas [18]. A simple explanation could be that the sponges rapidly occupy the open space left by the retreat of coral populations. A comprehensive survey of the coral reefs in the Florida Keys found that the giant barrel sponge *Xestospongia muta*, an important species in the region, increased their population density by over 30% from 2000 to 2006 [19]. In addition to these large barrel sponges that are highly visible, encrusting sponges that occupy the space on seafloor and within cracks of the reefs, may represent a large biomass that is easily overlooked and difficult to quantify by field survey. Human activities could contribute to the observed changes in the reefs. Increasing anthropogenic nutrient loading of N and phosphorus (P) might favor the heterotrophic filter feeding process over the more tightly regulated algal-coral symbiosis [6]. As a survivor through early earth history to the present [20], sponge species have evolved various body plans to adapt to changing environments. Sponges with high cell density of microbial symbionts thrive in clear water environments whereas sponges living in more turbid water tend to have body plans with lower microbial cell density, reflecting the differentiation driven by nutrient source coming primarily from photosynthetic and N-fixing symbionts or from filtration, respectively [21]. The development of versatile strategies could be the reason for the success of

sponges across a wide spectrum of nutrient concentrations [22]. Though the long-term effect of ocean acidification on sponges is still under debate, the trend of sponge dominance observed in many coral reef locations across the world suggests that the impact of sponges on the coral reef ecosystem might be greater in the future.

1.3 Microbial symbionts associated with marine sponges

1.3.1 Diversity of microbial communities associated with sponges

Depending on the density of microbial cells that reside in their mesohyls, sponges can be divided into two categories: High Microbial Abundance (HMA) sponges and Low Microbial Abundance (LMA) sponges (Fig. 1.4). The line that separates the two categories is the microbial density compared with that in the surrounding seawater. LMA sponges contain the same order of magnitude of microbial cells as the surrounding seawater whereas HMA sponges can host a microbial community 3-4 orders of magnitude greater in concentration than the surrounding water column [16]. LMA sponges also show lower microbial diversity compared with HMA sponges [23].

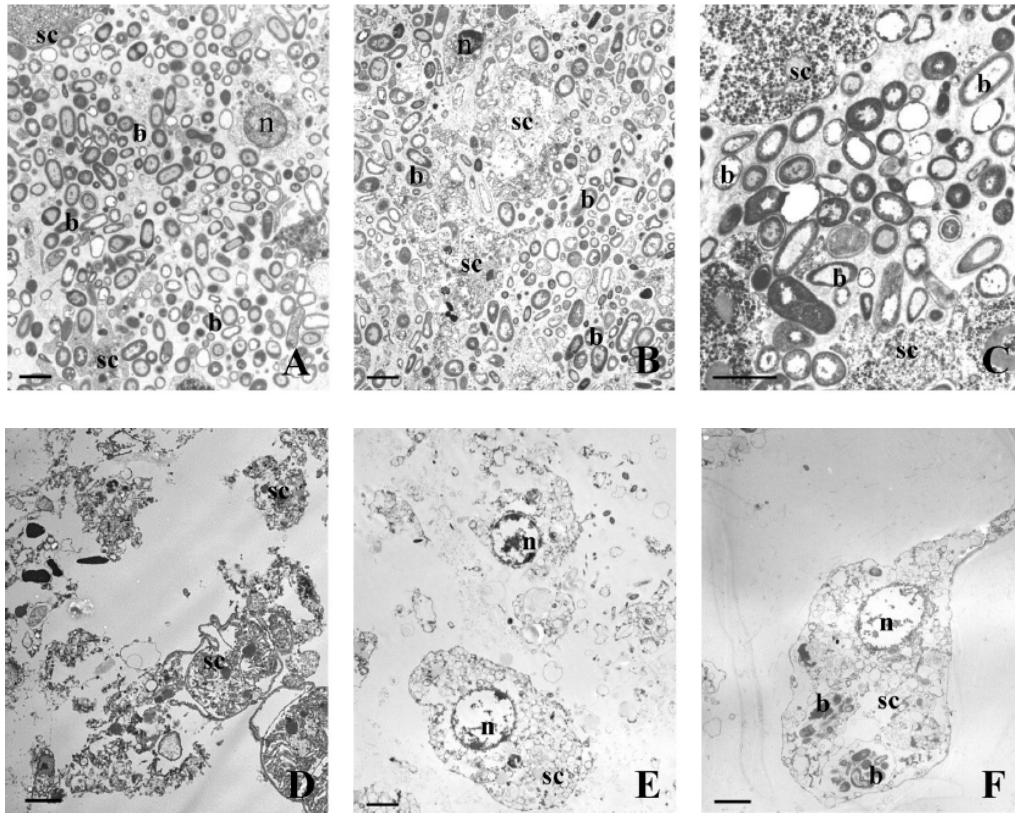


Figure 1.4. Transmission electron microscopy of selected high microbial abundance sponges (upper panel) and low microbial abundance sponges (lower panel): (A) *Aplysina aerophoba*, (B) *Spheciospongia vesparium*, (C) *Aiolochoiria crassa*, (D) *Amphimedon ochracea*, (E) *Axinella cannabina*, (F) *Acanthella acuta*. Scale bar, 2 μ m; b, bacteria; n, nucleus; sc, sponge cell. Adapted from Gloeckner et al. [23] with permission.

Initial studies on microbial diversity in sponges applied molecular techniques targeting the 16S rRNA gene as a convenient phylogenetic marker. Using PCR product cloning and Sanger sequencing, sponge microbiologists uncovered over 25 bacterial phyla from sponges worldwide. The most common phyla present including Acidobacteria, Actinobacteria, Chloroflexi, Bacteroidetes, Cyanobacteria, Firmicutes,

Planctomycetes, Proteobacteria and Verrucomicrobia [16, 24]. The community structure identified from sponges is distinct from that in the surrounding seawater environment, suggesting that sponges can actively select members that predominate in the host microbiome. Spatial and temporal studies show stability of the bacterial and archaeal communities over seasonal changes and under environmental stresses [25-27]. Rapid adoption of next generation sequencing (NGS) technology further revealed an even greater diversity of the sponge-associated communities and provided insights into key factors like host specificity and geographic locations that play a role in shaping sponge microbial communities [28-32]. Subsequent study found that the existence of sponge-derived sequences in many other marine environments [33], though in low abundance, echoing the Baas-Becking hypothesis that “everything is everywhere, but the environment selects” [34]. Scientists working in this area are increasingly using the term “sponge-enriched microbial community” rather than “sponge-specific microbial community” to better reflect this new understanding [35].

With the recent development of the Sponge Microbiome Sequencing Project [36], part of the Earth Microbiome Project, the bacterial communities associated with ca. 1500 sponge samples collected worldwide are being sequenced for 16S rRNA gene-based microbial diversity studies using Illumina HiSeq under the same standard protocol. This large collaboration of sponge microbiologists from around the world has already generated 19 million 16S rRNA gene- reads from sponge microbiomes, representing over 40,000 OTUs, and aims to provide a very large dataset to answer key questions of global scale in sponge microbiology. The questions include: what is the microbial community host specificity and stability? Are there biogeographic distribution patterns in sponge-associated microbes? So far, published results have shown that host identity is the key factor in shaping the symbiotic community structure [37].

1.3.2 Functional analysis of the symbiotic communities in sponges

Molecular tools and high throughput sequencing techniques have helped to overcome the constraints imposed by the difficulty in culturing many of these symbionts and expanded our knowledge of the sponge microbiome, revealing their functional connections with their hosts in chemical defense, immunity, metabolism, reproduction and development [38-41]. Marine sponges are sessile organisms on the seafloor and their ability to defend themselves against predators is crucial in their long-term survival and evolution. In addition to physical defense provided by siliceous spicules, many sponges excel at chemical defense by producing high levels of toxic bioactive compounds [16]. Molecular studies have shown that in many cases bacteria take part in the invertebrate host chemical defense by producing bioactive compounds. Bacterial symbionts retain the entire secondary metabolic synthesis pathway even in cases where their genomes have undergone substantial streamlining, a strong indication of the importance of this functional trait [42]. Because of the high incidence of bioactive compounds in sponges, they have been a major focus of natural product discovery efforts over the past decades [43, 44]. The extensive research in this field has greatly extended our knowledge of bioactive compound chemistry and promoted biomedical applications of sponge-derived compounds in anti-cancer applications and for many other diseases [45].

Sponges normally live in oligotrophic environments and could face food limitation [46]. The ability to obtain additional nutrients from microbial symbionts might provide a competitive edge favored by long-term evolution. Microorganisms are the major driving force in the element biogeochemical cycles [47]. In the sponge mesohyl, frequent water exchange brings in constant nutrient and oxygen (O) supply and internal respiration by sponge and symbiont cells can create an O₂ gradient. The

resultant heterogeneous microenvironment provides suitable niches for diverse symbiotic microorganisms. The high abundance of microbial cells and suitable conditions for their growth are likely to result in significant nutrient flux mediated by the microbial communities from sponges into the surrounding environment, which could be important for the local ecosystem. Several meta-transcriptomic and meta-proteomic studies have reported the functional genes and protein expression maps in the sponges, showing complex microbially mediated C, N and sulfur (S) cycles [38, 41, 48], which will be further discussed in the Section 1.4.

Within the sponge mesohyl, a high abundance of microbial cells live in the same matrix as sponge cells such as archaeocytes and choanocytes. The bacteria-bacteria and bacteria-sponge cell interactions are likely to be highly active. Diverse bacterial quorum sensing genes, including *luxS* and those in the *luxI-luxR* family, have been found in sponge-associated bacteria [49-51]. The quorum sensing pathway in a key sponge symbiont *Ruegeria sp.* KLH11 has been described in detail [49, 50, 52]. Strong circumstantial evidence for the importance of signaling within sponge-associated bacterial communities was provided by the direct detection of signaling acylhomoserine lactone (AHLs) molecules *in situ* within sponges [53]. In other invertebrates, bacterial symbionts can form close associations with the host and play an important role in host development [54]. Inter-kingdom “crosstalk” in sponges is receiving increasing research attention and insights into this process would strengthen our understanding of host-microbe interactions [55, 56].

Sponges may acquire bacterial symbionts by vertical transmission (inherited through the germline) or by horizontal transmission (through filtering of surrounding water which exposes sponges to very large numbers of bacteria). Sponge-associated bacterial communities have been shown in many studies to be markedly different

from the communities in the surrounding water, implying that sponges must have mechanisms to select specific bacteria that become enriched as symbionts. Several studies have shown vertical transmission of specific symbionts [57]. A dominant alpha-proteobacterium was cultured from the sponge *Rhopaloeides odorabile* [58] and proved to be vertically transmitted through the larvae [59]. Specific recognition mechanisms must function to maintain vertically-transmitted symbionts and to distinguish between “prey” and symbionts in those bacteria taken up by filtering of the surrounding water. The sponge symbiont recognition mechanisms could be crucial for understanding the development of host immunity. Recently, discovery of ankyrin and tetratricopeptide repeat proteins in the symbionts suggests a mechanism for sponges to discern between phagocytosis targets and bacteria that will be maintained as residents within the symbiont community [60, 61].

1.4 The role of sponge associated microorganisms in mediating nutrient cycles

1.4.1 Carbon cycle in sponges

Sponge pumping rate can vary greatly by morphology, size and temporal rhythm [62]. With non-stop water pumping rate at $0.2 \text{ ml (ml sponge)}^{-1}\text{s}^{-1}$, sponge can filter approximately 17,280 times its body volume in a single day [63]. With an extremely high particle removal efficiency of 80-90% in the size range from 0.2 to 50 μm , water flowing out of the sponge oscula is almost free of large particles. The sponge-symbiont holobiont shows an ability to take up and consume dissolved organic carbon (DOC) from the surrounding seawater, and HMA sponges tend to have higher DOM uptake ability than the LMA sponges, suggesting that bacteria might play an important role in DOM uptake [64]. Therefore, by their filter feeding behavior alone, sponges can quickly deplete particulate organic carbon (POC) and

DOC in the surrounding water column [22, 63]. Ingestion rates for sponges of 29-1270 mg C m⁻² sponge per day have been reported [65]. Stable isotope $\delta^{13}\text{C}$ tracer studies also showed strong indications of reef origins in the tissue of coral cavity sponges, suggesting an important source for C diet in these sponges [66]. Field measurements showed high sponge respiration rates, often exceeding C ingestion rates [67, 68]. Therefore, measured by increased volume or weight, the growth of sponges is generally slow at 5-60% per year for tropical and temperate sponges [69, 70]. The rate can drop dramatically for cold water sponges to 0.07% per year [71]. Although the gain in biomass can be minimal for many sponges, reports show that encrusting sponges living in the reef cavity tend to have short cell cycles and fast cell shedding, mimicking a “sponge pump” that converts ambient dissolved nutrients into particulate form, enhancing organic C cycling in the local reef environment and preserving nutrients in the sediments [14, 72]. Follow-up research suggests that other massive sponges can also be a significant source contributing to this pathway [73]. Additionally, the high abundance of microorganisms that lives inside the sponges can provide an additional carbohydrate source for the hosts through autotrophic C fixation. Net primary production was detected in sponges hosting cyanobacterial symbionts, and not in sponges without cyanobacterial symbionts, providing strong evidence for the importance of microbial symbionts in affecting the sponge C cycle [74]. Genomic studies on symbiotic archaea in sponges found gene clusters responsible for C fixation, suggesting that archaea can also contribute to the sponge holobiont C production [75].

1.4.2 Nitrogen cycle in sponges

N is a key limiting factor in primary production. The major marine sources in nature are N₂ fixation and upwelling of nitrate rich deep-sea water as ‘new

production' and bacterially mediated mineralization like ammonification that provide N resources from organic matter, known as 'regenerated production'. On the other hand, the major N sinks are identified as denitrification and anammox that happens in the O₂ minimum zone [76] and estuarine sediments [77]. N in nature exists in a wide range of redox states from -3 to +5. The transformation of these different N species between various redox states is conducted exclusively by prokaryotes so far as is currently known. Reports using micro-electrodes to measure O₂ penetration in live sponges found a complex and dynamic change in the redox state within sponges, resulting from changing pumping rate and O₂ consumption by internal respiration [78]. Most of the microbially mediated N cycle pathways known in nature so far have also been found in sponge symbiotic microbial communities, suggesting that the sponge mesohyl represents a miniature version of the vast ocean. Field N flux measurements found that most sponges, both HMA and LMA, are major sources of inorganic N. The chemical form of N released from LMA sponges are high in ammonia compared with nitrate that predominates in HMA sponges [21], indicating that microbial ammonia oxidation plays a role in the latter case. The fact that sponges are a source of inorganic N suggests that sponges as a whole might not be N limited, though the internal heterogeneity within sponges could create some N limited zones. N₂ fixation activity in sponges was first detected using the acetylene reduction assay, with results suggesting that sponges with cyanobacteria symbionts were positive in N₂ fixation whereas there was no N₂ fixation activity in sponges lacking cyanobacteria [79]. Latter studies showed inconsistent results from different sponge species [80]. Stable N isotope signatures are reliable indicators to track the source of N supply. By consideration of δ¹⁵N values and high/low microbial abundance, Caribbean sponges can be divided based into three major groups: HMA sponges with high δ¹⁵N, HMA

sponges with low $\delta^{15}\text{N}$ and LMA sponges with high $\delta^{15}\text{N}$. No differentiation of stable isotope fractions was observed on $\delta^{13}\text{C}$ values [21]. The low $\delta^{15}\text{N}$ values observed in some HMA sponges suggests that these sponges rely on biological N_2 fixation by symbiotic diazotrophs to fulfill their N demand. High $\delta^{15}\text{N}$ values in LMA sponges indicate that they obtain N mainly by filter feeding on organic particles and by dissolved N uptake.

N flux studies on some HMA sponges showed that these sponges are a source of nitrate and a sink in ammonia, indicating the presence of an active ammonia oxidation community in these sponges [21]. Also, loss of N through denitrification in sponges was first reported in *G. barretti* [81] and the potential anammox bacteria *Planctomyces* were found to be present in sponges *M. laxissima* and *I. strobilina* [82]. Functional pathways in N_2 fixation and ammonia oxidation will be further discussed in Chapter 2 and 3.

1.4.3 P, S, Si and trace element cycles in sponges

Many other important elements that may limit the ecosystem production have been far less studied than C and N. P is essential in building the backbone of DNA/RNA and lipid membrane for all living organisms. In his Scientific American essay “Mineral cycles” in 1970, ecologist Edward S. Deevey stressed the importance of P by stating “the photosynthetic fixation of C would be fruitless if it were not followed by the phosphorylation of the sugar produced” [83]. In contrast to the N cycle, the P cycle is thought to be controlled by chemical processes more than by biological processes [84]. The lack of study of the P cycle in sponge microbiology was emphasized in the review by Taylor et al. [16]. Field measurement of phosphate flux through sponges suggested that sponges may be a minor source for phosphate [85]. Bacterial isolates from sponge tissues were able to solubilize phosphate, potentially increasing the P cycle efficiency in their living habitat [86].

The existence of anaerobic niches in sponges opens the door for anaerobic chemoautotrophic microbial processes in the sponge-associated communities. Many S-cycle related processes are carried out by obligate anaerobes. Metagenomic and metatranscriptomic studies revealed S-related metabolic pathways in the symbiotic microbial communities [38, 87]. Functional gene studies and experiments in which bacteria were localized by fluorescent in situ hybridization (FISH) revealed the presence of S-reducing and -oxidizing bacteria in sponge tissues [78]. 16S rRNA sequences retrieved from sponges also indicate the presence of phototrophic S bacteria and sulfate-reducing bacteria in the communities [27, 88]. Harboring symbiotic S-oxidizing bacteria to utilize hydrogen sulfide in the environment might provide sponge hosts with a competitive advantage in sulfide rich sediment areas or under S toxicity situations [89].

Sponges use dissolved silicon to build their siliceous skeletons. A recent study estimated that silicon consumption by sponges could account for utilization of 20% of the total supply of silicon in the overlaying water column. The same study also showed that the maximum silicon uptake efficiency of the sponge *Axinella* occurred at concentrations of silicon two orders of magnitude higher than the concentration in ambient seawater. This is much higher than the concentration needed for maximum silicon uptake efficiency in diatoms, the present-day dominant silicon consumer, and might reflect an ancient heritage of sponges from the high silicon concentration in early Earth history [90]. With their high abundance and large biomass in coral reefs, sponge could have a large impact on the Si cycle in local reef habitats.

Transition metals like iron (Fe), copper (Cu), magnesium (Mg) and zinc (Zn) are absolutely required in building active sites for essential enzymes. For example, protein families that contain Fe₂S₂ and heme-binding domains account for approximately 50% of all metal-containing oxidoreductases that mediate redox reactions in living cells [91]. Limitation of these elements may greatly constrain the relevant metabolic pathways. A recent study provides a detailed trace element composition from 16 Red Sea sponge species, showing diverse trace metal accumulation patterns in different species and up to 200-fold increase in concentration compared with the surrounding sediment [92]. Another study found accumulation of copper, zinc, cadmium and chromium in the sponge *Halichondria*, suggesting that sponges can be indicators of heavy metal contamination [93]. These results suggest that sponges can have important roles in the cycle of these trace elements and the microbial community can play a role in selective enrichment of certain elements from the environment.

1.5 Scope of this dissertation

1.5.1 Nitrogen fixation and ammonia oxidation mediated by the microbial symbionts associated with sponges

Efficient nutrient cycling by the benthic community is key to understanding the success of coral reef ecosystems in retaining limited nutrients and maintaining substantial productivity and biodiversity. Sponges are important members in this community; their active filter-feeding and high abundant microbial symbionts can create strong nutrient fluxes connecting the water column and sediment. This dissertation focuses on microbially mediated nutrient cycling happening within the microbial-sponge symbiosis system. In Chapter 2, N-fixing bacterial symbionts in two Caribbean sponges are characterized using molecular and culture-based techniques, and temporal expression pattern of their nitrogenase genes is described. In Chapter 3, the ammonia oxidation archaea (AOA), a relatively understudied group, are described in sympatric sponges from the Caribbean and Mediterranean Sea. The stability of the AOA community and the role of sponge hosts and geographic locations in shaping the AOA community structure are assessed.

1.5.2 Discovery of a P sequestration pathway through microbial-sponge symbiosis

In Chapter 4, the accumulation of polyphosphate (polyP) granules in sponges is reported for the first time, in a study of three Caribbean sponges. The amount of polyP is determined by its fluorescence properties. Molecular and culture-based results suggest the bacterial origins of these granules. Based on these findings and our knowledge of sponge symbionts, a P sequestration pathway through microbial-

sponge symbiosis is proposed and the potential implications of this process on the P cycle in coral reefs, where P limitation is frequently observed, are discussed.

1.5.3 Discussion of the potential role played by marine sponges in N and P cycling in coral reef environments.

Nutrients include essential elements like C, N, P and various trace elements; these nutrients are building blocks for all life. Elemental cycles converge in nutrient cycles, and nutrient cycles converge in the metabolism of all organisms in the community. These tangled nutrient cycles can pose constraints on each other and can be examined in a comprehensive manner. In Chapter 5, an overview of the nutrient cycles in microbial-sponge symbiosis is summarized based on my graduate work and current progress in sponge microbiology research. Future productive research directions in the field are also discussed.

**Chapter 2: Characterization of diazotrophic communities
associated with two Caribbean sponges: *Iricinia strobilina*
and *Mycale laxissima***

2.1 Abstract

Sponges that harbor microalgal or cyanobacterial symbionts may benefit from photosynthetically derived carbohydrates, which are rich in C but devoid of N, and may therefore encounter N limitation. Diazotrophic communities associated with two Caribbean sponges, *Ircinia strobilina* and *Mycale laxissima*, were characterized using the molecular gene marker *nifH* that encodes nitrogenase. Representative diazotrophs were obtained from sponge samples through targeted culturing techniques, including cyanobacterial symbionts from enrichment culture. The temporal pattern of activity in the N-fixing community was studied in a time series during which three individuals of each sponge were collected in four time points (5:00, 12:00, 17:00, 22:00). *nifH* genes were successfully amplified from the corresponding gDNA and cDNA pools and sequenced by high throughput 454 amplicon sequencing. In both sponges, over half the *nifH* transcripts were classified as being derived from cyanobacteria and the remainder were from heterotrophic bacteria. I found various groups of bacteria actively expressing the *nifH* gene during the entire day-night cycle, an indication that the N₂ fixation potential was fully exploited by different N-fixing bacterial groups associated with their hosts. This study shows for the first time the dynamic changes in the activity of the diazotrophic bacterial communities in marine sponges. My study expands understanding of the diazotrophic groups that contribute to the fixed N pool in the benthic community. Sponge bacterial community-associated diazotrophy may have an important impact on the N biogeochemical cycle in the coral reef ecosystem.

2.2 Introduction

2.2.1 Nitrogen limited marine primary production

Coral reef ecosystems are well known for their high biodiversity and productivity, despite low ambient nutrient availability. Numerous studies on the N cycle in coral reefs have linked high local primary production to benthic biological activities, including the efficient recycling of N between algae and invertebrate hosts and benthic N-fixing communities contributing to the “new” N source [94-96]. In the sponge mesohyl, frequent water exchange between the sponge and the surrounding environment can create an O₂ gradient and brings in a supply of nutrients [78]; these conditions may facilitate the essential redox reactions required by N-fixing symbiotic microorganisms [97]. The high abundance of microbial cells and suitable conditions are likely to result in significant nutrient flux mediated by the microbial community, which could be important for the local coral reef ecosystem.

2.2.2 Nitrogen fixation in the benthic community

A classic early study showed the transfer of photosynthetic carbohydrates from symbiotic cyanobacteria to the sponges that hosted these symbionts [74]. The continuous influx of the photosynthetic product that is rich in C but devoid of N could trigger an imbalance in the C:N ratio in the symbiont-sponge system, leading to a N source deficiency. Field incubation experiments showed the uptake of ¹⁵N labeled ammonia and nitrate by both sponge cells and bacterial fractions and suggested the translocation of labeled N from bacteria to hosts [98, 99]. Coral reef ecosystems are characterized by low dissolved N availability in the water column, conditions that might cause the sponge holobiont to seek an alternative N source to balance their budget. N₂ fixation, an anabolic pathway carried out only by prokaryotes, accounts

for half of the reactive N supply that sustains ocean primary production [100]. This pathway requires an anaerobic microenvironment and significant energy supply for N₂ reduction. To provide suitable conditions for N₂ fixation, some diazotrophs like *Anabaena* develop heterocysts as a spatial compartment to create required anaerobic conditions; other groups like the unicellular cyanobacterium *Cyanothece* conduct N₂ fixation at night, temporally separated from oxygenic photosynthesis that occurs during the daytime [101, 102].

2.2.3 Current knowledge on nitrogen fixation in the two sponges

In a field study in 2007, the Hill laboratory reported consistently lower $\delta^{15}\text{N}$ values from tissues of the sponge *Ircinia strobilina*, indicating that these sponge individuals may obtain their N from N₂ fixation, whereas samples from the sponge *Mycale laxissima* showed higher $\delta^{15}\text{N}$ ratios, suggesting less reliance on N₂ fixation [82] (Fig. 2.1). Subsequent molecular studies demonstrated the presence of diverse *nifH* genes from cyanobacteria along with heterotrophic bacteria in both sponges. However, the only *nifH* gene transcripts were those belonging to cyanobacteria [103]. In the current study, I applied a high throughput sequencing method that allowed deeper coverage of the community, and expanded the sampling strategy to monitor the N-fixing activities during a diel cycle. I also applied more targeted approaches to culture diazotrophs from the sponges and used immuno-fluorescent assay to localize nitrogenase in the culture. In my research, I addressed major questions regarding the diazotrophic communities associated with sponge hosts. Is the symbiotic community species specific? How stable is the community in the long term? Do the members of the community that are active in N₂ fixation change over the diel cycle?



Figure 2.1. Caribbean sponge *I. strobilina* (left) and *M. laxissima* (right). Courtesy of Jan Vicente.

2.3 Materials and methods

2.3.1 Sampling strategy

Tissue samples of *M. laxissima* and *I. strobilina* were collected by SCUBA diving at a depth of 20 m from Sweetings Cay, Bahamas (26° 33.78'N, 77° 52.89'W) in July 2012. Surface water temperature in the collection site was 26.7°C. Prior to collection, three large (1-5 kg) individuals of *M. laxissima* and *I. strobilina* were tagged for recurrent sampling. For each individual, 1 cm³ piece of tissue was collected with a sterile scalpel at local time 5:00 (dawn), 12:00 (noon), 17:00 (dusk), and 22:00 (night) for one diel cycle. To reduce the impact of tissue damage during sampling, small individual samples were taken from distant locations of the same sponge for each time point. During each night dive glow sticks were used instead of dive torches to prevent photosynthetic activity from interfering with N₂ fixation. Samples for DNA and RNA extraction were preserved in RNAlater stabilization solution (Qiagen, Valencia, CA, USA) on board within 20 mins after underwater collection prior to long-term storage at -80°C. Three seawater samples (5-10 L) from

the sampling site were collected at noon in close proximity (1 m) to sampled sponges and filtered through 0.22 μm Sterivex filter units (Millipore, Billerica, MA, USA). Seawater samples were collected to compare the diversity of sponge N-fixing bacteria with those found in the surrounding environment.

2.3.2 Measurement of stable isotopes

Sponge samples for $\delta^{15}\text{N}$ measurement were collected from Sweetings Cay, Bahamas (26° 33.78'N, 77° 52.89'W) in July 2012 and from Conch Reef, Key Largo, Florida, USA, NE Caribbean (24° 57.11'N, 80° 27.57'W) in March 2010 and July 2011, prior to the collection of the sponge samples used in this study for the bacterial community analyses. Sponge samples for this purpose were drained and were rinsed three times with artificial seawater, then frozen at -20°C before processing. Three individuals of each sponge were lyophilized and grounded to fine powder. Samples (ca. 1.0 mg) were packed in tin capsules for shipping and analyzed for N isotope ratios by continuous flow isotope ratio mass spectrometry at the UC Davis Stable Isotope Facility as described previously [103].

2.3.3 Culture bacterial symbionts from sponge tissue

Cell separation by Percoll gradient centrifugation

Sponge tissue samples were finely chopped into small pieces with a sterile blade razor and suspend in calcium and magnesium free-artificial seawater (CMF-ASW) for 30 min to dissociate bacteria and sponge cells. These samples (8 ml) were layered on top of a Percoll (Sigma-Aldrich, St. Louis, MO, USA) gradient in 50 ml Falcon tubes. The discontinuous gradient was prepared at concentrations of 10%, 25%, 50% and 100% of Percoll (from top to bottom) in 8 ml volumes for each layer. The tubes were centrifuged in a swinging bucket rotor at 600 g for 20 min at 4°C.

After centrifugation, each layer was removed carefully using sterile needles to pierce through the tube wall and transferred to 15 ml Falcon tubes.

Culturing of anaerobic diazotrophs from sponges

Bacterial fractions separated by Percoll gradient centrifugation were first subjected to serial dilution in filtered seawater to achieve dilution ranging from 1/10 to 1/10000. All four dilutions were immediately transferred to a sealed glove bag (Glass-Col, Terre Haute, IN, USA) set up on site, evacuated of air, and re-inflated with N₂ gas. Residual O₂ was removed by the addition of a steel wool O₂ sink in an acidified copper sulfate solution [104]. Methylene blue anaerobic indicator strips (BBL Microbiology Systems, Cockeysville, MD, USA) were used as indicators to confirm a reduced atmosphere. The four dilution of Percoll fractions were inoculated under anaerobic conditions into 20 ml Hungate tubes with gas tight septa [105] filled with 10 ml of N-free medium [106]. The Hungate tubes containing N-free enrichment medium were prepared under anaerobic conditions before the field trip. Cultures were transported back to the laboratory and incubated at 25°C with shaking at 120 RPM for 4 days. After this enrichment step, 100 µl of inoculum from these enrichment cultures was injected to fresh media anaerobically in a glove bag and fresh cultures were incubated for another 4 days followed by a third round of enrichment with the same procedure.

Culturing of cyanobacteria from sponges

A top agar overlay method [107] was used for isolation of cyanobacteria from sponges. SN30 medium [108] with 2% agarose served as the bottom layer of plates. The same serial dilutions described above were used as inocula for isolation of cyanobacteria. Diluted sample fractions (1 ml) were added 1:1 to 0.5% of low melting agarose, and quickly spread on the plate as the top layers. The plates were

incubated on the bench covered by one sheet of shade cloth at ambient temperature under 14/10 h of light/dark cycle. Single colonies that grew on the plates were streaked onto fresh SN30 medium plates and transferred to liquid SN30 medium in polycarbonate tubes. These liquid cultures were subcultured at ca. monthly interval for more than two years. Total DNA from all enrichment cultures was extracted using the same methods as used for the environmental samples described below and the strains were identified by sequencing their 16S rRNA genes.

2.3.4 Catalyzed reporter deposition immunofluorescent assay (CARD-IFA) and fluorescent in situ hybridization (CARD-FISH)

Bacterial cultures were fixed by 2% paraformaldehyde in 1X PBS pH 7.4 at room temperature for 20 min, and dehydrated by an ethanol gradient from 25%, 50% to 100%. A drop of fixed bacterial cells was heat fixed on a Superfrost-Plus slide (Electron Microscopy Science, Hatfield, PA, USA), immersed in 1 ml of permeabilization buffer with 12.0 mg/ml lysozyme in 100 mM Tris and 50 mM EDTA, pH 8.0 and incubated at 37°C for 45 min in a humidified chamber. Endogenous peroxidases were inactivated with a 2% H₂O₂ solution for 5 min at room temperature in the dark. For CARD-FISH, slides were hybridized with eubacteria probe EUB338 conjugated with horseradish peroxidase, following the protocol described by Pernthaler et al. [109]. Non-EUB338 probe with complimentary nucleotides was used as a control. For CARD-IFA, samples were blocked (0.5% BSA in 1X PBS) for 30 min at room temperature. Slides were incubated with a 1:500 dilution of primary antibody chicken polyclonal antibody for NifH raised from chickens (Agriseria, Sweden) in blocking solution for 1 hr at room temperature. Slides without primary antibody were used as controls. Slides were washed 3 times with 1X PBS for 5 min before application of the secondary antibody (anti-chicken

IgY conjugated with horseradish peroxidase from Promega, Madison, WI, USA) at a 1:1000 dilution in blocking reagent and incubated at room temperature for 1 hr. Slides were washed 5 times with 1X PBS for 5 min. Tyramide signal amplification with fluorophore Cy5 (Perkin Elmer, Waltham, MA, USA) was performed following manufacturer's instructions. Slides were washed 2 times in 1X PBS for 5 min, 2 times in MilliQ water and counterstained by 1X SYBR gold (Life Technologies, Frederick, MD, USA) for 2 min in the dark, then washed with MilliQ water 3 times for 1 min, dried with a reverse graded ethanol series and stored in the dark. All slides were visualized using a Zeiss Axioskop microscope equipped with a Biorad Radiance 2100 laser scanning system.

2.3.5 Acetylene reduction assay

Enrichment cultures from sponges were transfer to 2.0 ml GC vials containing 500 µl N-free enrichment media under anaerobic condition. 100 µl of acetylene, generated from calcium carbide, were immediately injected into the GC vials. The vials were incubated at 25°C without shaking and sampled at 1.5, 12, 24 and 48 hr after inoculation. Acetylene reduction activities were measured by sampling 50 µl of headspace using a gas-tight syringe and analyzed by a HP5890 gas chromatograph (Hewlett Packard) equipped with a flame ionization detector and stainless steel column (0.32 x 182.88 cm). Purified ethylene at concentration of 100 ppm was used as the reference (Supelco, Bellefonte, PA).

2.3.6 Genomic DNA/RNA extraction and *nifH* gene PCR amplification

Total DNA and RNA from the three individuals of each sponge species collected during four time points from Sweetings Cay, Bahamas, July 2012 were extracted using a TissueLyser System (Qiagen) and an AllPrep DNA/RNA Mini Kit

(Qiagen), combined with RNAase-free DNase treatment steps (Qiagen) for RNA samples following the manufacturer's protocol. Total DNA from seawater samples was extracted using a Power Water Sterivex DNA isolation kit (Mo Bio, Carlsbad, CA, USA) following the manufacturer's protocol. Nested PCR was used to amplify *nifH* gene fragments from genomic DNA (gDNA) and the cDNA derived from RNA as described below. For gDNA samples, *nifH* gene fragments were amplified by first round primers nifH32F (5'-TGAGACAGATAGCTATYTAYGGHAA-3') and nifH623R (5'-GATGTTTCGCGCGGCACGAADTRNATSA-3')[110] at a concentration of 100 µM each because of the highly degenerate primers used for *nifH* genes covering 128 and 96 different combinations of nucleotide sequences. For RNA samples, the concentration of extracted RNA was measured using a Nanodrop spectrophotometer 2000 (Thermo Scientific, Waltham, MA, USA), and 100 ng of RNA template from each sample was added to RevertAid Reverse Transcriptase mix (Thermo Scientific) with primer nifH3 (5'-ATRTTRTTNGCNGCRTA-3') as described previously by Zani and colleagues [111]. After reverse transcription, cDNA was amplified using first round PCR primers nifH3 and nifH4 (5'-TTYTAYGGNAARGGNGG-3') at a concentration of 100 µM each. RNA samples without the RT step were included as PCR template to check for residual DNA in the RNA samples.

A total of 33 PCR product samples (three gDNA from filtered seawater, 24 cDNA samples from four time points of the six individuals and six gDNA samples for each individual, pooled from four DNA extractions done at each of the four time points) from the first round were sent to Research and Testing Lab (Lubbock, TX, USA) and subject to a second round of PCR targeting a variable region (360 bp) encoding dinitrogenase reductase subunit using barcoded primer sets nifH1 (5'-

TGYGAYCCNAARGCNGA-3) and *nifH*2 (5'-ADNGCCATCATYTCNCC-3') [112]. Subsequent amplicon pyro-sequencing by 454 Life Science GS FLX + platform (Roche Diagnostics, Branford, CT, USA) generated about 3000 raw sequencing reads from each tagged sample.

2.3.7 Sequence analysis pipeline and statistical analysis

Initial data were processed using the mothur software package, following the guidelines and recommendations in the mothur manual (www.mothur.org) [113] for sequence quality trimming, chimeric checking and denoising to generate a single fasta file. Sequence reads less than 300 bp, plus barcoding tag and primer information were subsequently removed using the 'trim.seqs' command. The cleaned sequences were pre-clustered using Simultaneous Alignment and Tree Estimation using default setting for nucleotide analysis [114]. Representative sequences from each cluster were blasted against the GenBank database using the blastn function to confirm sequence identity and non-*nifH* gene sequences were removed. In some of our cDNA samples, non-*nifH* gene sequences accounted for up to half of the total reads, and were classified as either 16S rRNA sequences from bacteria or 23S rRNA sequences from sponges. A possible explanation for this is that the RNA extraction included a large quantity of ribosomal RNA from microbial symbionts and the hosts, therefore the nested PCR used in my study could lead to reverse transcription and amplification of unintended rRNA sequences. After all corrections, I obtained 67,212 *nifH* sequences in 33 samples. Unique *nifH* sequences were translated into amino acid sequences (120 bp) using MEGA, then aligned to the reference *nifH* database from Marine Microbiology, University of California Santa Cruz (<http://pmc.ucsc.edu/~wwwzehr/research/database/>), built into a phylip-formatted

distance matrix and clustered into OTUs at the 90% similarity level in translated amino acid sequences with the nearest neighbor method. Representative *nifH* sequences from each OTU were deposited in the NCBI database under accession numbers KM083066- KM083092 and raw amplicon sequence data were deposited in the NCBI-SRA database under BioSample accession SAMN02869232. 24 cDNA samples were sub-sampled according to the sample with the minimum number of reads to enable diversity comparisons among individuals and time points. Translated amino acid sequences from representative OTUs and their top blast hits (GenBank database) were imported into ARB [115] for *nifH* gene phylogenetic analysis. Multiple sequence alignments were visually checked and improved manually using the ARB editor. The aligned *nifH* sequences (120 bp) were imported into PhyML 3.1 software package to construct a tree based on Maximum Likelihood method [116]. The robustness of the resulting tree topologies was evaluated by 1000 bootstrap replicates.

Diversity metrics (observed OTUs, coverage, Chao1 estimator, Shannon index and Simpson's inverse) were calculated for sequence data from sponge species and seawater. Diazotrophic communities in sponges were compared by nonmetric multidimensional scaling (nMDS). All analyses were performed using the mothur software package [113]. One-way and multiple factorial ANOVA was performed in the Statistica 7.0 (StatSoft, Tulsa, OK, USA).

2.4 Results

2.4.1 $\delta^{15}\text{N}$ signature of sponge samples

$\delta^{15}\text{N}$ values from *I. strobilina* samples were 1.01 ± 0.97 (SD, n=24), consistently lower than the values 3.86 ± 0.92 (SD, n=24) from *M. laxissima*

(ANOVA between sponge species, $p < 0.01$) and no significant difference (Multiple factorial ANOVA, $p > 0.05$) between the collection years (Fig. 2.2). I also measured the $\delta^{15}\text{N}$ data during the diel cycle. The outcome confirmed the difference in species level, but did not reveal any patterns over a 24 hours time span.

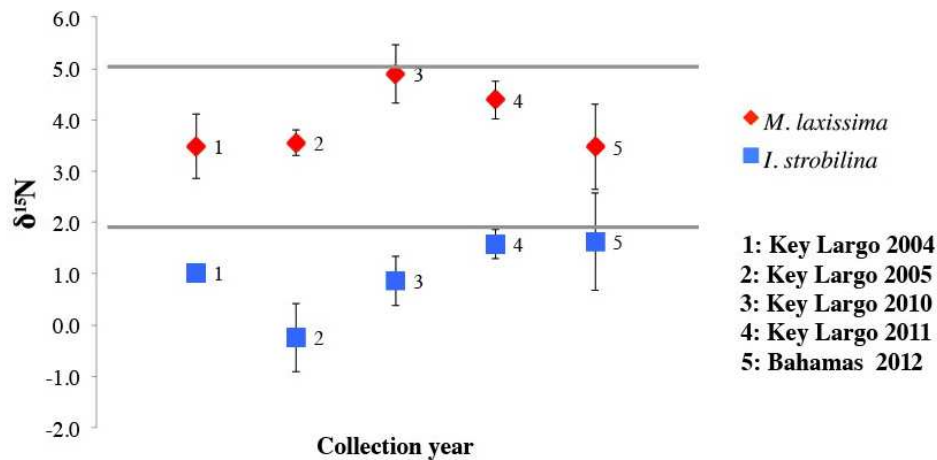


Figure 2.2. $\delta^{15}\text{N}$ values of sponges: *I. strobilina* (IS, square in blue), *M. laxissima* (ML, diamond in red). Each point represents the mean $\delta^{15}\text{N}$ value taken from three sponge individuals of the same species during different years. Error bars indicate the standard deviation of each mean calculation.

2.4.2 Temporal changes in the active diazotrophic community in a diel cycle

The diversity of the bacterial diazotrophic communities revealed by *nifH* gene sequences amplified from total DNA was quite similar between the two sponge species. Deep sequencing of *nifH* amplicons revealed a greater diversity of N-fixing groups in the communities. Based on 90% amino acid sequence similarity, I recovered 22 OTUs from the sponge *I. strobilina*, and a slightly higher diversity (24 OTUs) from *M. laxissima* (Table 2.1). The communities were dominated by cyanobacteria, alpha-proteobacteria and gamma-proteobacteria. Strict anaerobes

belonging to delta-proteobacteria were also found. To distinguish them from autotrophs such as cyanobacteria, all proteobacteria and Verrucomicrobia are referred to as heterotrophic bacteria in this study. The diversity of *nifH* genes in the surrounding seawater was lower than in the sponges with 17 OTUs, all from heterotrophic bacteria (Fig. 2.3) and OTU composition was different from those detected in sponge samples (ANOVA, between sponges and seawater $P < 0.001$). Community compositions based on sequence reads from individuals were consistent in gDNA source, with no significant difference in both individuals (ANOVA between individuals, $p > 0.05$) and species level (ANOVA between sponge species, $p > 0.05$). Detailed individual community compositions are shown in Figure 2.4. The dominant cyanobacterial OTUs were found in all sponges, regardless of location and time of collection. In fact, the representative sequence from OTU1, belonging to the cyanobacterial genus *Leptolyngbya*, shared 100% identity with the DNA sequences found in the same sponge species collected in 2004 and 2005 at Conch Reef, Key Largo [82].

Table 2.1. Richness and dominance metrics for diazotrophic communities in sponges and seawater based on *nifH* gene sequences (OTU = 90% amino acid sequence similarity).

Source	Observed OTUs (Sobs)	Number of reads	Expected OTUs (Chao1)	Simpson Inverse Index	Shannon Index
<i>I. strobilina</i>					
gDNA	22	8290	26 (17-33)	12.2	3.2
cDNA	16	17855	18 (12-20)	6.9	2.6
<i>M. laxissima</i>					
gDNA	23	7383	24 (16-33)	12.5	3.2
cDNA	14	18537	17 (11-22)	6.1	2.5
Seawater					
gDNA	17	5147	21 (17-30)	9.2	2.9

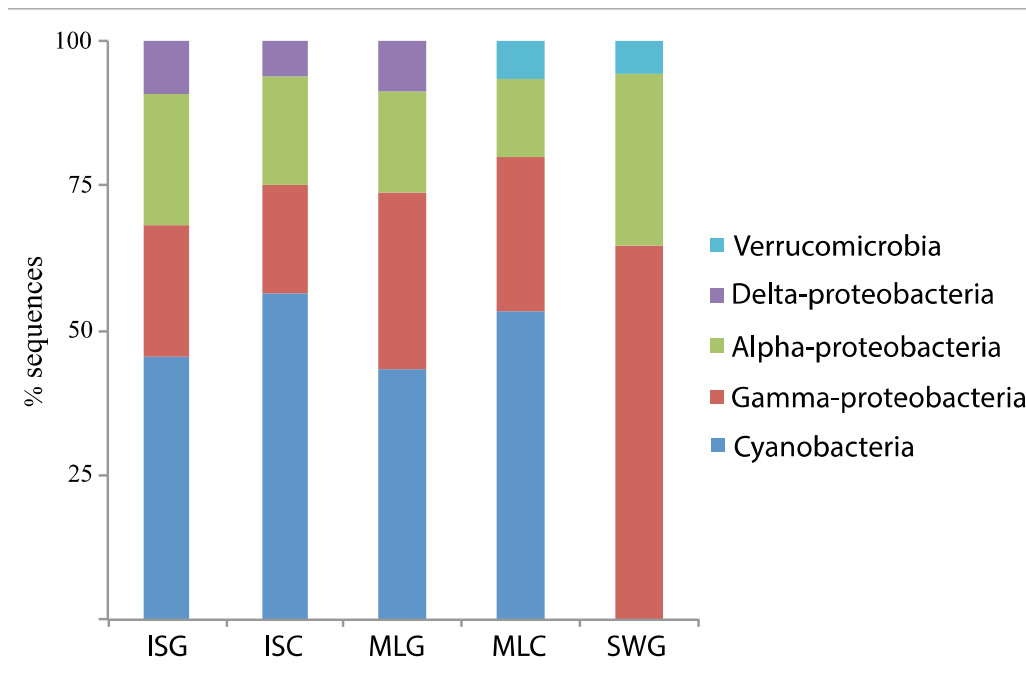


Figure 2.3. Community structure of *I. strobilina*, *M. laxissima* and seawater based on 90% translated amino acid sequences similarity of *nifH* genes from gDNA and cDNA sources. (ISG: gDNA from *I. strobilina*, ISC: cDNA from *I. strobilina*, MLG: gDNA from *M. laxissima*, MLC: cDNA from *M. laxissima*, SWG: gDNA from seawater).

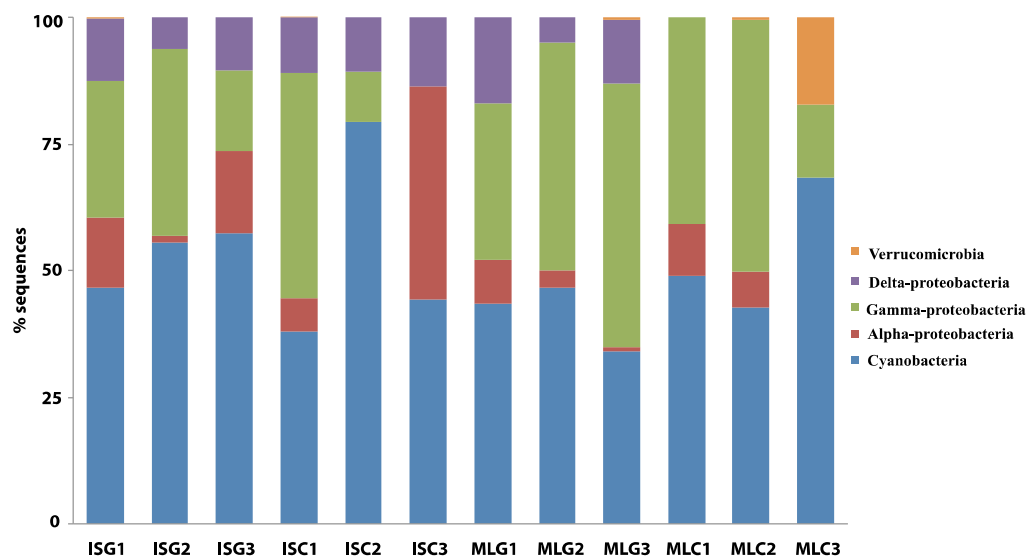


Figure 2.4. Community structure of *I. strobilina*, *M. laxissima* and seawater based on 90% translated amino acid sequences similarity of *nifH* genes from gDNA and cDNA sources. (ISG1: gDNA from individual 1 of *I. strobilina*, ISG2: gDNA from individual 2 of *I. strobilina*, ISG3: gDNA from individual 3 of *I. strobilina*, ISC1: cDNA from individual 1 of *I. strobilina*, ISC2: cDNA from individual 2 of *I. strobilina*, ISC3: cDNA from individual 3 of *I. strobilina*, MLG1: gDNA from individual 1 of *M. laxissima*, MLG2: gDNA from individual 2 of *M. laxissima*, MLG3: gDNA from individual 3 of *M. laxissima*, MLC1: cDNA from individual 1 of *M. laxissima*, MLC2: cDNA from individual 2 of *M. laxissima*, MLC3: cDNA from individual 3 of *M. laxissima*).

In the cDNA dataset, transcripts from cyanobacteria, alpha-proteobacteria and gamma-proteobacteria accounted for at least 80% of sequence reads in each sample. The diversity of *nifH* genes from the cDNA libraries was lower than from the gDNA libraries. 13 OTUs were shared between sponge species, although two gamma-proteobacterial OTUs were found exclusively in *M. laxissima* cDNA samples, and one cyanobacterial and one gamma-proteobacterial OTU were found only in *I. strobilina* samples. Cyanobacterial transcripts were found from filamentous cyanobacteria, including heterocyst-forming genera like *Anabaena*, and non-heterocyst forming genera like *Leptolyngbya* and from unicellular cyanobacteria, closely related to *Cyanothece*. However, no group showed a consistent expression pattern corresponding to particular times in the light/dark cycle. Most transcripts from heterotrophic bacteria were classified either as alpha-proteobacteria closely related to aerobic genus *Xanthobacter* or as gamma-proteobacteria closely related to facultative anaerobe *Klebsiella* (Table 2.2). A complete list of OTUs found in this study is provided (Appendix Table S2.1) and their phylogenetic relationships with cultured N fixers and closest environmental clones are presented in the tree shown in Figure 2.5.

Table 2.2. *nifH* gene OTUs found in high abundance in *I. strobilina* and *M. laxissima* sponge samples and their closest BLAST sequence matches.

Sponge- derived 90%-OTUs	No. of reads in each OTU per source				Closest BLAST match (accession no., % identity, source)	Closest cultivated microorganism (accession no., % identity, source)
	ISG	ISC	MLG	MLC		
OTU01	673	3580	1310	4320	EU594242.1 (100%) Sponge RTMLH02	KC256775.1 (88%) <i>Leptolyngbya minuta</i>
OTU02	173	1967	989	2315	HM601491.1 (92%) Florida key reef water	AB264111.1(84%) <i>Cyanothece sp</i>
OTU03	338	2215	282	627	EU594072.1 (96%) Sponge IS15S	HQ906641.1 (99%) <i>Mastigocladus testaurum</i>
OTU04	122	855	1688	3152	KF657100.1 (88%) Coral clone	FR669148.1 (84%) <i>Klebsiella sp.</i>
OTU05	428	558	155	663	GU594006.1 (95%) Freshwater lake	DQ439648.1 (95%) <i>Anabaena sphaerica</i>
OTU06	305	1458	198	1130	EU594012.1 (93%) Sponge IS3H07	CP000781.1 (98%) <i>Xanthobacter autotrophicus</i>

ISG = gDNA from *I. strobilina*; ISC = cDNA from *I. strobilina*; MLG = gDNA from *M. laxissima*; MLC = cDNA from *M. laxissima*

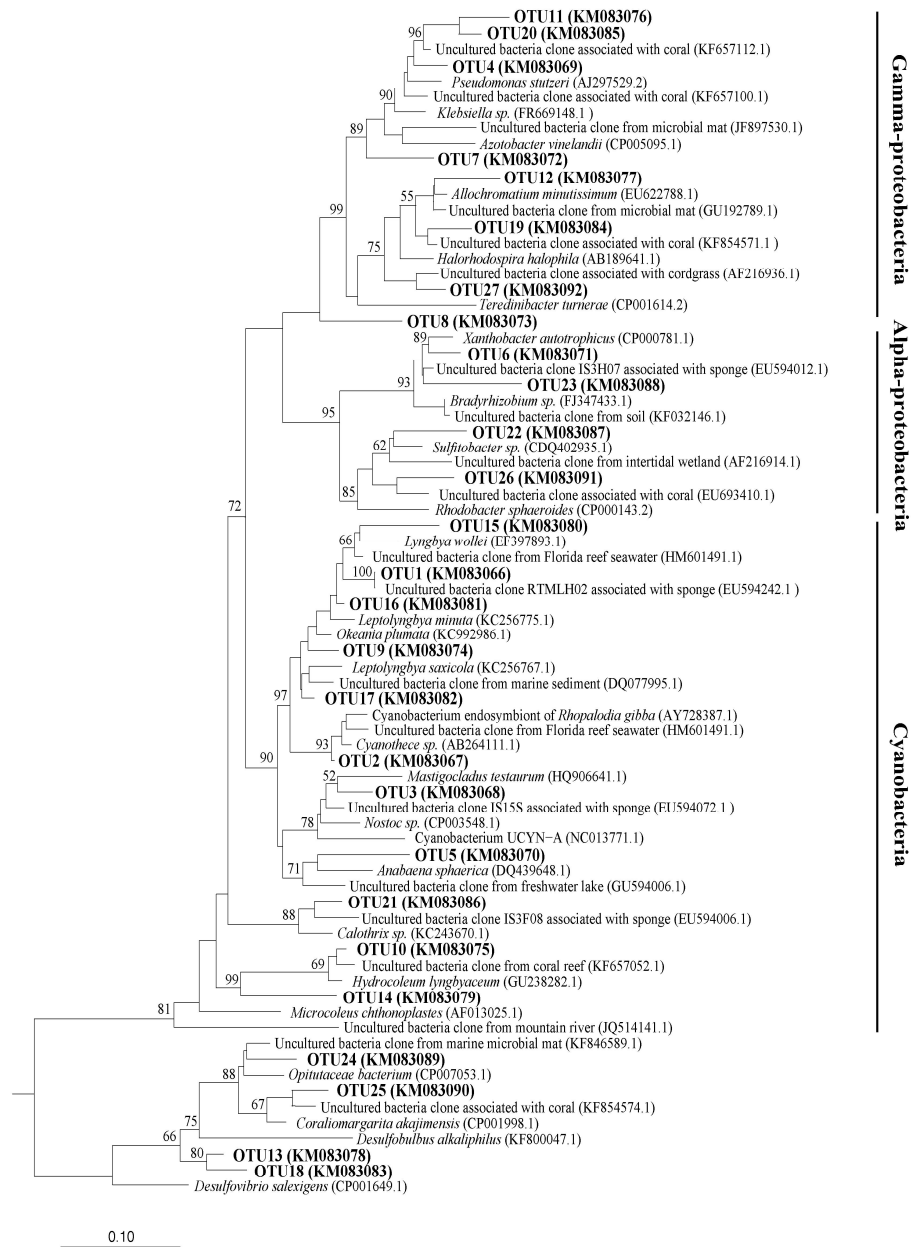


Figure 2.5. Phylogenetic relationships of diazotrophic communities in sponges and water column based on *nifH* gene. Sequences from this study are highlighted in bold. Tree topology constructed using maximum likelihood method with bootstrap values (>50%) indicated at the branch nodes. Archaeal *nifH* gene sequence from *Methanocaldococcus jannaschii* DSM 2661 (SAMN02603984) was used as outgroup.

I selected sequences from three major groups: cyanobacteria, gamma-proteobacteria and alpha-proteobacteria. For these groups, I normalized sequence reads by minimum sample reads across all samples and compared the composition of the actively transcribed components of the community during day and night. When I combined transcript reads from six individuals of the two sponge species I found that cyanobacterial transcripts were dominant in the daytime, accounting for $94.1 \pm 10.7\%$ (SD, n=6) of the total sequence reads from both sponge species. The percentage pattern changed significantly at night (One-way ANOVA, $p < 0.01$), with $72.8 \pm 37.4\%$ (SD, n=6) of transcripts deriving from heterotrophic bacteria (Fig. 2.6 and 2.7). In the species level, day/night difference was more significant for *I. strobilina* (ANOVA between symbiont species during day/night, $p = 0.04$) and less significant for *M. laxissima* (ANOVA between symbiont species during day/night, $p = 0.11$), largely due to a relative high proportion of cyanobacterial transcripts found in the nighttime sample of the third *M. laxissima* individual.

Within sponge samples, cyanobacterial transcripts were consistently dominant at dusk and heterotrophic bacterial transcripts were more abundant at dawn. In contrast, community structure at noon and at night showed variation among different individuals at each time point (Fig. 2.6 and 2.7). For example, two *I. strobilina* individuals showed dominance of heterotrophic bacterial transcripts at 10:00 PM, whereas individual 2 at the same time point had transcripts exclusively from cyanobacteria (Fig. 2.6).

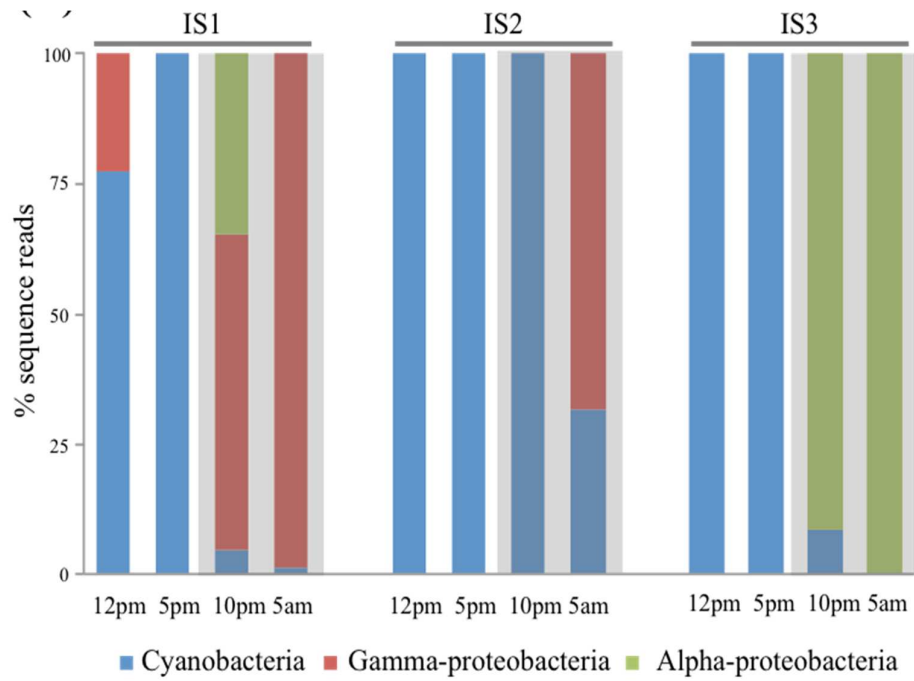


Figure 2.6. Relative abundance of transcript reads from three individuals of *I. strobilina* in a diel cycle. IS1, IS2 and IS3 represent three individual sponges.

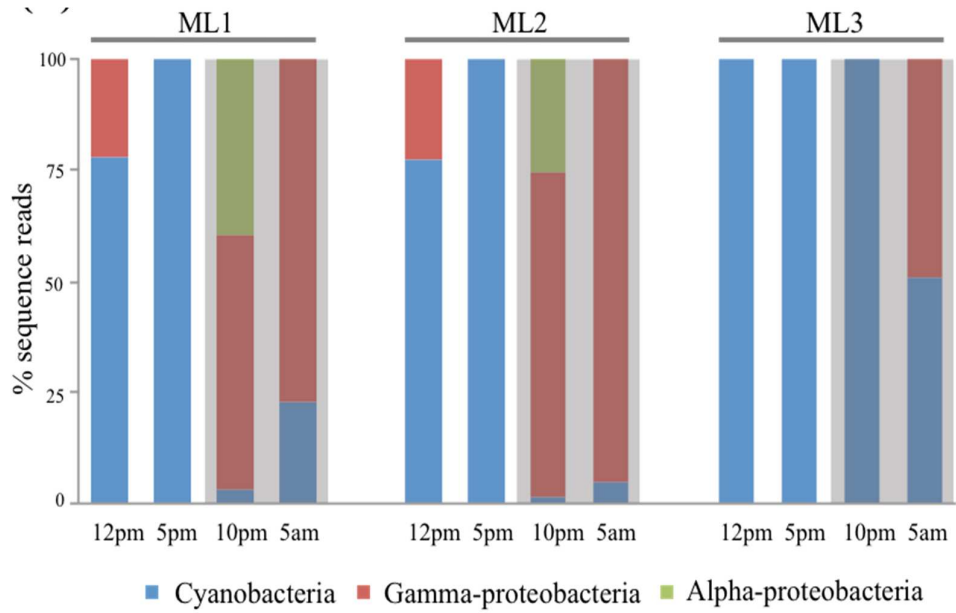


Figure 2.7. Relative abundance of transcript reads from three individuals of *M. laxissima* in a diel cycle. ML1, ML2 and ML3 represent three individual sponges.

In the nMDS plot of community structure, *nifH* gene sequences derived from sponge samples clustered together with no obvious distinction in sequences between the two sponge species, whereas *nifH* gene sequences derived from seawater samples were clearly separated from the sponge-derived sequences (Fig. 2.8).

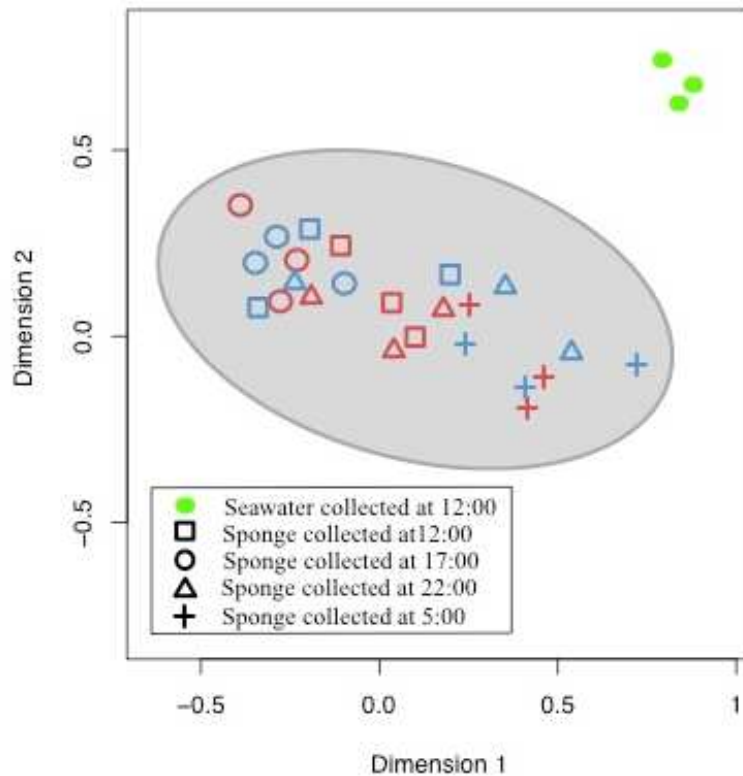


Figure 2.8. Non-metric multidimensional scaling (nMDS) plot based on Bray-Curtis distances between cDNA samples from four different time points: 12:00 (squares), 17:00 (circles), 22:00 (triangles) and 5:00 (crosses). Blue color symbols represent samples from *I. strobilina*, red color symbols represent samples from *M. laxissima* and green color solid oval symbols represent gDNA samples from surrounding seawater (Stress value= 0.10, $R^2=0.86$).

2.4.3 Cultivation of diazotrophs from sponges

Percoll gradient centrifugation was successfully used to enrich microbial cell fractions with similar density and to separate these bacterial cells from sponge cells. Microscope examination of the Percoll fractions showed that most microbial cells were enriched in the lower density portion of the gradient between 0-25% of Percoll concentration. Sponge cell aggregates and spicules were predominantly found pelleted at the bottom of the tubes. I found that microbial cells containing chlorophyll were enriched in the layer between 10-25% of the Percoll concentration (Fig. 2.9). The presence of chlorophyll was revealed by auto-fluorescence of these cells when visualized by epifluorescent microscopy. By using separated cell fractions from each layer as inocula, followed by targeted cultivation method, I was able to isolate microbes that would likely be missed by traditional culturing techniques.

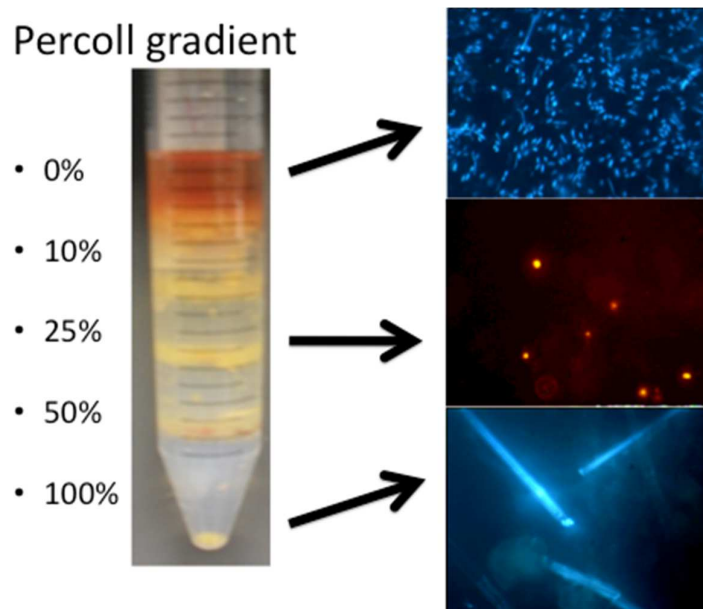


Figure 2.9. Percoll gradient of *M. laxissima* tissue and microscopic examination of fractions showing separation of microbial cell types and sponge spicules.

By enriching in N free medium under anaerobic conditions, I aimed to obtain a stable anaerobic diazotrophic community from the sponge microbiome and to isolate specific sponge-associated N-fixing bacteria. After three rounds of enrichment culture transfer, *nifH* genes were amplified from the enrichment culture. Sequence information showed that the diazotrophs in the enrichment cultures belong to gamma-proteobacteria. The closest relative is *Photobacterium* and *Vibrio* for enrichment cultures from *M. laxissima* and *I. strobilina* respectively.

Using an overlay method, six cyanobacterial strains were isolated from the two sponges and maintained in the laboratory. Two of these cultures are filamentous cyanobacteria. The rest are unicellular cyanobacteria affiliated with the genus *Synechococcus*. Two of these cyanobacterial enrichments were found to carry *nifH* genes by direct sequencing of the *nifH* PCR product amplified from the corresponding genomic DNA. Both of these enrichments were dominated by single strains but were not entirely axenic. Microscopic examination revealed bacteria with different morphology to the dominant strains. One of these strains, designated as ISC5, is a red filamentous cyanobacterium classified as *Leptolyngbya* based on both 16S rRNA and *nifH* gene sequences analysis. The strain grows slowly, tends to attach to the surface of the culture tube, and some aggregates can adjust their position by floating to the medium surface. A small filament was transferred to fresh SN30 medium approximately every month for over two years to enrich the dominant strains. The other *nifH* gene carrying strain, designated as ISC4, is a cyanobacterium in the genus *Synechococcus* (Table 2.3).

Table 2.3. N-fixing bacterial strains enriched from sponges *M. laxissima* (ML) and *I. strobilina* (IS).

	Host	Method	Closest culture relative	Identity (%)	Class
ISN1	IS	Anaerobic	<i>Vibrio parahaemolyticus</i>	99	<i>Gammaproteobacteria</i>
MLN1	ML	Anaerobic	<i>Photobacterium</i> sp.	99	<i>Gammaproteobacteria</i>
ISN2	IS	N free medium	<i>Rhizobium</i> sp.	98	<i>Alphaproteobacteria</i>
ISC5	IS	SN medium	<i>Leptolyngbya</i> sp.	98	<i>Oscillatoriales</i>
ISC4	IS	SN medium	<i>Synechococcus</i> sp.	97	<i>Chroococcales</i>

In order to visualize bacteria containing nitrogenase enzyme in the sponge-derived enrichment cultures and tissue sections of sponges, CARD-FISH was used to target the bacterial universal 16S rRNA gene and CARD-IFA was used to target nitrogenase. To validate these two imaging techniques, I collaborated with Ryan McDonald, in Dr. Harold Schreier's laboratory, in his studies of the diazotrophic microbial community in the gut of Amazonian wood-eating catfish. I was able to establish the protocol for CARD-FISH in anaerobic enrichment culture from fish gut inocula (Fig. 2.10) and in thin sections of the gut (Fig. 2.11). CARD-IFA was successfully used to localize nitrogenase in anaerobic enrichment culture from fish gut (Fig. 2.12) and thin section of the gut (Fig. 2.13). Applying these protocols, I was able to successfully visualize the nitrogenase in anaerobic enrichment cultures from the sponge *M. laxissima* (Fig. 2.14). Importantly, the nitrogenase was also shown to be present in the filamentous cyanobacterial strain ISC5 (Fig. 2.15). I was unable to visualize the nitrogenase in the strain *Synechococcus* ISC4, although the presence of *nifH* gene in this strain was shown by PCR amplification. Various attempts were made to localize nitrogenase in sponge tissue sections, but I did not obtain consistent results showing co-localization of bacterial cells and nitrogenase-positive signals.

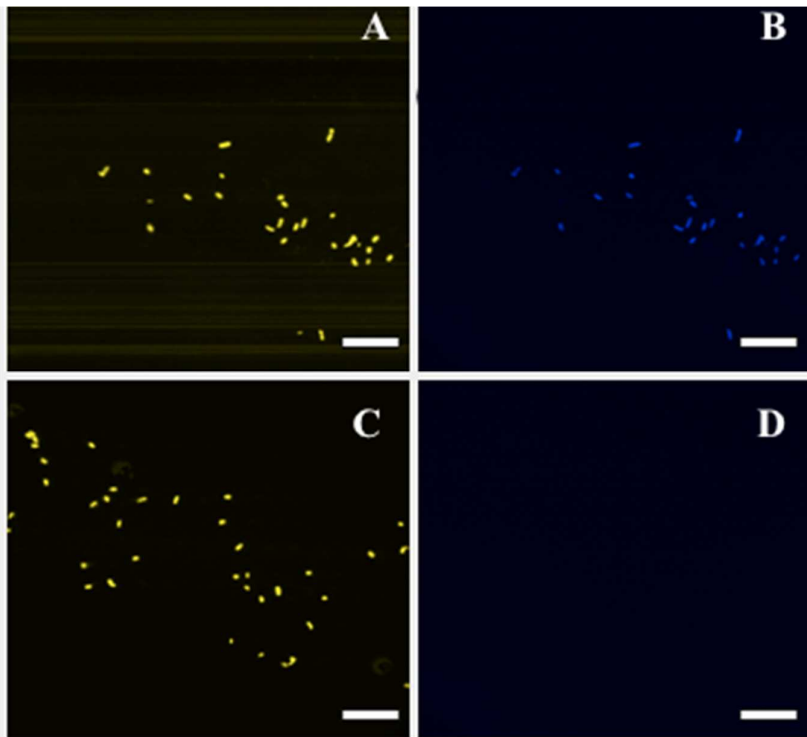


Figure 2.10. Validation of CARD-FISH method using anaerobic enrichment culture from the fish gut. (A) Counter-staining with SYBR gold. (B) Hybridization with CARD-FISH Eub-338 probe. (C) Counter-staining with SYBR gold. (D) Hybridization with CARD-FISH non-Eub-338 probe as negative control. Bar = 10 μm .

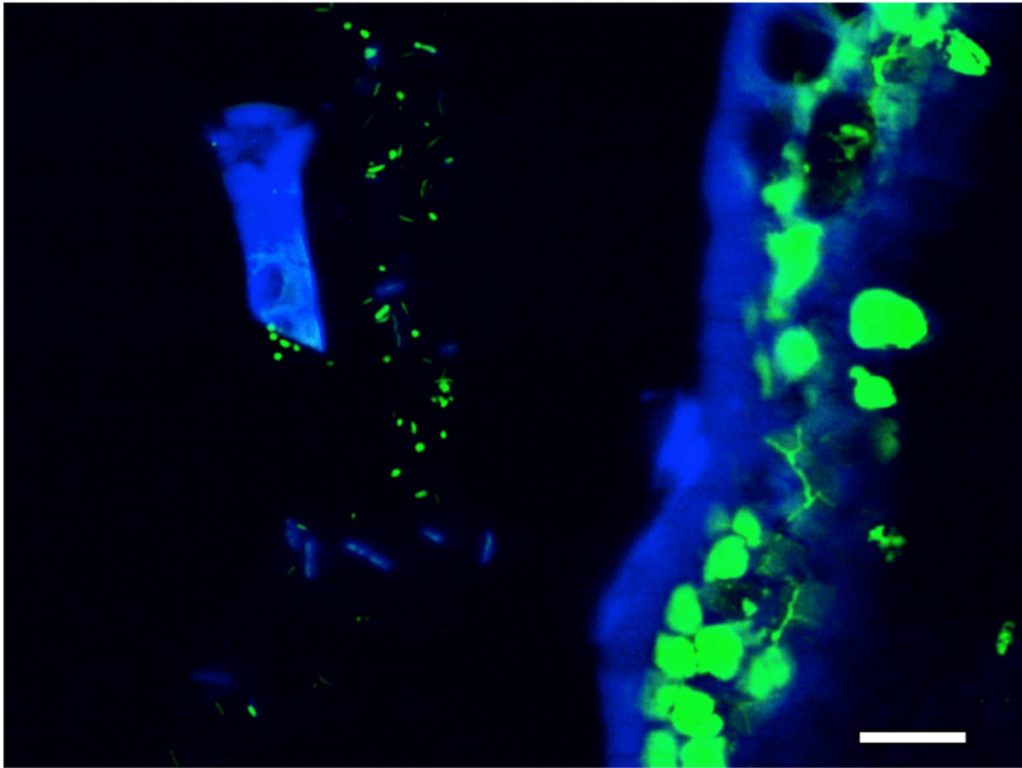


Figure 2.11. CARD-FISH: Bacteria were visualized in the thin section of the fish GI tract using EUB338 eubacteria probe (Blue). The sample is counterstained with SYBR gold to visualize cell nuclei (Yellow). Bar = 10 μm .

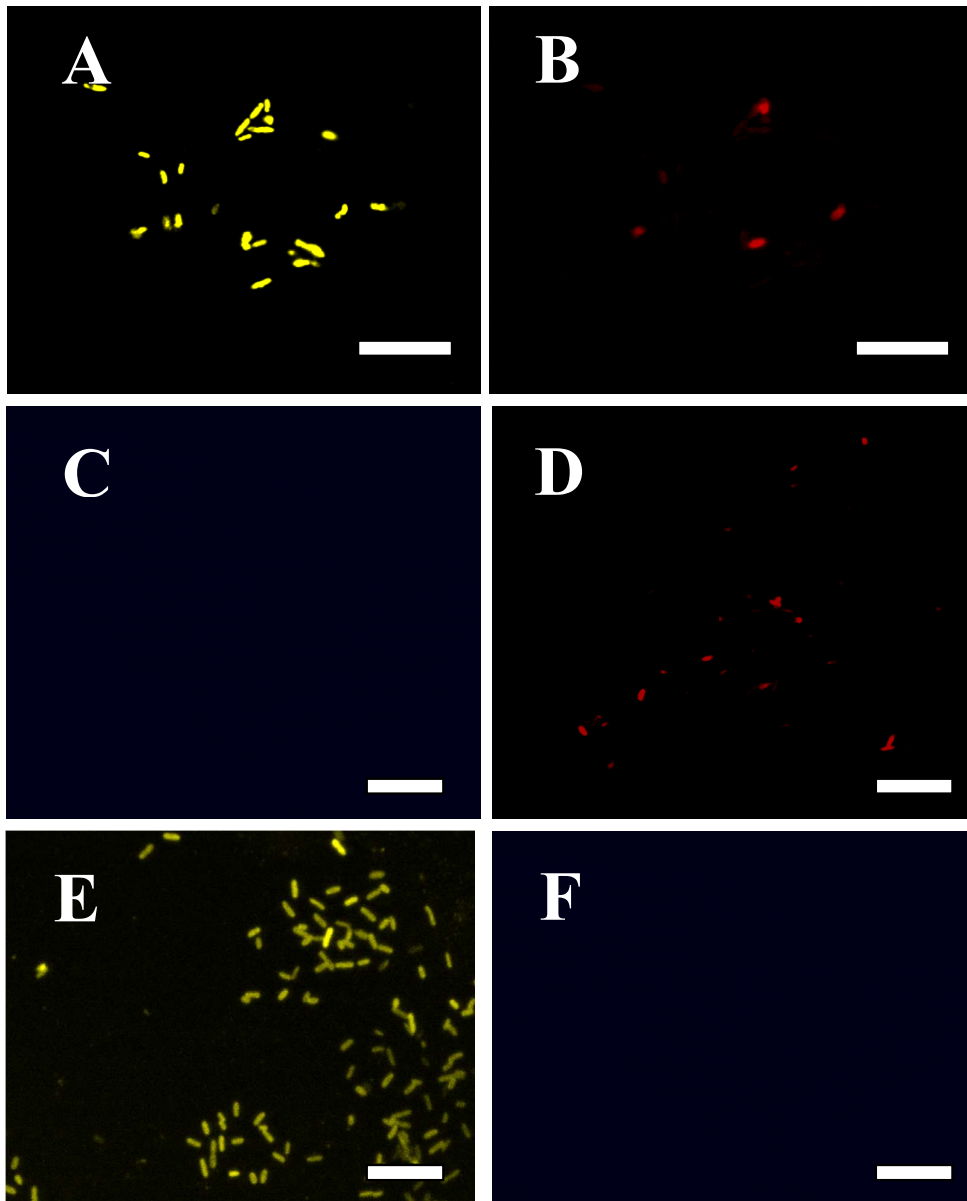


Figure 2.12. Validation of immuno-fluorescent assay for nitrogenase detection in anaerobic enrichment culture from fish gut. Hybridization with Eub-338 probe conjugated with 6-FAM (A) and applied primary anti-nitrogenase antibody (CY5) (B). Hybridization with non-Eub338 probe conjugated with 6-FAM as negative control (C) and applied primary anti-nitrogenase antibody (CY5) (D). Hybridization with Eub338 probe conjugated with 6-FAM (E) and no primary anti-nitrogenase applied as negative control (F). Bar = 10 μ m.

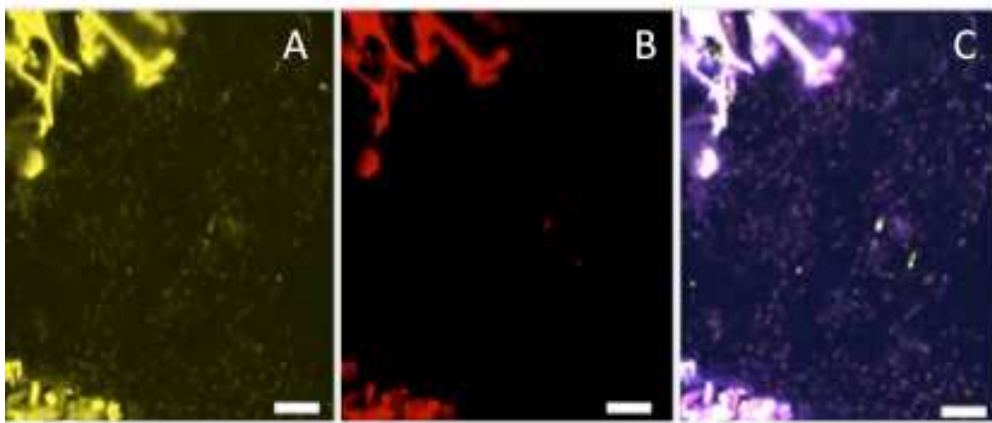


Figure 2.13. N-fixing bacteria were identified in the thin section of fish gut by anti-nitrogenase antibody. (A) The thin section of fish gut was counterstained by SYBR gold (yellow). (B) Anti-nitrogenase antibody conjugated with HRP was applied for nitrogenase detection. Deposition of CY5 fluorophore (red) catalyzed by HRP indicated the detection of nitrogenase in corresponding areas. (C) Merge images from channel A and B showed the co-localization of nitrogenase signal and bacterial cells. Bar = 10 μm .

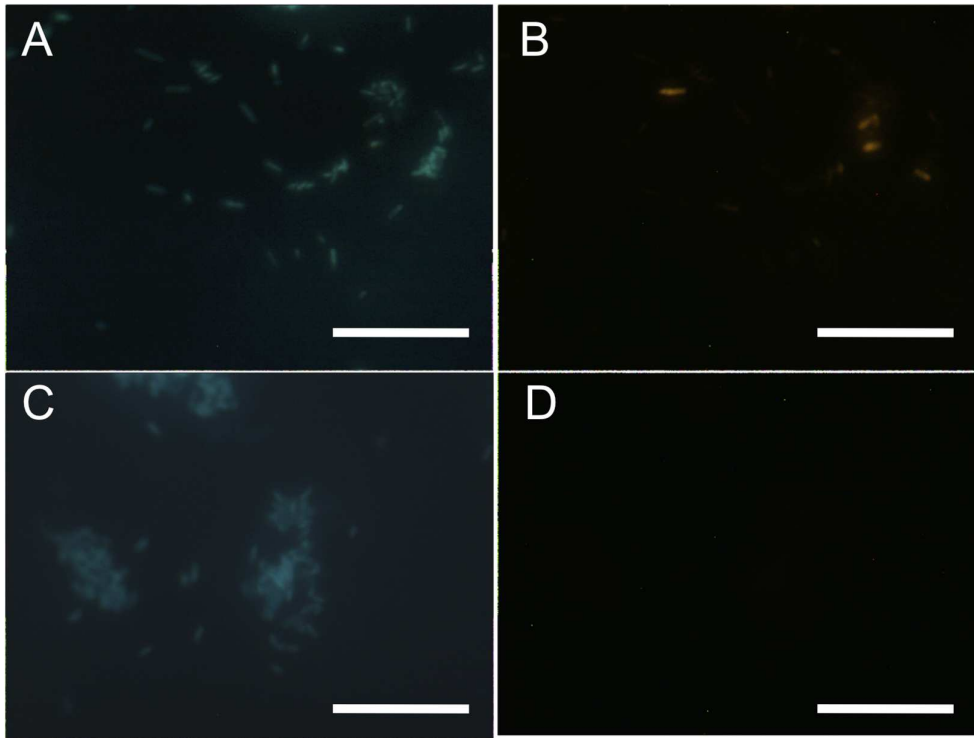


Figure 2.14. Anaerobic enrichment culture from sponge *M. laxissima* stained with DAPI and primary antibody under UV excitation (A) and same field with green excitation (B). The same culture examined without applying primary antibody in UV excitation (C) and green excitation (D). Bar = 10 μm .

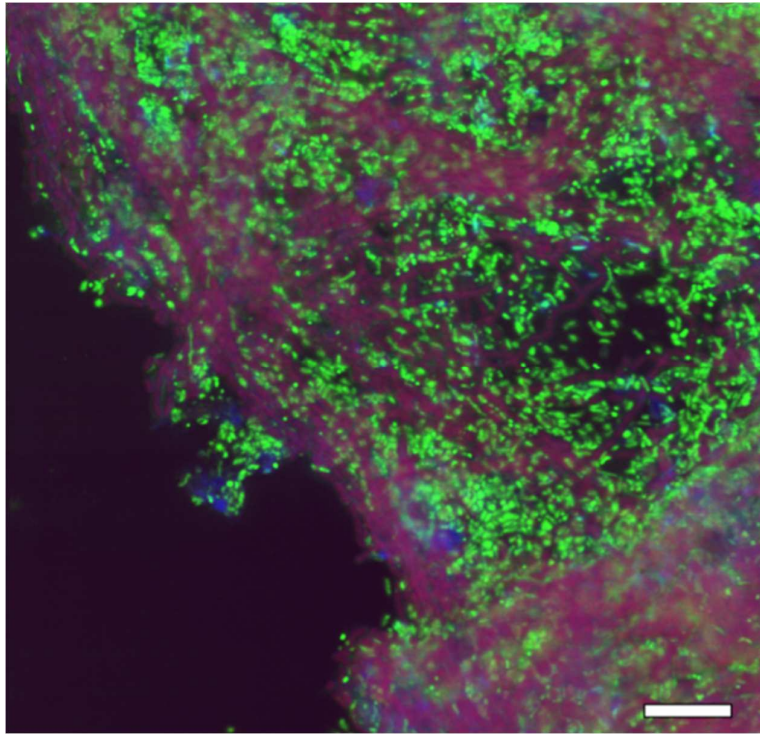


Figure 2.15. Fluorescent microscope image of filamentous cyanobacteria ISC5. Red color is the autofluorescence from the cyanobacteria under green excitation and yellow color shows the bacteria associated with cyanobacteria stained by SYBR gold. Blue color indicates nitrogenase localized by HRP conjugated antibody after Cy5 fluorophore deposition. Bar = 10 μm .

2.5 Discussion

2.5.1 Biological nitrogen fixation is important in some sponges

The $\delta^{15}\text{N}$ values in the two sponges *M. laxissima* and *I. strobilina* were consistent over two sampling periods 8 years apart, suggesting that the sources of fixed N for each of these sponge species remain the same over long periods. The fact that *I. strobilina* is considered to be a high microbial abundance (HMA) sponge and

M. laxissima to be a low microbial abundance (LMA) sponge [67, 70] is consistent with a relatively higher prokaryote activity for processes such as N₂ fixation in *I. strobilina*. Based on field observation, adult *I. strobilina* individuals are generally denser than *M. laxissima* and show lower pumping rates. This could result in less efficient O₂ penetration of the mesohyl in *I. strobilina* thus providing more anaerobic niches for N₂ fixation by non-heterocyst cyanobacteria and heterotrophic bacteria. The $\delta^{15}\text{N}$ values likely reflect the combined effect of biologically available fixed N input and loss in the holobiont. Studies on nutrient flux through sponges showed that sponges serve as a net source of nitrate [81, 99, 117], although not much nitrate and ammonia release were reported for *M. laxissima* and *I. strobilina* [21]. This observation is potentially contradictory to the hypothesis that there is N deficiency in the sponge microbiome community that requires the activity of N-fixing bacteria to provide additional fixed N. However, the net export of nitrate does not reflect the spatial heterogeneity in the sponge mesohyl [118, 119]. Whether the total sponge mesohyl is a N limited environment or not, carbohydrate input from photosynthesis and inorganic N species removal by ammonia oxidation and denitrification [82] likely requires localized replenishment of fixed N to the bacterial community. Previous research found similar low $\delta^{15}\text{N}$ values in spongin fractions derived from sponge tissue, indicating the transfer of N to the hosts [120]. Measurement of $\delta^{15}\text{N}$ values of bacteria and sponge cells separated from the same sample could provide more direct evidence on whether the hosts benefit from fixed N produced by microbial symbionts.

2.5.2 Dramatic changes in active nitrogen fixers and importance of cyanobacteria

In agreement with the previous study [82], I found similar N-fixing communities in the two distantly related sponge species, suggesting that the difference in $\delta^{15}\text{N}$ values observed in these two species might reflect differences in the rates of N-fixation rather than being the consequence of different N-fixing communities. In order to confirm the relatively lower $\delta^{15}\text{N}$ data observed in *I. strobilina*, indicating a greater role for N_2 fixation in the sponge, quantitation of *nifH* transcripts using real-time PCR could provide insights into the relative N_2 fixation potential of the two similar N-fixing communities found in the two sponge species.

Studies of coral reef ecosystems have shown that N-fixing bacteria are widely distributed in the water column and corals [121, 122]. My results show a consistent presence of N-fixing cyanobacterial groups in marine sponges from two geographic locations of the Caribbean coral reef (Sweetings Cay, Bahamas and Key Largo, Florida). The fact that cyanobacterial-derived *nifH* transcripts are dominant in sponge samples collected during the daytime, suggests that cyanobacteria are potential key N-fixing symbionts in the two sponges. The coexistence of heterocyst-forming filamentous cyanobacteria and unicellular diazotrophic cyanobacteria in both sponges also suggests that, in order to protect nitrogenase from an oxidative environment, both spatial and temporal separation strategies might be adopted by the community. However, when comparing the presence of heterocyst-forming filamentous and unicellular cyanobacterial transcripts under light/dark conditions, neither group showed a consistent diel pattern in this study.

A previous study on *nifH* gene diversity associated with corals showed a high proportion of heterotrophic bacteria in those communities [122]. In my study, high

throughput sequencing revealed rare phyla like delta-proteobacteria that were not detected by the previous study which used a cloning and sequencing method. Unlike the cyanobacteria, N-fixing heterotrophic bacteria seem to be more active in N₂ fixation at night. The heterotrophic bacterial communities were not well conserved between the two sponge species or between sponges of the same species collected at each location, suggesting that the heterotrophic diazotrophic bacteria may not be as closely associated with their host as the cyanobacterial groups. The fact that, based on phylogeny (Fig. 2.6), the closest environmental clones of many heterotrophic bacterial OTUs found in this study are connected with the benthic community (i.e. associated either with coral, sea-grass or from the marine sediment) suggests that the heterotrophic N-fixing bacterial selection may be controlled by a “first come first served” process [123], which proposes that local geographic factors matter the most in shaping some bacterial communities.

A recent study on the natural bacterial community in the Hawaii Ocean Time-Series station found a diel expression pattern in which photosynthesis related transcripts from *Prochlorococcus* peaked at dawn or dusk [124]. Although not directly influenced by sunlight, N₂ fixation is regulated by local environmental factors include pH, O₂, NH₄⁺ and organic C availability which is strongly impacted by a photosynthetic process [125]. A study of diazotrophs in the open ocean found temporal patterns of *nifH* transcript abundance in different cyanobacteria phylotypes but no obvious pattern for heterotrophic gamma-proteobacteria [126]. Though the existence of horizontal gene transfer of *nifH* in proteobacteria [127] could potentially complicate the phylogenetic assignment, so far no HGT of *nifH* gene between cyanobacteria and heterotrophs has been reported, thus the multiple taxon *nifH* gene expression in my study likely reflected bacterial responses to sporadic and transient

environmental cues inside the hosts. Sponge mesohyl can undergo spatial gradients that fluctuate through active pumping [78]. The associated diazotrophic community must control N₂ fixation under these oscillating environmental conditions. I detected diurnal patterns in those members of the bacterial community that are actively expressing N₂ fixation genes. I speculate that this pattern may reflect a combined effect of energy supply from photosynthesis and oxic states in localized regions of the holobiont during the light/dark cycle. The low light intensity at dusk, at the end of the light cycle, may be a time at which energy is still available from the day-time photosynthesis to power N₂ fixation in cyanobacteria while accumulated O₂ could limit N₂ fixation in heterotrophic bacteria. Conversely, at dawn, at the end of the dark cycle, the energy gained from photosynthesis by autotrophs may be exhausted, and bacterial respiration may create an anoxic state by O₂ consumption, favoring N₂ fixation from heterotrophic bacteria. The other two time points in my dataset may reflect the intermediate state between the two scenarios described above, thus resulting in individual variation in active N fixers. Meanwhile, some sampling constraints might limit the interpretation of the current results. First, the stress effects incurred by each time of tissue collection on the sponge hosts and associated microbial communities are worth considering. Though the actual stress impacts are difficult to assess, an additional sampling point during the daytime showing the dominance of cyanobacterial *nifH* transcripts immediately after the dark cycle, would strengthen my hypotheses. Alternatively, the detection of the expression of stress gene marker like *hsp70* gene for the host [128] or *dnaK* gene [129] for symbiotic cyanobacteria over the time course in future studies might provide insights into stress effects in the sponge during the course of sampling.

The imbalance of the global N budget indicates a potential underestimation of biological N₂ fixation [130]. N₂ fixation by non-cyanobacterial groups has been overlooked and could play an important role in marine environments [131]. N₂ fixation by non-cyanobacterial groups in sponges may also contribute to N input from the benthic community to nutrient limited coral reef ecosystems.

My study shows that cyanobacteria are a dominant and consistent group in the diazotrophic community within sponges, and various heterotrophic bacteria groups can be important components in the community. Composed of “core” cyanobacteria and flexible heterotrophic bacteria, N fixers in sponges represent an optimal combination to replenish the N pool.

2.5.3 Diazotrophic enrichment cultures from sponges

Many groups of bacteria that are widely found in environmental samples including in sponges by molecular approaches can not be cultured by traditional cultivation techniques, and are referred to as “the uncultured microbial majority” [132]. Comparing the culture dependent and independent microbial communities often generates conflicting results. Molecular gene markers like *nifH* can reveal the dynamic changes in the community structure in the natural environment, but culture independent techniques can not monitor the gene expression and N₂ fixation rate under well controlled environmental conditions, which are useful in predicting the contribution of the sponge microbial symbionts to the N budget of coral reef environment. The ability to culture key representative of the N-fixing bacteria associated with sponges in the laboratory could fill these missing gaps by enabling physiology experiments on the behavior of symbionts under condition of nutrient limitation and environmental stress. Also, the cultures would allow direct genome sequencing with high coverage and confidence in assembly to obtain genomic

information for further understanding their metabolic capacity. I successfully used an enrichment method to isolate two strains of cyanobacteria that harbor *nifH* genes, although the strains are not axenic. I was able to amplify the *nifH* gene from the genomic DNA of filamentous cyanobacterial *Leptolyngbya* strain ISC5, the expression is confirmed by *nifH* gene amplicon from cDNA. The presence of the nitrogenase enzyme was confirmed by in situ localization of nitrogenase. However, the acetylene reduction assay failed to detect the enzyme activity for the enclosed culture. It is possible that due to their low growth rate, the nitrogenase activity might fall below the instrument detection limit.

**Chapter 3: The role of symbiotic archaea in marine sponges
show stability and host specificity in community structure
and ammonia oxidation functionality**

3.1. Abstract

Archaea associated with marine sponges are active and influence the N metabolism of sponges. However, little is known about their occurrence, specificity and persistence. We aimed to elucidate the relative importance of host specificity and biogeographic background in shaping the symbiotic archaeal communities. We investigated these communities in sympatric sponges from the Mediterranean (*Ircinia fasciculata* and *Ircinia oros*, sampled in summer and winter) and from the Caribbean (*Ircinia strobilina* and *Mycale laxissima*). PCR cloning and sequencing of archaeal 16S rRNA and *amoA* genes showed that the archaeal community composition and structure was different from that in seawater and varied among sponge species. We found that the communities were dominated by ammonia-oxidizing archaea closely related to *Nitrosopumilus*. The community in *M. laxissima* differed from that in *Ircinia* spp., including the sympatric sponge *I. strobilina*; yet geographical clusters within *Ircinia* spp. were observed. Whereas archaeal phlotypes in *Ircinia* spp. were persistent and belong to 'sponge-enriched' clusters, archaea in *M. laxissima* were closely related with those from diverse habitats (i.e., seawater and sediments). For all four sponge species, the expression of the archaeal *amoA* gene was confirmed. Our results indicate that host-specific processes, such as host ecological strategy and evolutionary history, control the sponge-archaeal communities.

3.2. Introduction

Marine sponges are one of the most significant groups in benthic ecosystems in terms of diversity, abundance and function [14, 48]; notably, they are key players in the recycling of nutrients such as nitrogen [21, 117]. Early incubation experiments provides evidence of strong nitrification rate by sponges [133]. Interestingly, their contribution to nutrient cycles in the ecosystem relies on the complex microbiota associated with them, including bacteria and archaea [82, 118, 134]. Archaea have been detected in sponges from different oceans [135-137] and belong mostly to the newly-defined kingdom Thaumarchaeota, previously known as Marine Group 1 Crenarchaeota [138, 139]. Based on genomic information and physiology experiments, members of this phylum of archaea are chemolithoautotrophic ammonia oxidizers; i.e., they can convert ammonia to nitrite and fix CO₂ using the energy obtained from ammonia oxidation. Archaeal members of sponge symbiotic microbial communities may play an important role in the N cycle of the ocean.

Despite the interesting metabolism of these organisms, the symbiotic archaeal communities in sponges have received less attention than bacterial communities and little is known about the specificity, persistence or resilience of archaea in sponges. With one exception [29], sponge symbiotic archaeal communities have been found in low diversity, are metabolically active and may reach high densities in the mesohyl in some sponge species [118, 135, 140]. The first study of archaea in sponges found a sole archaeal phylotype persistently associated with *Axinella mexicana* that was named *Cenarchaeum symbiosum* [141]; and, since then, closely-related phylotypes have been found in other sponge species [35, 142]. The presence of archaea in sponge larvae suggests a tight link between archaeal symbionts and the sponge host phylogeny [143, 144]. Conversely, environmental factors (e.g., seasonal changes in

seawater conditions) or host biogeography may also affect the structure of archaeal symbionts in marine sponges [142].

Our study aims to elucidate the relative importance of host species-specific factors and biogeographic background in shaping sponge-associated archaeal communities. To achieve our objective, we investigated the archaeal communities in congeneric sponges and distantly-related sympatric sponges. Two Mediterranean species, *Ircinia fasciculata* and *Ircinia oros* (Fig. 3.1) and two Caribbean species, *I. strobilina* and *M. laxissima* were targeted. Mediterranean species were sampled in summer and winter, as this region shows a marked seasonality in seawater conditions [25]. We used cloning and Sanger sequencing techniques to study archaeal 16S rRNA and *amoA* genes as phylogenetic markers as well as *amoA* transcripts to assess the active ammonia oxidizing archaea communities. The archaeal *amoA* gene encodes the α -subunit of ammonia mono-oxygenase, which is essential in the nitrification process and is well conserved. The archaeal communities from seawater were also included in the analysis.

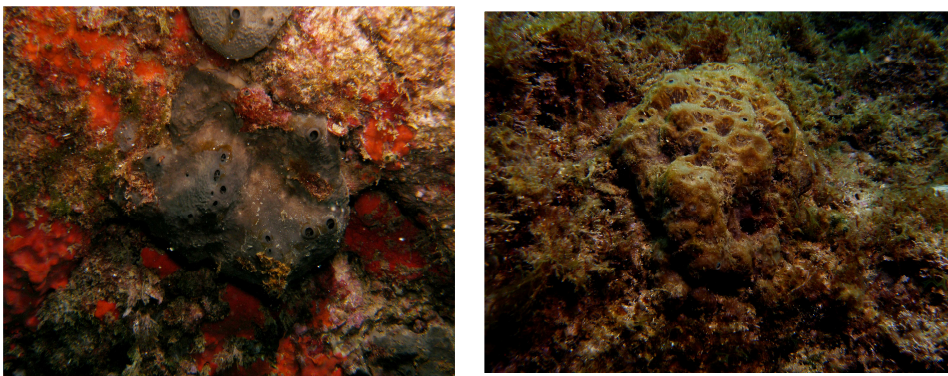


Figure 3.1. Mediterranean sponge *I. oros* (left) and *I. fasciculata* (right). Courtesy of Lucia Pita.

3.3 Material and methods

3.3.1 Sampling strategy

All sponge and seawater samples were collected by SCUBA diving. Tissue samples from three *I. fasciculata* individuals and three *I. oros* individuals were collected at Tossa de Mar (Girona), NE Spain, NW Mediterranean Sea (41°43.23'N, 2°56.45'E) during the summer (September 2012) and winter (March 2013) seasons. Three *M. laxissima* individuals and three *I. strobilina* individuals, were collected from Conch Reef, Key Largo, Florida, USA, NE Caribbean (24° 57. 11' N, 80° 27. 57' W) in July 2011. Three seawater samples from Key Largo (3 L for each sample) were collected simultaneously in close proximity (1 m) to sampled sponges and filtered through 0.22 µm Sterivex filter units (Millipore, Billerica, MA, USA). In all cases, intact sponges were held in the seawater in which they were collected, maintained at the ambient temperature, and transported rapidly (within one hour) back to a shore laboratory for processing. Seawater was drained and the sponges were rinsed three times with sterile artificial seawater. Tissue samples (1-cm cubes) were sterilely excised from sponges. Samples for DNA and RNA extraction were preserved in RNAlater stabilization solution (Qiagen, Valencia, CA, USA) on board, transferred to a -20°C freezer on site then kept in a -80°C freezer for long-term storage.

3.3.2 16S rRNA and *amoA* gene clone library construction

Total DNA and RNA from sponges and the seawater samples from Key Largo were extracted using a TissueLyser System (Qiagen) and AllPrep DNA/RNA Mini Kit (Qiagen) with RNAase-free DNase treatment steps (Qiagen) for RNA samples following the manufacturer's protocol. Reverse transcription of RNA was performed using RevertAid Reverse Transcriptase (Thermo Scientific, Waltham, MA, USA)

with random primers and following manufacturer's protocol. RNA samples without the RT step were included as PCR template to check for residual DNA in the RNA samples.

We constructed archaeal 16S rRNA gene clone libraries from total DNA for each sponge specimen and seawater samples from Key Largo. Partial archaeal 16S rRNA gene sequences (ca. 950 bp) were amplified using the archaea-specific primer set 21F: (5'-TTC CGG TTG ATC CYG CCG GA-3') and 915R: (5'-GTG CTC CCC CGC CAA TCC-3') at 0.2 μ M each [135]. 50 μ l of PCR mix included: 5 μ l of 10x high-fidelity PCR buffer (Invitrogen Life Technologies, Carlsbad, CA, USA), 2 μ l $MgCl_2$, 1 μ l of a mix of deoxynucleoside triphosphates (dNTP) at 0.2 mM, 0.2 μ l of Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen), and 1 μ L of each primer. Thermocycler parameters were set to 95°C for 5 minutes; 30 cycles of 94°C for 45 seconds, 56°C for 60 seconds, and 72°C for 60 seconds; then, a final extension at 72°C for 15 minutes. Amplification products were analyzed by electrophoresis in 1.0% (w/v) agarose gels in 1x TAE buffer. PCR products were ligated into PCR-XL-TOPO vectors and transformed into ONEShot TOP10 chemically competent *Escherichia coli* cells using the TOPO XL PCR Cloning Kit (Invitrogen). Plasmid DNA was isolated from individual clones, purified using Mini prep spin kit (Qiagen) and sequenced by using an ABI PRISM 3130XL genetic analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were screened for chimeras in mothur software [113].

3.3.3 Phylogenetic analysis of archaeal 16S rRNA and *amoA* genes

All archaeal 16S rRNA gene sequences were aligned in ARB [115] and ascribed to 97% operational taxonomic units (OTUs) using the mothur software

package [113]. Diversity metrics (observed OTUs, coverage, Chao1 estimator, Shannon index and Simpson's inverse) were calculated for sponge species and seawater. The structure of the archaeal community in each sponge species was compared by nonmetric multidimensional scaling (nMDS). All analysis were performed using mothur software package [113].

All DNA sequences obtained here and their top BLASTn hits (GenBank database) were imported into ARB [115] for 16S rRNA and *amoA* gene phylogenetic analysis. For 16S rRNA phylogenetic analysis we also included the archaeal sequences from Galand et al. [145], who characterized the archaeal assemblages in coastal seawater over seasons in the NW Mediterranean Sea. Sequences were aligned based on homologous regions for each gene (dataset included in the software for the 16S rRNA gene, and based on ca. 2000 environmental sequences kindly provided by Dr. A. Santoro for the *amoA* gene). Multiple sequence alignments were visually checked and improved manually using the ARB editor. The aligned archaeal 16S rRNA gene (699 bp) or *amoA* gene (489 bp) were imported into PhyML 3.1 software package to construct a tree based on Maximum Likelihood method (Jukes-Cantor correction). The robustness of the resulting tree topologies was evaluated by 100 bootstrap replicates for archaeal 16S rRNA and 1000 for *amoA* gene [116].

Quality-checked archaeal 16S rRNA and *amoA* gene sequences obtained in this study were deposited in GenBank under the accession numbers KJ504270-KJ504352, KJ526740-KJ526772 and KM042426-KM042428 respectively (Appendix Table S3.1 and Table S3.2).

3.4 Results

3.4.1 Diversity and structure of archaeal communities

281 archaeal 16S rRNA gene sequences obtained in our study were ascribed to 14 OTUs (97% sequence identity). Archaeal communities in Caribbean sponges were less rich than in seawater samples, and not all the diversity in seawater was covered (Table 3.1, Fig. 3.2). For the sponge species, rarefaction curves of Chao1 estimator reached the asymptote (Appendix Fig. S3.2), so we proceeded to compare the diversity metrics and structure of their archaeal communities. In terms of diversity (Shannon and inverse Simpson indices, Table 3.1), the community in *I. fasciculata* and *I. oros* was similar and stable over seasons. The archaeal 16S rRNA clone libraries derived from *I. strobilina* (3 individuals and 74 clones analyzed) were composed of only one OTU (OTU001, Table 3.2) at the 97% cut-off level. In contrast, the sympatric Caribbean sponge *M. laxissima* presented the highest richness among sponge samples; yet the archaeal diversity in this sponge is low (Shannon and inverse Simpson indices, Table 3.1), due to the community being dominated by one OTU (OTU002, Table 3.2). In the nMDS plot, *Ircinia*-derived archaeal communities appeared closer to each other than to *M. laxissima* (Fig. 3.2). In each Mediterranean *Ircinia* species, archaeal communities from different seasons clustered together and closer to the other Mediterranean species than to the Caribbean *I. strobilina* sponges. The relative abundance of OTUs in sponge species is depicted in Figure 3.3. Within the total 14 OTUs recovered in this study, 6 OTUs were retrieved in at least one sponge sample. Mediterranean *Ircinia* sponges showed the same archaeal community composition, although relative abundances of OTUs slightly varied from *I. fasciculata* to *I. oros* (Table 3.1, Fig. 3.3). The only OTU in *I. strobilina* (OTU001) was also present in the other two *Ircinia* species but absent in the sympatric sponge *M.*

laxissima and surrounding seawater (Table 3.2). In contrast, the archaeal communities in *M. laxissima* were dominated by OTU002 (99.3% identity to the cultivated archaeon *Nitrosopumilus koreensis*), accounting for more than 90% of the sequences recovered from this species (Table 3.2). OTU002 was retrieved once from the NE Caribbean seawater (Table 3.2). Similarly, another common OTU in *M. laxissima* (OTU016) was also observed in Caribbean seawater samples (Table 3.2).

Table 3.1. Richness and dominance metrics for archaeal communities in sponges and seawater based on 16S rRNA gene sequences (OTU = 97% sequence identity).

	Nb of sequences	Observed OTUs (Sobs)	Coverage	Expected OTUs (Chao1)	Inverse Simpson Index	Shannon Index
<i>I. fasciculata</i>	46	3	1	3 (3-3)	2.1 (1.8-2.6)	0.8
September	23	3	0.957	3 (0-3)	2.2 (1.8-2.9)	0.9
March	23	3	0.957	3 (0-3)	2.1 (1.7-2.9)	0.9
<i>I. oros</i>	46	3	1	3 (0-3)	1.7 (1.3-2.2)	0.7
September	22	3	1	3 (3-3)	1.5 (1.1-2.3)	0.7
March	24	3	1	3 (3-3)	1.9 (1.4-3)	0.8
<i>I. strobilina</i>	74	1	1	1 (1-1)	1 (1-1)	0
<i>M. laxissima</i>	78	4	0.974	5 (4-17)	1.1 (1.0-1.3)	0.4
Seawater						
Key Largo	37	10	0.865	13 (11-32)	4.9 (3.5-8.2)	2.1

Lower and upper 95% confidence intervals are shown in parentheses where available.

Table 3.2. Archaeal OTUs (16S rRNA gene) found in at least one sponge sample and their closest BLAST sequence matches.

Sponge-derived 97%-OTUs	No. of clones in each OTU per sponge species and seawater					Closest BLAST match (accession no., %identity, source)	Closest cultivated microorganism (accession no., %identity, source)
	ML	IST	IF	IO	SWK1		
OTU001	0	74	27	35	0	HM101089 (98.0%) sponge	NR_102913 (96%) <i>Nitrosopumilus maritimus</i>
OTU002	73	0	2	4	1	JQ227250 (100%) seawater	NR_102904 (99.3%) <i>Nitrosopumilus koreensis</i>
OTU007	0	0	17	7	0	AY192632 (98.4%) sponge	NR_102904 (96.9%) <i>N. koreensis</i>
OTU016	3	0	0	0	2	AB611676 (99.8%) seawater	NR_102904 (95.5%) <i>N. koreensis</i>
OTU023	1	0	0	0	0	EF069366 (99.5%) seawater	NR_102904 (93%) <i>N. koreensis</i>
OTU025	1	0	0	0	0	EF367493 (97.0%) sediment	NR_102904 (94.6%) <i>N. koreensis</i>

ML = *M. laxissima*; IST = *I. strobilina*; IF = *I. fasciculata*; IO = *I. oros*; SWK1 = Seawater from Key Largo, Caribbean Sea

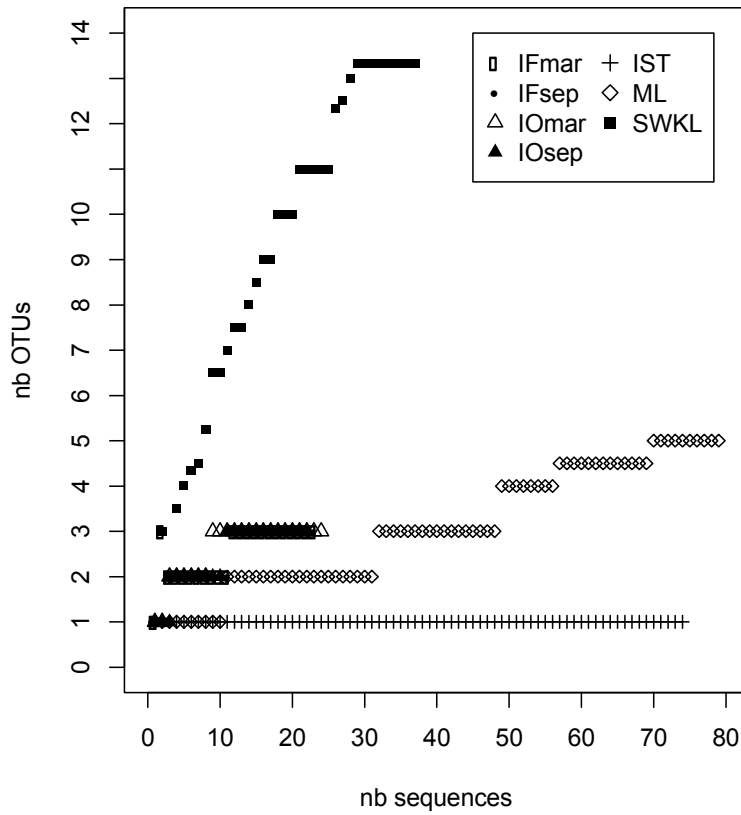


Figure 3.2. Rarefaction curves for Chao1 estimator of the archaeal 16S rRNA gene sequences obtained from *I. fasciculata* samples collected in March (IFmar), and September (IFsep), *I. oros* samples collected in March (IOmar) and September (IOsep), the Caribbean sponges *I. strobilina* (IST) and *M. laxissima* (ML), and seawater samples from Key Largo, Caribbean Sea (SWKL).

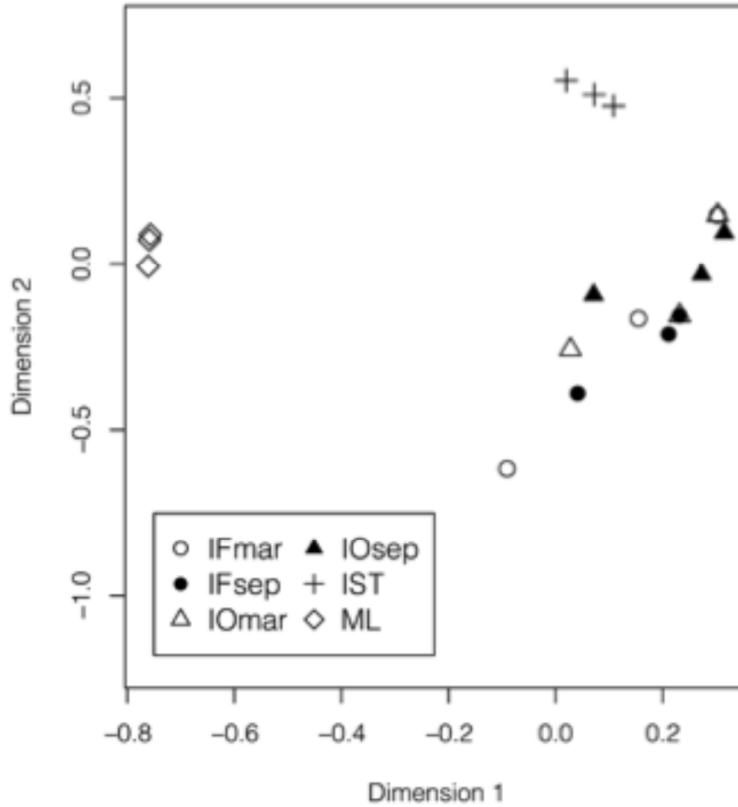


Figure 3.3. Non-metric multidimensional scaling (nMDS) plot based on Bray-Curtis distances between samples. IOsep, *I. oros* from September (summer); IOmar, *I. oros* from March (winter); IFsep, *I. fasciculata* from September (summer); IFmar, *I. fasciculata* from March (winter); IST, *I. strobilina*; ML, *M. laxissima*; SWKL, seawater from Key Largo (NE Caribbean). Stress value= 0.08; $R^2= 0.978$.

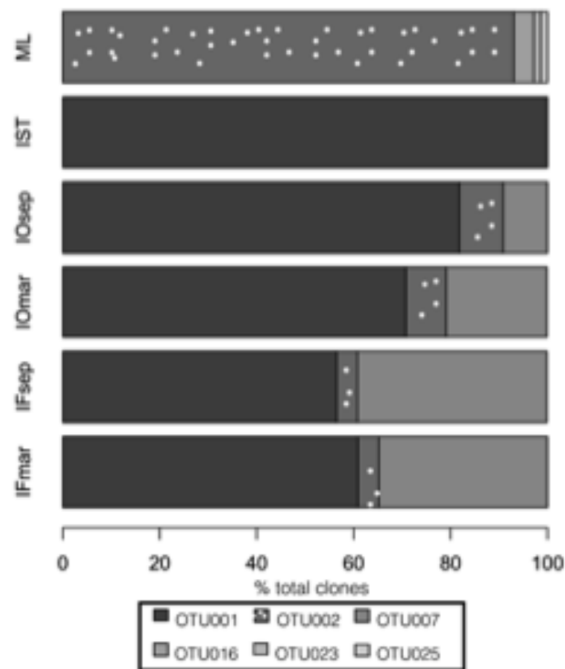


Figure 3.4. Relative abundance of archaeal OTUs (97% identity) in sponge samples, derived from 16S rRNA gene clone libraries. ML, *Mycale laxissima*; IST, *I. strobilina*; IOsep, *I. oros* from September (summer); IOmar, *I. oros* from March (winter); IFsep, *I. fasciculata* from September (summer); IFmar, *I. fasciculata* from March (winter).

3.4.2 Archaeal community phylogeny

All the sponge-derived archaeal 16S rRNA gene sequences obtained in this study fell into the recently proposed kingdom Thaumarchaeota, previously known as Marine Group 1 Crenarchaeota [138, 139] and were closely related to *Nitrosopumilus* sp. Specifically, the dominant OTU001 of archaeal 16S rRNA sequences recovered from the three *Ircinia* species formed a separate branch in the tree topology (Fig. 3.5), supporting the existence of sponge-enriched archaeal clusters, sensu Moitinho-Silva et al. [146]: sequences in this OTU clustered together with archaeal sequences

(GenBank database, $\geq 98\%$ identity) derived from other sponges species, including diverse genera and geographically distant locations (Great Barrier Reef, East China Sea, coastal of Indian Ocean). So far, this OTU has not been reported from seawater samples. OTU007, recovered from only Mediterranean *Ircinia* species, was also related with a sponge-derived sequence (Fig. 3.5, Table 3.2). In the seawater samples analyzed in this study, archaeal communities were dominated by Marine Group 2 Euryarchaeota.

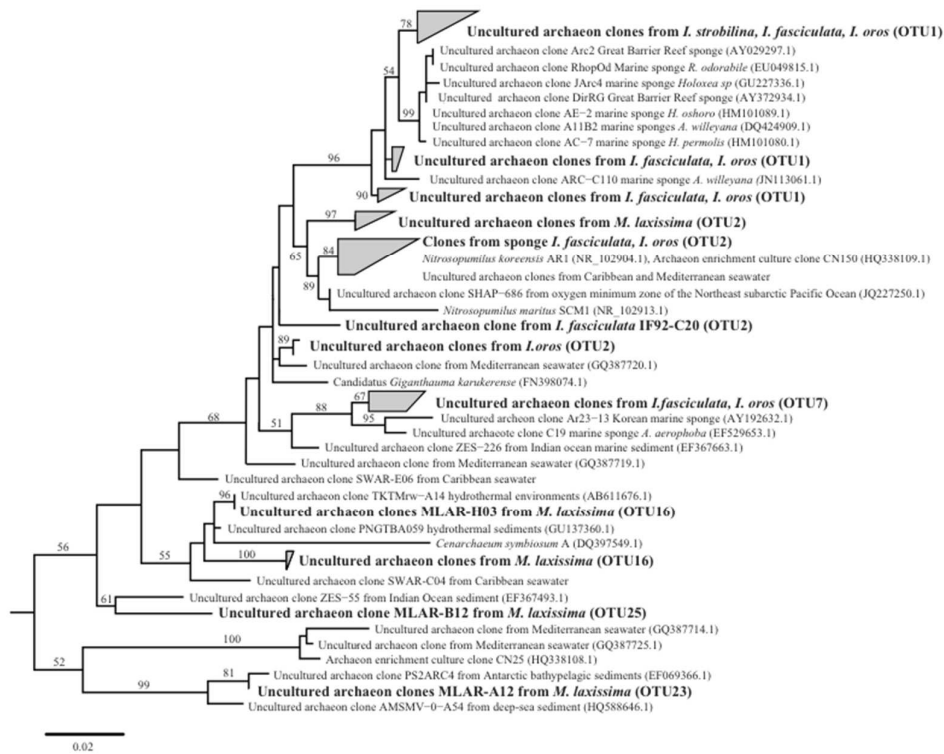


Figure 3.5. Phylogenetic relationships of archaeal communities in sponges based on archaeal 16S rRNA gene. Sequences from sponges in this study are highlighted in bold and shaded rectangles represent sequences from same sponge species or locations. Tree topology constructed using maximum likelihood method, with bootstrap values ($>50\%$) indicated at the branch nodes. 16S ribosomal RNA gene from uncultured euryarchaeote clone B0803_E3A (GQ387923.1) was used as outgroup.

3.4.3 Ammonia-oxidizing archaea (AOA)

A 635 bp fragment of the *amoA* gene was amplified from both DNA and cDNA extracts of all sponge species and seawater, which indicates the presence and activity of AOA in sponge-derived archaeal communities. We subsequently constructed clone libraries from DNA extracts for all four species and cDNA clone libraries for each Caribbean species (one sample per sponge species). We were able to amplify *amoA* gene PCR products from cDNA for all Mediterranean sponges but clone library construction was not successful. In total, 68 archaeal *amoA* gene and transcript sequences were obtained in our study and ascribed to 11 OTUs (97% sequence identity), with 6 total OTUs found in sponge samples (Appendix Table S3.3).

In the case of the Caribbean sponges, the *amoA* genes found to be expressed fell into the same OTUs that dominated the *amoA* assemblage amplified from the corresponding DNA samples. Specifically, in *M. laxissima*, *amoA* gene fragments amplified from DNA all fell into OTU006; the cloned fragments from the cDNA sample also fell into OTU006. Similarly, for *I. strobilina*, almost all *amoA* gene fragments amplified from DNA fell into OTU004 and all clones derived from this cDNA sample also fell into OTU004 (Appendix Table S3.3).

Those OTUs obtained from sponge samples in this study were closely related with *amoA* gene sequences found in other sponge species (BLASTn search, >88.2% similarity), with the only exception of OTU003 (formed by one sequence retrieved from *I. fasciculata*) that was related to a sequence from a sand filter (87.9% similarity) (Appendix Table S3.3). Phylogenetic analysis placed *amoA* sequences from sponges in separate branches based on the host genus (Fig. 3.6). The AOA sequences obtained from *Ircinia* species fell into the same cluster, together with a

sequence derived from the sponge *Rhopaloeides odorabile* (89% sequence identity), whereas the sequences from *M. laxissima* formed a different cluster together with sequences from the sponge *Luffariella* sp., a coral species and free-living archaea (Fig. 3.6).

Table 3.3. Archaeal OTUs-97% similarity (*amoA* gene) found in sponge samples in this study and their closest BLAST sequence matches.

amoA gene 97%-OTUs	No. of clones in each OTU per source							Closest BLAST match (accession no., % identity, source)	Closest cultivated microorganism (accession no., % identity, source)
	ML	MLC	IST	ISTC	IF	IO	SWKI		
OTU001	0	0	0	0	0	8	0	EU049829 (88.2%) sponge	CP003843 (84.2%) Candidatus <i>Nitrosopumilus</i> sp.
OTU002	0	0	0	0	5	0	0	EU049829 (88.6%) sponge	CP003843 (83.4%) Candidatus <i>Nitrosopumilus</i> sp.
OTU003	0	0	0	0	1	0	0	HQ455773 (87.9%) sand-filter	AB546962 (87.2%) Candidatus <i>Nitrosopumilus</i> sp.
OTU004	0	0	17	5	0	0	0	EU049829 (89.4%) sponge	CP003843 (82.0%) Candidatus <i>Nitrosopumilus</i> sp.
OTU005	0	0	1	0	0	0	0	EU049829 (89.4%) sponge	CP003843 (83.0%) Candidatus <i>Nitrosopumilus</i> sp.
OTU006	10	5	0	0	0	0	0	EU049830 (98.2%) sponge	KF957665 (87.0%) Candidatus <i>Nitrosopumilus</i> sp.
Total high-quality clone sequences	10	5	18	5	6	8	16		

ML = genomic DNA from *M. laxissima*; MLC = cDNA from *M. laxissima*; IST = genomic DNA from *I. strobilina*; ISTC = cDNA from *I. strobilina*; IF = genomic DNA from *I. fasciculata*; IO = genomic DNA from *I. oros*; SWKI = genomic DNA from Seawater, Key Largo, Caribbean Sea

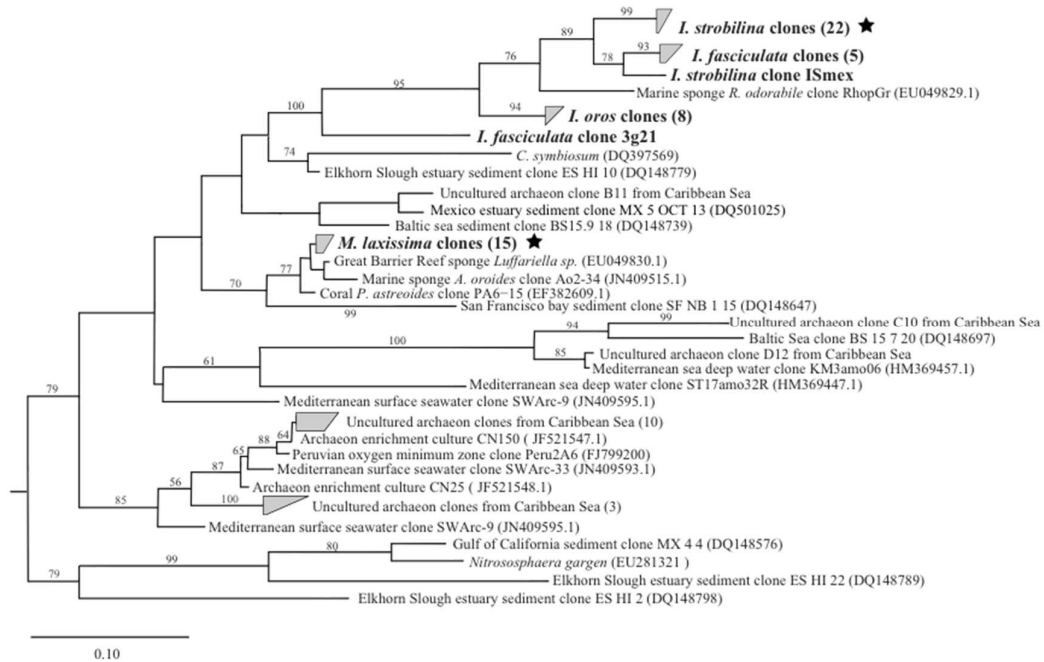


Figure 3.6. Phylogenetic relationships of archaeal communities in sponges based on archaeal *amoA* gene. Sequences from sponges in this study are highlighted in bold. Number in parentheses indicated the quantity of clones from corresponding DNA clone libraries. The star symbol indicated sequences from corresponding cDNA clone libraries. Shaded rectangles represent samples from the same sponge species or locations. Tree topology constructed using maximum likelihood method with bootstrap values (>50%) indicated at the branch nodes. Archaeal putative *amoA* gene sequence from candidatus *Nitrosocaldus yellowstonii* strain HL72 (EU239961.1) was used as outgroup.

3.5 Discussion

In this study, we analyzed the sponge-associated archaeal communities in sympatric sponges from the Mediterranean Sea (*I. fasciculata* and *I. oros*) and the Caribbean Sea (*I. strobilina* and *M. laxissima*) to elucidate the relative importance of

host phylogeny and biogeographic background in structuring these communities. Although we did not capture all of the diversity of the archaeoplankton in the surrounding seawater (Fig. 3.2), our results show that sponge species harbor symbiotic archaeal communities different from the archaeoplankton, in agreement with previous studies [147]. In all four sponge species, archaea closely related to *Nitrosopumilus* dominated the archaeal symbiotic communities and phylogenetic analysis detected “sponge-enriched” archaeal clusters. Our results, based on archaeal 16S rRNA gene sequences, *amoA* gene and transcript sequences, showed low diversity and host genus-specificity of the sponge-derived archaeal communities in *Ircinia* spp. from the Mediterranean and the Caribbean Seas.

The expression of archaeal *amoA* genes was confirmed for all species, and sequence information from cDNA libraries of the two Caribbean sponges showed expression of the same OTUs as found in DNA libraries, which suggests that dominant members in some communities were metabolically active AOA. Interestingly, the *amoA* OTUs were almost all closely related with *amoA* gene sequences found in other sponges and were quite closely related to *amoA* gene sequences from the cultured microbe *Candidatus Nitrosopumilus* sp., indicating that close relatives of this archaeon may be quite ubiquitous in sponges. However, the experimental difficulties in obtaining clone libraries from cDNA of the Mediterranean sponges limited our ability to further compare the active communities among these sponge species.

Archaeal communities in Mediterranean species were persistently recovered from samples collected during different seasons (i.e., summer and winter) despite the marked seasonality in seawater conditions of temperature and irradiance [25], supporting the stability of sponge-archaea associations [136, 141, 148]. Archaeal 16S

rRNA gene OTU001 (present in all *Ircinia* spp.) and OTU007 (present in Mediterranean *Ircinia* spp. but absent in *I. strobilina*) were consistently recovered over seasons and clustered with sponge-derived sequences in all our analysis, suggesting that these two OTUs correspond to sponge-enriched archaea.

Based on 16S rRNA gene analysis at community level, the archaeal community in sponges depended on the host species considered. The community in *M. laxissima* differed from those in *Ircinia* spp., including the sympatric Caribbean sponge *I. strobilina*, and presented the highest archaeal richness of all four sponge species, although dominated by a single archaeal OTU. Phylogenetic analysis of 16S rRNA and *amoA* gene sequences confirmed the host genus-specificity of archaea in sponges. In addition, the archaeal phylotypes dominant in *Ircinia* spp. were mostly closely related to archaea found in other sponge species, whereas archaeal phylotypes in *M. laxissima* seem to be more closely related to environmental samples. Within the same genus, the archaeal communities in Mediterranean *Ircinia* spp. (*I. fasciculata* and *I. oros*) were more similar to each other than to *I. strobilina*. Indeed, the communities in *I. fasciculata* and *I. oros* were composed of the same archaeal phylotypes, in contrast to the species-specificity of their bacterial communities [149]. Interestingly, *I. strobilina* and *I. fasciculata* are phylogenetically closer to each other than to *I. oros* [150]. It appears that the archaeal 16S rRNA gene did not reflect a coevolution of sponge hosts within a same genus with their archaeal symbionts. Our analysis suggests the influence of biogeographic background. However, the phylogeny based on *amoA* gene showed a different picture. The AOA sequences from *I. strobilina* and *I. fasciculata* were more closely related to each other than to AOA from *I. oros*, in accordance with their host phylogenetic relationships. The information discrepancy observed with these two phylogenetic markers suggests that

genes involved with functional characters like ammonia oxidation might be subjected to higher selection pressure during host evolution than ribosomal genes.

The different host specificity of archaeal phylotypes found in *Ircinia* species (dominated by sponge-enriched archaea) and *M. laxissima* (more closely related to environmental archaea) suggests a tighter link in *Ircinia* species that may reflect the different ecological strategies adopted by HMA sponges like *Ircinia* spp. [149, 151] and LMA sponges like *M. laxissima* [67], as shown for sponge bacterial communities [146, 151, 152]. Considering the low pumping rate in many HMA sponges [153, 154], the activity of AOA may provide an efficient way to remove ammonia waste secreted by the host and prevent toxic ammonia accumulation, strengthening the tie between symbionts and HMA sponges [118].

The archaeal communities in sponge hosts studied herein were specific and persistent within the same sponge species. At least some of the sponge archaeal symbionts were metabolically active AOA, suggesting that these symbionts play a key role in ammonia detoxification for their hosts, and could significantly impact the N cycle in the ecosystem. However, the archaeal community composition and structure varied depending on the sponge considered. Whereas archaeal phylotypes in *Ircinia* spp. seemed to belong to sponge-enriched clusters, geographical clusters within the genus were also observed. Comparatively, archaeal phylotypes in *M. laxissima* were closely related to sequences from diverse habitats (i.e., seawater, sediments). Our results indicated that host-specific processes, such as host ecological strategy and evolutionary history, determine the sponge-archaeal communities in some species. Persistent sponge-specific archaeal groups may provide a good target for future studies comparing sponge-associated and free-living AOA, and the interaction of bacteria and archaea within the sponge host.

Chapter 4: Phosphorus sequestration in the form of polyphosphate by microbial symbionts in marine sponges

4.1 Abstract

Marine sponges are major habitat-forming organisms in coastal benthic communities and have an ancient origin in evolutionary history. Here I report significant accumulation of polyphosphate (polyP) granules in three common sponges species of Caribbean coral reefs. The identity of the polyP granules was confirmed by energy-dispersive spectroscopy and by the fluorescence properties of the granules. Microscopy images revealed that a large proportion of microbial cells associated with sponge hosts contained intracellular polyP granules. Cyanobacterial symbionts cultured from sponges were shown to accumulate polyP. I also amplified polyphosphate kinase (*ppk*) genes from sponge DNA and confirmed that the genes were expressed. Based on these findings, I propose here a potentially important P sequestration pathway through symbiotic microorganisms of marine sponges. Considering the widespread sponge community and abundant microbial cells associated with them, this pathway is likely to have a significant impact on the P cycle in benthic ecosystems.

4.2 Introduction

4.2.1 P limitation in marine environment

In the euphotic zone of the open ocean, driven by excess solar energy supply, nutrients in the water column are normally depleted by organisms to build up biomass. Imbalance in the element supply and consumption creates direct nutrient constraints on the standing biomass. Based on Liebig's law of the minimum, life in the oligotrophic upper ocean would be most likely limited by N, P or Fe. Over the

decades, several field studies covering large-scale ocean transects observed a combination of nutrient limitation from these three elements, suggesting case-specific scenarios that are difficult to generalize [155-157]. It is a continuous debate between marine biogeochemists and geologists on which element is the ultimate limiting factor over the long term in marine ecosystems. One group proposes the notion that the N cycle controls marine primary production in the long run. They argue that the relative strength of N₂ fixation and denitrification control N availability in the oceans, consequently determining the efficiency of the biological pump, and Fe supply acts as an invisible hand behind that regulates the N cycle [158]. In contrast, the other group described P as the master regulator of the primary production in the ocean [159]. The theory is based on a simple argument: N shortage caused by denitrification would promote the N₂ fixation process within the microbial communities to increase internal supply, eventually reaching equilibrium in the long term but P demand can only be fulfilled through external physical processes or recycling of existing resources, making it difficult to balance the supply and demand by biological activities in the ecosystem. Many field studies have shown that surface oceans are more deficient in N than P, it is likely that N limits primary production in the short term and external P input controls the production in the longer term [160]. However, several reports on nutrient availability in the coral reef environment indicate a P limitation rather than N limitation in these ecosystems [10, 161].

4.2.2 Microbial activity related with the P cycle in the marine environment

Microorganisms are direct consumers competing for limited P. Selection pressure has forced them to evolve various strategies to adapt to low P conditions. In the Sargasso sea, phytoplankton were found to substitute P with S in the membranes, thereby reducing the cell quota for P [162]. Other than traditional P sources such as

phosphate, a large fraction of marine bacteria are able to utilize a wide spectrum of P-containing molecules with reduced P redox, such as compounds with C-P bond [163]. Abundance of alkaline phosphatase activities and transcripts was found in field measurements and meta-transcriptomic studies, indicating P scavenging behavior in the microbial community. One enigma in the ocean is the excess methane in the surface ocean, well above the equilibrium of gas level in the atmosphere, suggesting an in situ biological source. Methane production was originally considered to be an anaerobic process. The finding that heterotrophic bacteria can utilize methylphosphonate (MPn) for P acquisition and their ubiquitous presence in the environment revealed an important source of methane and a convincing explanation for the abundance [164].

Below the sunlit surface, the absence of solar energy poses a major constraint on biomass growth. Microbial communities in the dark rely on the oxidation of organic matter sinking down from the euphotic zone or take advantage of redox gradients in chemical species as their main energy sources. With use limited by energy supply, nutrient resources begin to accumulate with increasing depth. P availability tends to increase with depth below the euphotic zone, correlated with decline of O₂ availability in the O₂ minimum zone, reflecting a shifting balance toward respiration over photosynthesis. In this transitional thermocline layer, where redox state and P availability gradient are formed by mixing between surface and deep seawater, microbial activities related with the P cycle display a dramatic change. For example, the formation of phosphorite, P containing minerals, is a key sink in the P biogeochemical cycle to remove excessive P accumulation below the euphotic zone [155]. P mineralization can convert biologically active P such as phosphate to refractory minerals such as apatite, acting as a lid on marine primary production

[159]. It is believed that this P removal flux is largely controlled by physical and chemical processes, during which upwelling phosphate-rich water from deep sea result in a high concentration of P in pore water of the sediments, triggering mineralization under suitable redox conditions [165]. Microorganisms in thermocline layers can concentrate P by active uptake and enhance release of P from organic matter through phosphatase secretion. These microbially mediated activities could accelerate internal P cycle, potentially explaining phosphorite formation in the sediments of upwelling [166, 167] and some non-upwelling regions [168]. Compared with the N cycle, P is considered to be a redox insensitive molecule and not actively involved in microbially mediated elemental cycling and chemical transformation. Recent discoveries in the field begin to change this notion. Reports of microorganisms in the sediments and upwelling area reveal the existence of bacteria in the redox gradient in the ocean. These novel discoveries indicate that P can have dynamic changes in the redox state with suitable redox conditions including oxidation of phosphite by sulfate reduction [169].

The other P paradox is, compared with samples from surface water, the alkaline phosphatase (APase) activity per cell is found to be 100 fold higher in the deep water column [170], where the concentration of phosphate stays in the micromolar level, a thousand times higher than in the surface water. It is puzzling to see high APase expression in the P replete environment, contradictory to the notion that high APase presence indicates P stress. A recent study suggested the APase might serve to break down the sinking DOM for C skeletons rather than for P acquisition [171]. Therefore caution is needed in using APase as a gene marker for P limitation. P uptake gene like *pstA* might be a better candidate to indicate P limitation.

Not limited to the microbial community, P is of course also present in viruses. Element stoichiometry analysis of the viral community found that the N:P ratio in the viral particles ranged from 5:1 to 8:1. This is relatively low in N content compared to heterotrophic bacteria and cyanobacteria in which the ratio is 16:1 and 10:1 respectively. This is due to their relative richness in nucleotides in viruses. These P rich particles with high natural abundance may play an important role in P cycle in the ocean [172].

4.2.3 PolyP and the polyphosphate kinase gene

PolyP is a polymer of phosphate with tens to thousands of monomers, all connected by high-energy phosphoanhydride bonds (Fig. 4.1). The ubiquitous presence of polyP in all cell types in nature and their abundance in prebiotic Earth suggests this molecule could be a possible precursor before DNA and RNA. Besides serving as a P and energy reservoir for microorganisms in adverse conditions, polyP is involved in regulating many basic bacterial metabolic pathways. In most prokaryotes, two conserved genes are directly connected to polyP metabolism. The polyP synthesis kinase (*ppk*) gene is responsible for polyP synthesis from phosphate using ATP; exopolyphosphatase (*ppx*) gene is responsible for polyP hydrolysis to phosphate and generation of ATP. Both genes are constitutively expressed, thus the accumulation of polyP is controlled by the relative activity of the two enzymes.

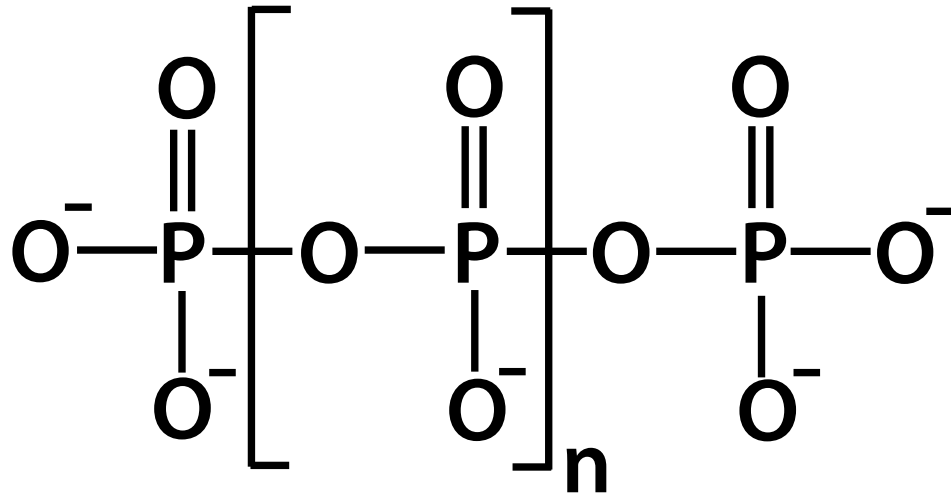


Figure 4.1. Composition of inorganic polyphosphate (PolyP). The value of n determines the number of phosphate unit in the polyP molecule with various lengths.

The construction of genetic mutants and isolation of the corresponding enzymes enabled further characterization of the biochemistry of this process [173]. Genetic studies showed that *ppk* plays a key role in the bacterial stress response, especially when cells are shifted to nutrient poor conditions. The accumulation of polyP triggers the ribosomal protein degradation [174]. This could be an important trait for microbial survival in the resource-limited open ocean. With no homologs found in mammalian cells, the Ppk protein was considered to be a promising antibacterial target in controlling human pathogen virulence.

PolyP metabolism has also been extensively studied in sewage treatment facilities as an effective way to remove excess phosphate from wastewater through polyP accumulating bacteria, a process called enhanced biological phosphorus removal (EBPR) [175]. The process starts with an anaerobic phase to select enrich polyP accumulating bacteria (PAO) in the community, then treatment is switched to an aerobic phase to facilitate the uptake of phosphate from the water column by PAOs

and storage as polyP granules (Fig. 4.2). With intracellular high-density polyP granules, PAOs sink down to the bottom as sludge at the end of the aerobic phase, thus phosphate is removed from the water column. So far, researchers can neither confirm the identity of the PAOs nor culture them from the community [176]. The sludge obtained is inoculated as seed in the anaerobic phase of the next round as a continuous cycle. Some treatments take advantage of the high negative charge of the polyP molecule that makes it an ideal chelating agent to attract heavy metals like mercury and uranium for bio-remediation purpose [177].

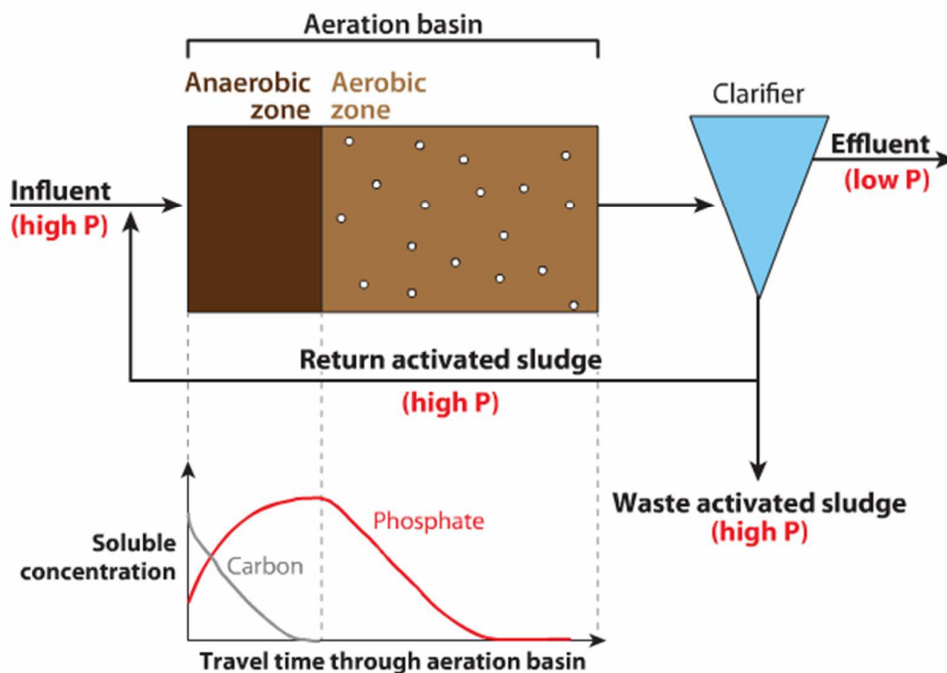


Figure 4.2. Flow diagram of a typical enhanced biological P removal activated sludge wastewater treatment system as presented by McMahon et al. [175] with permission.

Studies on polyP in the marine ecosystem face the challenge of establishing a specific, rapid and quantitative method to detect polyP in environmental samples.

Staining cells with toluidine blue can give polyP granules a purple color under light microscopy [178]; the electron density granules are also visible as dark spots in transmission electron microscope [179], but these methods of visualization provide no quantitative information. P-31 nuclear magnetic resonance (P-31 NMR) can detect polyP by distinguishing the bonding pattern of the nuclei of interest, providing a bulk and non-destructive manner to determine relative abundance of P compound like polyP, phosphonate and P esters [180]. However, due to existence of complex P compounds in nature, it is difficult to distinguish polyP in the environmental sample by bonding pattern alone [181]. A photometric method provides a single and sensitive solution to quantify polyP in the environment. The fluorescent dye 4',6-diamidino-2-phenylindole (DAPI) can bind to polyP and shifts the peak DAPI emission wavelength from 475 nm to 525 nm under UV excitation. The fluorescence intensity is proportional to the concentration of polyP [182]. Recent studies optimized the experimental protocol to enable a low detection limit at 0.5-3 μM [183, 184]. An electron microscope equipped with Energy Dispersive Spectroscopy (EDS) can provide element compositional information on sample surfaces, but the technique provides only element relative abundance and not chemical species information. Rigorous sample preparation by fixation and dehydration might limit the application in soft biological samples. With the advance of synchrotron X-ray spectromicroscopy techniques, it is now possible to simultaneously capture quantitative information of different P compounds with sub-micron spatial resolution with minimum sample treatment [185]. However, this technique is limited to a few national laboratories with synchrotron generation capacity.

4.3 Material and methods

4.3.1 Sample collection and sponge section protocol

Marine sponge samples of *X. muta*, *I. strobilina* and *M. laxissima* were collected from Conch Reef, Key Largo, Florida (24° 57. 11' N, 80° 27. 57' W) in July 2011 by SCUBA diving. Sponge tissue samples were rinsed three times with artificial seawater, fixed with 4% paraformaldehyde (weight/vol) at room temperature for 2 h, rinsed with 20 mM Tris buffer (pH 7.0) and then transferred to 70% ethanol at -20°C until use. Fixed tissues were embedded in Cryomold® with Tissue-Tek® O.C.T medium (Sakura) and solidified on dry ice. Sponge sections of 6 to 10 μM were made on a cryostat microtome (Sakura Tissue-Tek® Cryo3) under -30°C and mounted on Superfrost Plus slides (Electron Microscopy Science). Percoll density gradient centrifugation was used to separate microbial cells from the sponge tissue. Paraformaldehyde fixed sponge tissue preparations were loaded on top of discontinuous gradients containing 10%, 25%, 50% and 100% percoll respectively, then centrifuged at 800g for 15 min at 4°C. The gradient allowed the enrichment of microbial cells in the 10% and 25% fractions and sponges cells and spicules were enriched in the 50% and 100% portion of the gradient tube.

4.3.2 Extraction of total polyP from sponges samples, DNA extraction, PCR amplification of *ppk* gene and clone library

The extraction and measurement of polyP followed the protocol described by Martin and Van Mooy as their “core” protocol [186]. Lyophilized sponge tissue (100 mg) from three individuals of each species was finely ground and dissolved in 20 mM Tris buffer (pH 7.0), homogenized by vortexing, sonicated for 15 s, immersed in boiling water for 5 min, then sonicated again for 15 s. The samples were treated with

40 unit/ml DNase (New England BioLabs) and 400 unit/ml RNase (New England BioLabs) at 37°C for 10 min to remove DNA and RNA. Proteinase K (Fermentas) was added to a concentration of 20 mg/ml and samples were incubated at 37°C for 30 min and centrifuged at 16,100 g for 1 min. Supernatants were collected and the remaining pellets were subjected to four additional successive extractions following the same protocol (Fig. 4.3). Supernatants collected from the first 4 extractions were pooled for polyP fluorometric measurement. The last extraction was used to determine the background fluorescence generated by the interaction between DAPI and other substances in the extracts. Matrix effect was evaluated by adding 2 nmol polyP standard into the extraction solution. This effect inflated the signal from 5-12%. Fluorescence data were adjusted accordingly and polyP concentrations calculated based on the equation in the supplemental information of Martin and Van Mooy [186]. Total P was determined using standard chemical techniques [187] by the Nutrient Analytical Service Laboratory (HPL-UMCES).

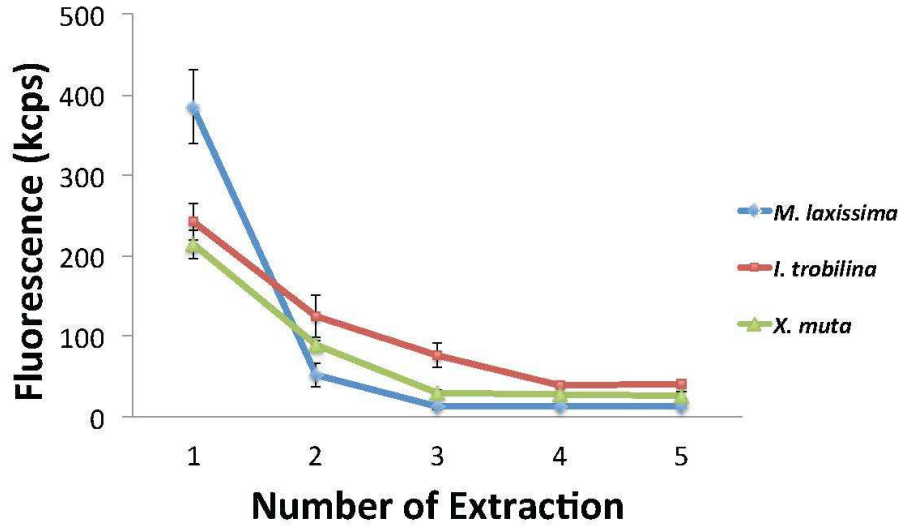


Figure 4.3. Fluorescence reading from 5 successive polyP extractions from lyophilized samples of three sponge species *X. muta* (green), *I. strobilina* (red) and *M. laxissima* (blue) stained with DAPI in triplicate. Error bars indicate ± 1 standard deviation.

The binding of polyP to DAPI shifts the peak DAPI emission wavelength from 475 nm to 550 nm under UV-light excitation, and the fluorescence intensity at this shifted wavelength is proportional to the concentration of polyP. Fluorescence measurements were carried out on a FluoroMax-4 spectrofluorometer (Horiba Scientific). The excitation wavelength was set to 415 nm with a 3 nm slit to minimize the interfering signal from free DAPI in the solution; emission wavelength was 550 nm with a 3 nm slit. Fluorescence emission spectra were recorded from 425 nm to 600 nm in 1 nm increments. PolyP standard was purchased from Sigma-Aldrich (S4379). Aliquots were dissolved in 20 mM Tris buffer (pH 7.0). The working solution for DAPI staining was 100 μ M, the solution was added to the samples at a 1 to 10 dilution to give a final DAPI concentration of 10 μ M. Samples were mixed by

vortex, incubate in room temperature for 5 min, mixed again by pipetting and measured in a quartz fluorescent cuvette. The quantification of polyP is based on the units of orthophosphate in solution, that is, the moles of polyP presented indicate the moles of orthophosphate regardless of the chain length of different polyP species (Fig. 4.4). The fluorescence signal is presented as thousands of counts per second (kcps) in all related figures and tables.

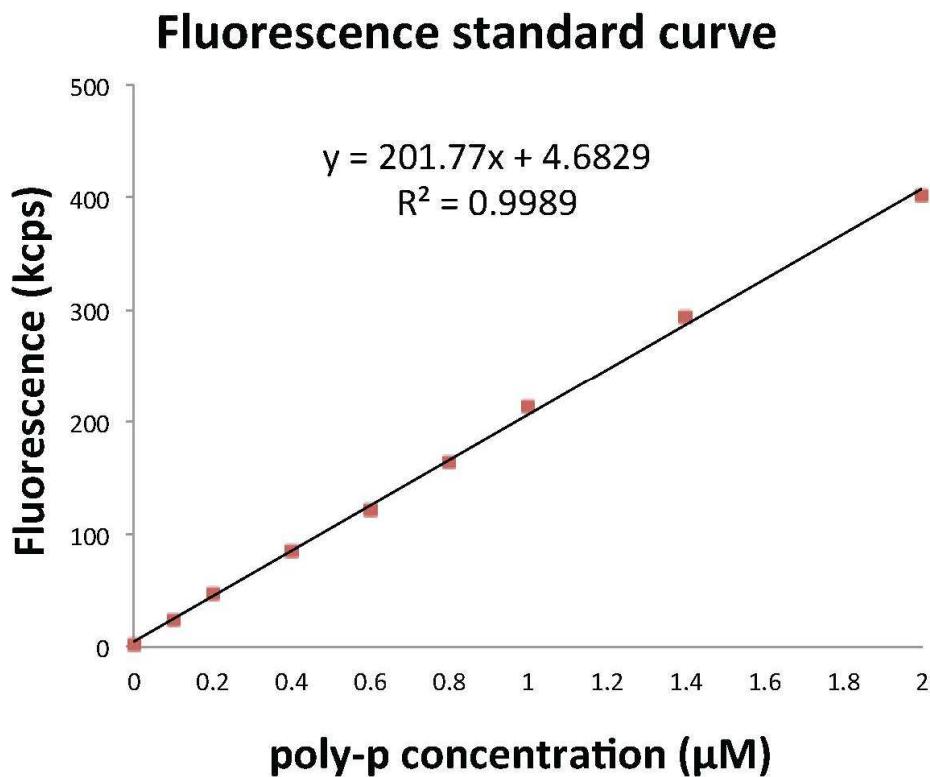


Figure 4.4. Standard curve of the fluorescence signal versus the concentration of polyP calculated in orthophosphate equivalents.

Total genomic DNA and RNA from three sponge species were extracted using a TissueLyser System (Qiagen) and an AllPrep DNA/RNA Mini Kit (Qiagen) with a RNAase-free DNase (Qiagen) treatment step for RNA samples. Reverse transcription

of RNA was performed using RevertAid Reverse Transcriptase (Thermo Scientific) with random primers and following the manufacturer's protocol. The *ppk* genes were amplified from genomic DNA and cDNA using primers designed by McMahon and colleagues [188] with the following conditions: 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 50 °C for 45 s and 72 °C for 2 min; and final extension at 72 °C for 12 min. RNA samples without RT step were included as PCR template to check for residual DNA in the RNA samples. PCR products of about 1,300 bp in length were ligated into PCR-XL-TOPO vectors, and transformed into ONEShot TOP10 chemically competent *E. coli* cells using the TOPO XL PCR Cloning Kit (Invitrogen Life Technologies). Plasmid DNA was extracted from individual clone and purified by Mini prep spin kit (Qiagen) and sequenced by an ABI PRISM 3130XL genetic analyzer (Applied Biosystems).

4.3.3 Visualization of polyP granules by fluorescence microscopy and scanning electron microscopy with energy dispersive spectroscopy (EDS)

The sponge tissue sections on Superfrost Plus slides were stained by 10 µM of DAPI solution for 2 mins, washed with MilliQ water three times, dehydrated by ethanol then imaged under a Zeiss LSM510 duo inverted confocal microscope equipped with a 40x Zeiss plan neofluar objective lens (1.3NA) in the Department of Physiology, University of Maryland, Baltimore. Sample sections were excited by a 405 nm laser light source, fluorescent emission signals were separated by filter NFT515 (NebenFarbTeiler 515) into two channels. Channel 1 collected emission wavelength from 420 nm to 515 nm, representing nucleotide-DAPI signals. Channel 2 was set up to collect emission wavelength above 530 nm, representing a shifting peak of 550 nm for polyP-DAPI signals. Figure 4.5 shows an image merged from both channels.

Bacterial cells fractions obtained from sponge tissue and cyanobacterial enrichment culture were stained by 10 μ M of DAPI solution for 2 mins, filtered onto a 0.2 μ m polycarbonate filter (Millipore), washed with MilliQ water three times and imaged by Axioplan universal microscope (Zeiss) equipped with a UV-G 365 filter set and a X-Cite series 120Q light source (Lumen Dynamic).

The Percoll fraction enriched with polyP signal under epi-fluorescence microscopy was selected and filtered onto a 0.2 μ m black polycarbonate filter. The filter samples were mounted on the graphite substrate, coated by C and imaged under JEOL 5700 Scanning Electron Microscope under voltage of 10 kV. Energy dispersive X-ray spectroscopy was used to determine element composition on focus point. Cyanobacteria enrichment culture was chemically fixed as described above. P signal heat map of cyanobacteria filament and corresponding SEM images were obtained using Hitachi SU-70 Ultra High Resolution FEG-SEM under 10 kV.

4.4 Results

4.4.1 Visualization and quantification of polyP from sponge tissues

Samples of three abundant marine sponges *X. muta*, *I. strobilina* and *M. laxissima* from Conch Reef, Key Largo, Florida were collected during June 2011. Sponge tissues sections of \sim 8 μ m in thickness were stained with 10 μ M of the fluorescent dye DAPI. Fluorescence microscopy images showed an abundance of yellow fluorescent granules (Fig. 4.5), corresponding to the shifting fluorescent emission of polyP after DAPI staining [184]. In order to further confirm the identity of the granules, Scanning Electron Microscope-Energy Dispersive Spectroscopy was applied to the granules separated from sponge tissue by Percoll density gradient (It is reported that polyP granules are not well penetrated by embedding reagents and tend

to be removed by microtome blades under traditional TEM thin section protocols [189]). Most of the granules were oval in shape with various sizes from 0.5 to 3 μm . They were visible as white, reflective bodies under SEM. A typical element point analysis on the granules yielded strong P signals, with a signature X-ray wavelength emitted from innermost shell of the atom ($K\alpha$) peak at 2.01 keV (Fig. 4.6 A). The spectra for some granules showed association of the P signal with cation ions such as calcium (Ca) with signature peak at 3.69 keV (Fig. 4.6 B), suggesting possible formation of apatite or phosphite precursors in the sponge mesohyl.

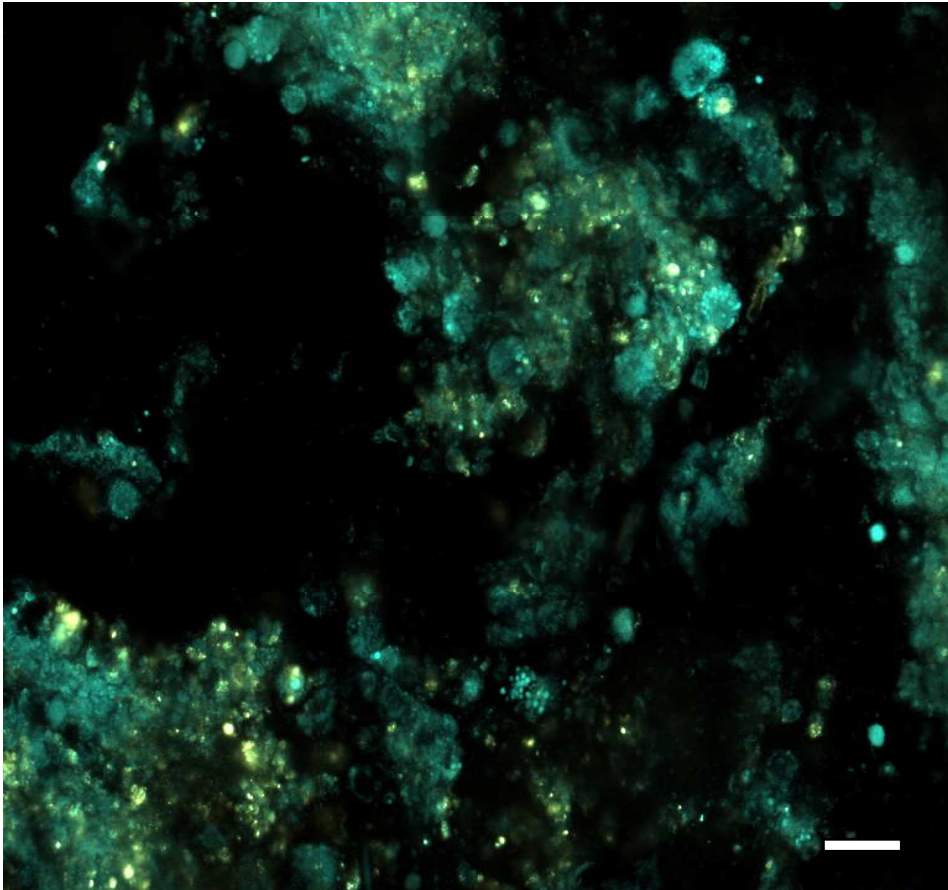


Figure 4.5. Yellow fluorescent granules observed in DAPI-stained samples. *X. muta* tissue section under confocal fluorescent microscope. Scale bar: 20 μm .

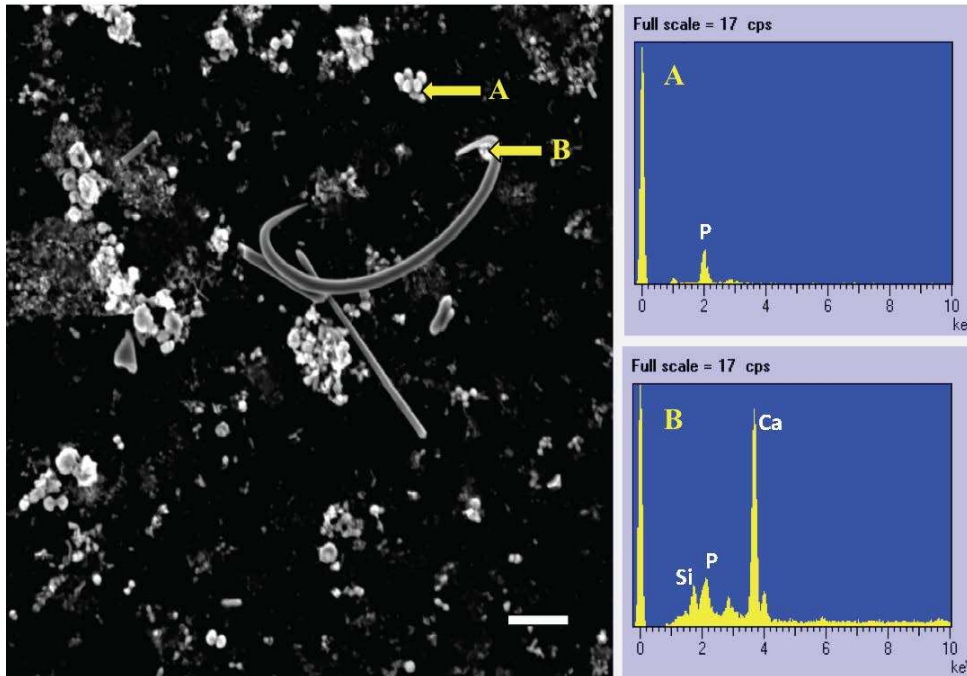


Figure 4.6. SEM image of sponge Percoll fraction obtained from *M. laxissima* and the energy dispersive X-ray spectra from the corresponding spots indicated by white arrow. Scale bar: 10 μm . (A) PolyP granules (Spectra peak at 2.01keV indicate P signal). (B) PolyP granules close to a spicule (Three major spectra peaks were identified: 1.74keV peak for Si, 2.01keV peak for P, 3.69keV peak for Ca).

The amount of polyP in the tissues of three sponge species was determined by extraction and measurement of fluorescent emission with DAPI staining [186]. To make direct comparisons, I calculated the proportion of P in the form of polyP to the total P (Table 4.1). Results showed the polyP pool could account for up to 40% of total P in sponge tissue. At the sampling site, the concentration of P in the water column ranged from 0.1-0.5 μM with a high N:P ratio, an indication of possible P limitation [161]. It was recently reported in oligotrophic Sargasso Sea, accumulation of polyP in phytoplankton enhanced the recycling of P in local environment [190] and polyP metabolism can be important in the ocean [191]. Considering the prevalence of

sponges in the coral reef and the very low P concentration in the surrounding seawater, the amount of P sequestered within sponges is likely to be a significant proportion of the total P in the ecosystem.

Table 4.1. PolyP and total P weight fraction in marine sponges, n = 3 for each sample.

Sponge sample	PolyP % dry wt \pm SD	Total P % dry wt \pm SD	PolyP: Total P
<i>X. muta</i>			
Epidermal layer	0.080 \pm 0.010	0.325 \pm 0.042	24.6%
Mesohyl layer	0.075 \pm 0.011	0.298 \pm 0.034	25.2%
<i>I. strobilina</i>			
Whole sponge	0.092 \pm 0.012	0.342 \pm 0.116	26.9%
<i>M. laxissima</i>			
Whole sponge	0.126 \pm 0.015	0.314 \pm 0.038	40.1%

4.4.2 PolyP granules located in microbial community associated with sponges

PolyP is found in almost all organisms from bacteria to plants and animals [173]. The biochemistry of polyP has been extensively studied in prokaryotes where its major roles are considered to be storing P and serving as an energy reserve [173]. The main polyP synthesis gene in prokaryotes is polyphosphate kinase (*ppk*). Polyphosphate kinase reversibly polymerizes phosphate to form high energy bond polymers and is widespread in diverse prokaryote species [173]. The gene responsible for the synthesis of polyP in eukaryotes was identified as the integral

membrane vacuolar transporter chaperone complex (*vtc4* gene) in yeast and diatoms [192]. I searched the genome of sponge *Amphimedon queenslandica* for potential *ppk* and *vtc4* genes. Blast results gave only one hit for the *ppk* gene (NCBI accession no. NW_003549918.1). The sequence has no intron and is closely related to sequences from chemolithoautotrophic bacteria at around 60% amino acid similarity.

I separated the microbial community from host tissues by Percoll density gradient centrifugation, and found a high proportion ($38 \pm 11\%$, $n=12$) of microbial cells possessed intracellular polyP granules (Fig. 4.7). Interestingly, I also obtained an enrichment culture of diazotrophic cyanobacteria from sponge tissue with the ability to accumulate polyP (Fig. 4.8). EDS mapping showed P hot spots in bacterial cells and a spectra on the point of interest showed major K α peaks for the elements P, O and Ca (Fig. 4.9). I was also able to amplify the *ppk* gene from the DNA of all three sponge species and from cDNA of *X. muta* and *I. strobilina*, indicating the existence and expression of *ppk* gene in those samples. Amplicons of *ppk* genes from sponge *I. strobilina* were cloned. Sequence results showed that those genes were related to autotrophic N-fixing cyanobacteria, aerobic anoxygenic phototrophic bacteria and S cycle related proteobacteria (Table 4.2), suggesting that these microbial symbionts might be able to obtain energy through phototrophic or chemotrophic pathways, potentially providing the energy needed for polyP synthesis.

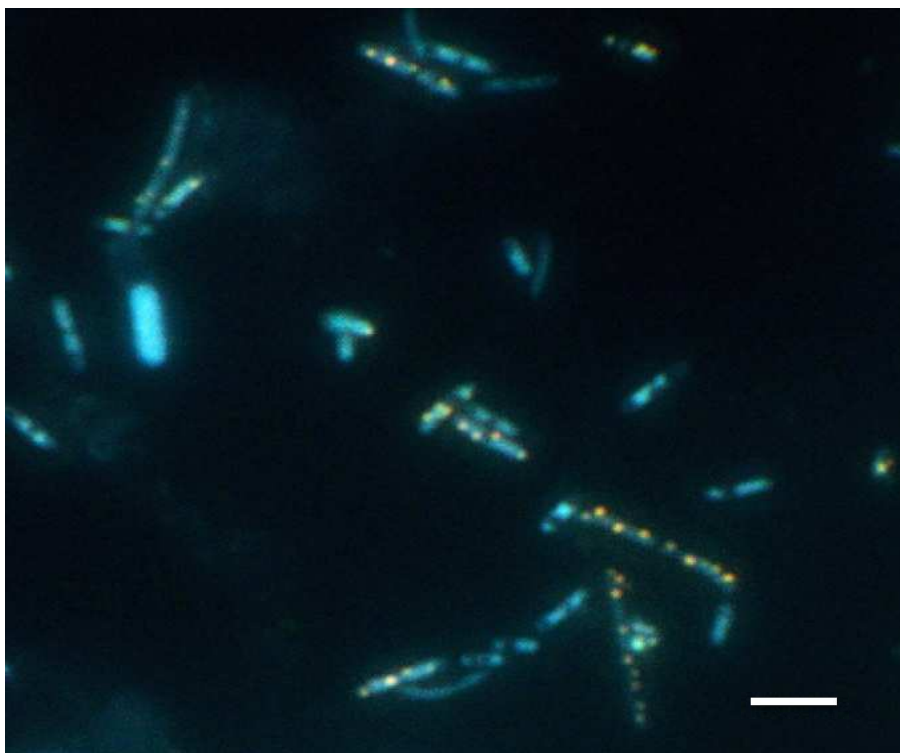


Figure 4.7. Bacterial cell fraction of sponge *X. muta* under epifluorescent microscope. Scale bar: 1 μm .

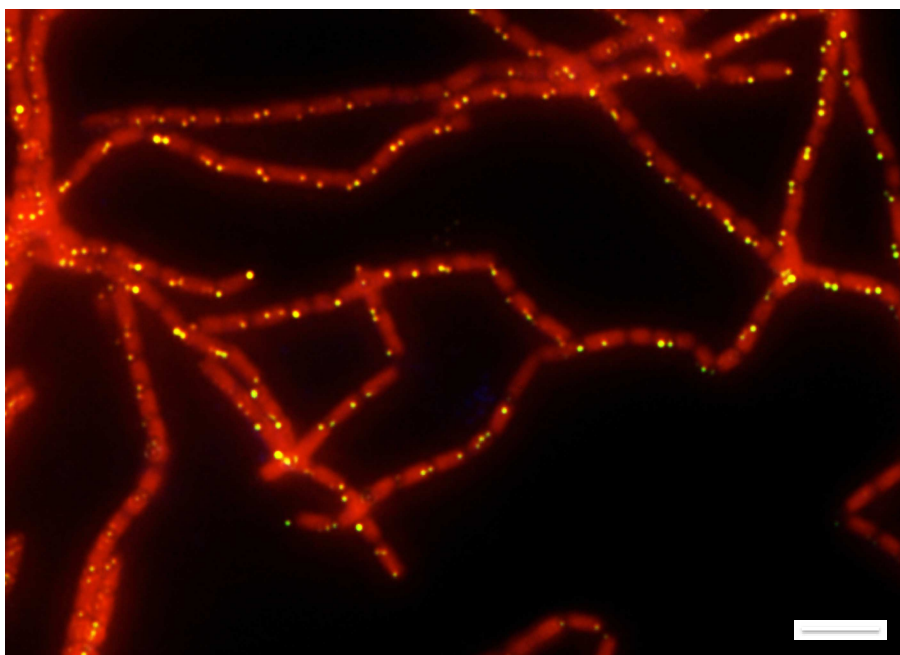


Figure 4.8. Enrichment culture of filamentous cyanobacteria *Leptolyngbya* isolated from sponge *I. stroblina* tissue. Scale bar: 1 μm .

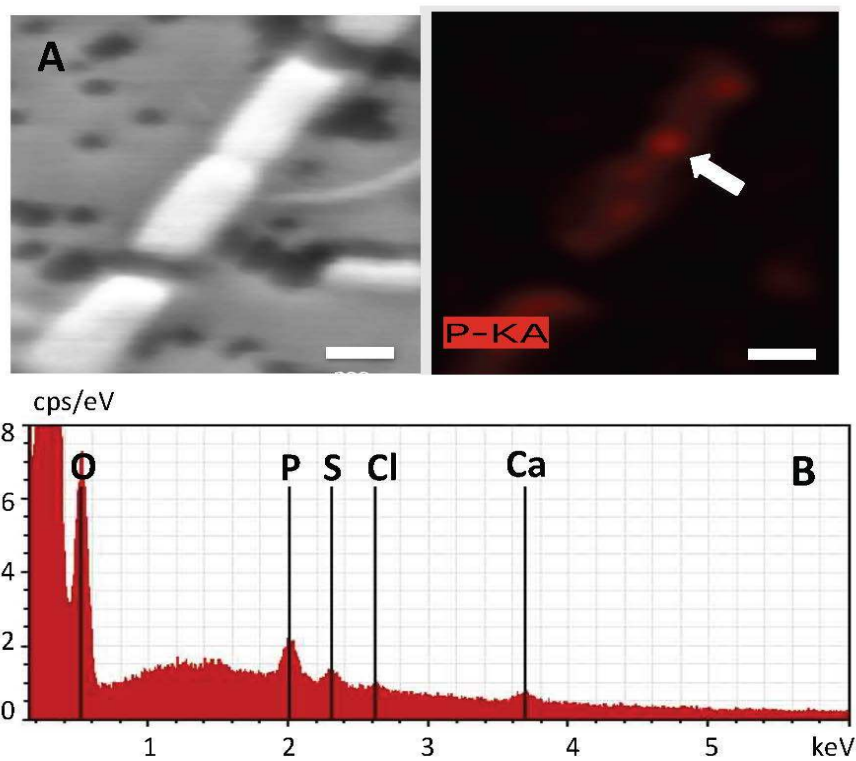


Figure 4.9. SEM image of a cyanobacterial enrichment culture from sponge *I. strobilina* and energy dispersive X-ray elemental analysis on corresponding area.

(A) Left: SEM image of the filamentous cyanobacteria *Leptolyngbya* sp. and Right: corresponding heat map of P signal. Scale bar: 500 nm.

(B) EDS spectra of point of interest (white arrow) shows a P signal along with signature peaks from other elements like Ca, Cl, S and O.

Table 4.2. Representative clones of *ppk* genes found in sponge *I. strobilina* and their closest BLASTx sequence matches.

<i>ppk</i> gene clones (accession no)	Closest cultivated microorganism (accession no., %identity, names)	Phylogenetic classification and biogeochemical cycles involved
IS-ppk-M2 (KP233195)	WP_006516974.1 (78%) <i>Leptolyngbya</i> sp. PCC 7375	N-fixing cyanobacteria
IS-ppk-M3 (KP233196)	WP_029066418.1 (90%) <i>Labrenzia</i> sp. DG1229	Phototrophic alpha-proteobacteria
IS-ppk-M6 (KP233197)	AHM02737.1 (84%) <i>Roseibacterium elongatum</i> DSM19469	Phototrophic alpha-proteobacteria
IS-ppk-M7 (KP233198)	WP_002682470 (52%) <i>Beggiatoa alba</i>	Sulfide oxidation gamma-proteobacteria
IS-ppk-M8 (KP233199)	WP_010943936.1 (56%) <i>Geobacter sulfurreducens</i>	Sulphur reducing delta-proteobacteria

4.5 Discussion

The presence of polyP and calcium phosphate granules in sponge mesohyl and the polyP accumulation in their microbial symbionts suggests that there is a potential efficient P sequestration mechanism in the sponge holobiont. Phosphate minerals in marine sediment are a major sink for the removal of reactive phosphate from water column [193]. However, the mechanism responsible for the formation of those minerals is not fully characterized. One important factor for apatite formation is a high concentration of the component ions in the local environment. Research suggests that a pulse of phosphate accumulation in sedimentary pore water could trigger the formation of apatite [193]. It is known that biogenic apatite formation is also promoted by coupling with Fe chemistry and polyP cycling in suitable redox gradients [167]. Previous studies suggested that giant S bacteria in the upwelling environment [166, 194] and diatoms in the water column [195] significantly contribute to this process through polyP accumulation.

Combining my observations and known factors in P-containing mineral formation, I here propose a working model of P mineralization through the sponge-symbiont system in the benthic environment (Fig. 4.10). First, filter-feeding by sponges efficiently removes particles between 0.2 to 10 μm in the water column, a fraction dominated by marine pico- and nano- plankton [13]. Limited nutrient flux studies compared dissolved phosphate content before and after water flow through sponge. Results suggested that sponges are a minor source of phosphate [48]; a large proportion of particulate P is therefore likely retained by the sponge holobiont. With the high affinity phosphate transporters and phosphatases that are commonly found in bacteria [196], sponge microbial symbionts may have the “luxury” of acquiring excess phosphate. Constant water flow facilitates O_2 penetration in sponge tissues

and creates a redox gradient. During periods of active pumping, the mesohyl is oxygenated, and the bacterial symbionts can convert the excess phosphate to the polyP storage form. When pumping activities cease, the O₂ level in the mesohyl can be quickly depleted [197], which could lead to polyP degradation for energy production, generating a pulse of phosphate release. The induction of polyP release as redox conditions shift from oxic to suboxic/anoxic conditions is well demonstrated in modern activated sludge sewage treatment plants where the microbially mediated phosphate release and polyP accumulation is controlled by alternating aerobic and anaerobic phases [175]. Phosphate released to the water column by sponge-associated microbes will be quickly diluted and consumed by P starved microorganisms. In contrast, the porous matrix within sponges might increase the chance of local ion accumulation. The transformation to apatite may be promoted in porous micro-environment within the sponge mesohyl where pulses of phosphate release from polyP degradation in anaerobic conditions may result in high concentrations of dissolved phosphate in tiny compartments, a similar scenario in the sediment where elevated phosphate concentration in pore water triggers mineral formation [198]. Alternatively, high concentrations of polyP combined with calcium in the sponge mesohyl can act as a nucleating template to form apatite [199]. This production of apatite from polyP in sponges may be one of the sources of the granules of biogenic apatite commonly observed in marine sediments [166, 195].

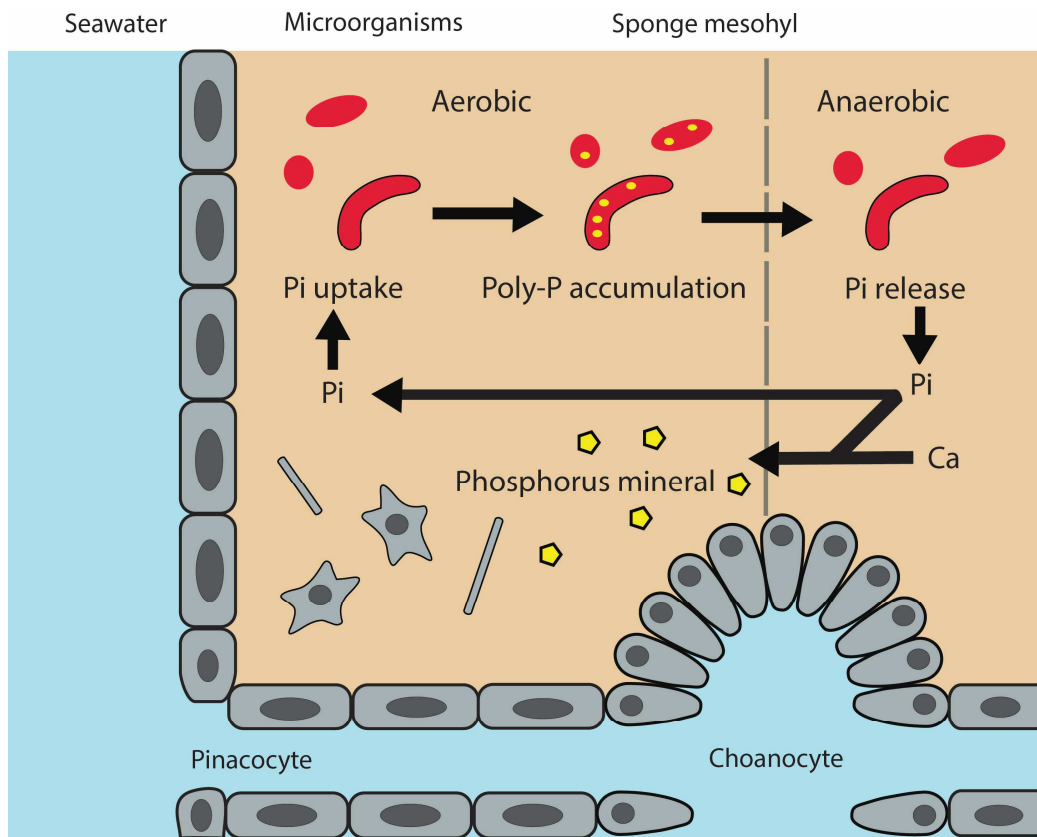


Figure 4.10. Conceptual diagram illustrating the uptake, accumulation, release of phosphate (Pi) and potential P bio-mineralization through polyP granules formation.

I propose in the conceptual diagram (Fig. 4.10) that the cycling of P inside sponges might favor bio-mineralization over biological activities, divert phosphate from re-entering the water column, and increase the burial efficiency of P in the form of apatite resulting in long-term geological sequestration. This P sequestration pathway raises several questions for future investigation: is the polyP pool in sponges transient with spikes in dissolved P pulse being released in the sponge “effluent”? Are polyP granules released from sponges, thereby resulting in sequestration of particulate P in the surrounding sediments? If so, how significant is this removal by sponges of previously bioavailable P relative to other fluxes of particulate P to the sediment? Is apatite formation in sponges a significant pathway for marine P

sequestration?

Marine primary production is thought to be constrained by N availability in the short term but subject to P limitation on longer geological time scales, based on the assumption that biological N₂ fixation should be able to keep pace with the P supply in the long run. P in marine ecosystems is supplied by riverine input and eventually lost to sedimentation in the ocean floor [200]. With increasing P input from anthropogenic mining, bacterial polyP accumulation in sponges can create an important avenue that removes excessive P from the water column to marine sediment burial. Repeating oscillation between anoxic and oxic state in the sponge mesohyl might drive selection of polyP accumulating organisms within the symbiotic community. These bacterial assemblages in sponges may serve as a new source to explore the diversity of phosphate-accumulating microorganisms and to discover novel *ppk* genes with potential for bioremediation applications.

As one of the most primitive animals on Earth [20], sponges can maintain growth under very low O₂ conditions [201], an environment that likely prevailed in continental seafloor during the Early Neoproterozoic era. It has been proposed that marine sponges may have played a vital role in the removal of phosphate to marine sediment and the expansion of the oxic zone at the ocean bottom, eventually paving the way for animal evolution [202]. Based on my finding, microbial symbionts in sponges may have made significant contributions to this benthic P cycle in early Earth history. The recently proposed concept of a “sponge loop” suggests that sponges can efficiently convert dissolved nutrient to particle forms through shedding cells, thus enhancing elemental cycles and retaining nutrient in the local environment [5, 14]. My study suggests that microbial symbionts can contribute to this process through polyP metabolism. With the wide distribution of sponges in the oceans and their

increasing population in many coral reef sites [18], sponges and their diverse microbial communities can play a significant role in mediating element cycling in the interface between the water column and marine sediment.

Chapter 5: Conclusions and future directions

5.1 Sponge microbiology: an intersection between ecology and symbiosis

The enormous diversity of microbial cells in the environment encompasses a myriad of metabolic capabilities to transform chemical species and mediate global biogeochemical cycles [47]. Their ecological impact on the marine ecosystem is well described in the “microbial loop”, where microorganisms can break down organic matter, take up inorganic nutrients and enhance elemental cycling [203]. Multiplied by the activities of billions of microbial cells in every liter of seawater in the euphotic zone, an enormous impact on nutrient biogeochemical fluxes can be expected from the microbial communities. Riding on the wave of the revolutionary advance in molecular biology and sequencing technology, which removed a major hurdle in microbial ecology, the inability to culture the majority of microorganisms, marine microbial ecologists have unveiled the major microbial players like unicellular cyanobacteria, archaea and marine viruses in marine ecosystems [204-206]. These findings have stimulated great interest in the scientific community and led to a reevaluation on the C and N biogeochemical cycles on the global scale.

Interestingly, in the past decade, another major breakthrough in science also placed microorganisms in the spotlight. In 2007, the National Institutes of Health initiated the Human Microbiome Project. The goal of this high profile project is to understand how the microbiome can affect human health by sequencing the metagenomic microbial communities associated with human gut, skin, mouth, lung and vagina. Scientific findings generated by this project so far have changed our perception of the impact of microbiome on human development and health. High throughput sequencing on large human populations unveiled distinct microbial variation in the human study groups with different ages, diet and biogeographic origins [207]. Spurred by the surging interest in the human microbiome, subsequent

studies uncovered underlying connections between human microbial symbiosis and diseases like gastrointestinal and developmental disorders with great implications in therapeutic development [208, 209]. Sponge microbiology research can benefit greatly by learning from the recent discoveries in these two fast evolving fields of molecular microbial ecology and human microbiome research. Studies of sponge symbiosis can help to bridge the gap between marine microbial ecology and microbial symbiosis, and test microbial ecological concepts in the context of microbial-animal symbiosis relationships.

Genome plasticity in bacteria through frequent horizontal gene transfer enables bacteria to acquire new genes and respond to environmental stress at a fast pace [210]. Unlike unicellular prokaryotes, multicellular eukaryotes tend to maintain genome stability and are less likely to acquire new skills by incorporating exogenous genes directly to their genomes. It makes sense for them to adopt a different strategy: partner with microbes in mutualistic or commensal relationships [56]. Various nutritional demands by the holobionts can be met by frequent exchange with the outside environment or by internal generation. The ability to obtain organic molecules from unlimited sources like CO₂ and N₂ through photosynthesis and N-fixing activities of bacterial symbionts would certainly provide a competitive advantage to the organisms that harbor these symbionts. With the genetic repertoire acquired from the bacterial partners, hosts can greatly expand their metabolic capacity and gain competitive edges in nutrient acquisition and defense against enemies, two essential themes to survive and evolve in the highly competitive natural environment. Nutrient acquisition and chemical defense do have strong underlying connections. The electron rich small molecules such as carbohydrates, peptides and lipids provide energy and building blocks necessary for the synthesis of large and complex defense

molecules like toxins and antibiotics. The benefits of chemical defense in microbial-sponge symbiosis have been demonstrated by a plethora of research articles in past decades. In this dissertation, I focused on the microbially mediated nutrient cycles happening inside sponges that have important implications for the host and for the entire coral reef ecosystem.

5.2 Microbially mediated nutrient cycles reflect differentiation in symbiotic strategies

In microbial symbiosis with insects, the two parties form an obligate symbiotic relationship, with evidence showing that either the hosts or the associated microorganisms lose redundant functions and depend on each other for nutrient exchange [211]. In contrast to the relatively simple symbiotic microbial groups in insects and some marine invertebrates [54], sponges harbor a far more diverse and less stable microbial community. Though some heritable symbionts have been observed in sponges [57, 59], other studies indicate that uptake by filter feeding is the dominant pathway for sponges to acquire symbionts, and the surrounding environments like the water column and sediments can serve as a “seed bank” to provide the best candidate for the tasks [16]. Therefore it is likely that the symbiotic relationships are tailored by specific metabolic demand from the host; spatial and temporal dimensions should also be included in consideration. In this sense, the sponge microbiome does share some similar traits with the human microbiome: both symbiotic systems show some overlaps in functional redundancy between the host genome and associated microbiome. Thus sponges can serve as a model system to study complex microbial symbiosis, which might include both obligate and facultative symbionts in the holobiont.

A high profile study found a higher bacteroidetes relative to firmicutes abundance in “obese” mice gut compared with the “lean” ones. Further metabolic analysis revealed that bacteroidetes can extract more energy from the same diet, a key factor contributing to the obesity of the host [212]. Perhaps, sponge symbionts can have a similar benefit to their sponge hosts in achieving higher energy efficiency from the same diet, thus provide a selective advantage for the partnership. The nutrient cycles studied in this dissertation are N₂ fixation, ammonia oxidation and polyP accumulation. They are all exclusively microbial metabolisms that are complimentary to the host physiology and expand the nutrient acquisition ability of the holobiont. The ability to acquire reduced N by symbiotic N₂ fixation is a good example. This benefit can be acquired by hosting cyanobacteria and heterotrophic bacteria carrying the nitrogenase gene cluster. Following a previous study on the same sponge species from Key Largo, Florida [103], I found that some *nifH* gene sequences from the most dominant OTU in the transcript dataset belong to the filamentous cyanobacteria *Leptolyngbya* and shared 100% identity in DNA sequences with the previous study. Sponge samples in the two studies were collected with a time interval of five years and spatial separation for nearly 200 miles apart in sampling sites. The highly conserved cyanobacterial *nifH* sequences suggest that cyanobacteria are key symbionts in the sponges studied. The importance of cyanobacteria in the sponge microbiome is well documented in the literature, from unicellular *Synechococcus spongiarum* in many *Xestospongia* species [213] and species specific filamentous *Oscillatoria spongeliae*, with genetic distinction from their planktonic counterparts in the water column [214]. The diazotrophic cyanobacteria, with their ability to perform C and N₂ fixation and P accumulation, might play a central role in regulating the major nutrient traffic in the sponge

mesohyl. In contrast, diazotrophic heterotrophs recovered in the two studies did not share high similarity in *nifH* gene sequences, suggesting that these symbionts might be recruited by the hosts from the surrounding water column or sediments.

Unlike the high diversity in *nifH* genes, archaeal *amoA* genes in two Caribbean sponges and two Mediterranean *Ircinia* sponges showed very low diversity and high stability. With no expression of the bacterial *amoA* gene detected in our study, it is likely that archaea were the main ammonia oxidizer for these sponges, implying that the archaeal community retains a tight association with the host. Community diversity analysis based on archaeal 16S rRNA and *amoA* gene phylogeny shows higher similarity in sponges from the same genus than from the same biogeographic location. The results support the hosts and symbionts co-evolution hypothesis, suggesting that the host identity exerts greater impact in shaping the archaeal community than biogeographic factors. The strategic differentiation in the two N cycles may reflect differential selection pressure imposed by host physiological demands. One explanation could be that while additional N input is the “icing on the cake”, the need to detoxify metabolic waste such as ammonia from animals can be essential for host survival. Considering the existence of anaerobic regions in the sponge mesohyl, anammox bacteria could be a crucial partner to remove of ammonia toxin when host subject to anaerobic conditions [82]. Forging a relationship with reliable partners responsible for ammonia oxidation under either aerobic or anaerobic conditions could be a priority in long-term evolution.

The N₂ fixation gene cluster is carried by diverse bacterial phyla and is found to be active in both aerobic and anaerobic conditions. Broader niche adaption means fewer constraints on the symbionts selection and may contribute to the higher variation in the diazotrophic community structure than the archaeal community

responsible for ammonia oxidation. The microbial community inside the sponge mesohyl is shaped by a series of combined factors including the substrate availability (ammonia, nitrate, phosphate), energy input (photosynthesis, carbohydrate respiration, chemoautotrophic) and O₂ supply. Spatial information provided by in situ localization experiments can be particularly useful in inferring the function of the sponge symbionts in specific locations.

Coral reefs are highly productive ecosystems that raise a conundrum called “Darwin’s paradox”: How can high production flourish in low nutrient conditions? This dissertation contributes to the understanding of the underlying mechanisms that govern the operation of the coral reef ecosystem by providing evidence that microbial symbionts can enhance elemental cycling in the habitat-forming sponge hosts and expand their nutrient acquisition capabilities. In my dissertation, I showed for the first time that the activity of the diazotrophic communities in marine sponges can actively change during a diurnal cycle, suggesting that temporal dimension needs to be factored in when estimating the contribution of benthic N₂ fixation to the overall N budget in this ecosystem. The successful cultivation of key cyanobacterial symbionts from the two sponges is important for subsequent work to study the response of symbionts under various environmental conditions. By comparing the archaeal communities associated with sympatric sponges separated by the Atlantic Ocean, I found that host phylogeny exerts a greater impact in shaping the symbiotic communities than the ambient environment. My study corroborates the view on tight association of microbial symbionts with their hosts, implying that symbiotic relationships can vary greatly, driven by specific functional roles. I also showed that, in three abundant Caribbean sponges, the granules, which have been commonly observed in sponge tissues for decades, are polyP granules. These granules can

account for up to 40% of the total P in sponge tissue. This finding has important implications for understanding P sequestration and recycling in the reef environment. I provide evidence that these granules are of bacterial origin and propose a P sequestration pathway through microbial symbionts and their sponge hosts. Considering the ancient origin of both partners, this process may have impacted the P cycle in Earth's early history.

5.3 Future directions

Numerous studies provide evidence that sponge hosts benefit from microbial symbiosis in chemical defense and nutrient acquisition. In return, sponge hosts might provide optimal shelter and metabolic intermediates for the growth of bacterial symbionts. More research is needed to explore the benefit from the perspective of microorganisms. To achieve this goal, the ability to culture microbial symbionts from sponges can be instrumental in understanding the metabolic needs of the microbes. Unsurprisingly, since most of the symbiotic microorganisms are difficult to culture, microbial community structure derived from culture-based method can draw a very different picture from 16S rRNA based molecular methods [215]. This is a common finding in microbiological studies in the open ocean environment, where the dominant community members are considered to be adapted to extreme low nutrient conditions, while isolation on traditional marine media would favor the growth of bacteria adapt to copiotrophic life [216]. The “dilution to extinction” method proved to be successful in isolating oligotrophic bacteria from the oceans. Representatives include *Sphingopyxis alaskensis* and *Pelagibacter ubique* (SAR11) [217-219]. Genomic information retrieved from these pure or enrichment cultures further elaborate the distinction at the molecular level between copiotrophic and oligotrophic life strategy. Sponges are surrounded by oligotrophic seawater, but filter feeding might provide

abundant nutrients in their mesohyl, therefore, it is very likely that microbial symbionts associated with sponges possess the ability to shift and adapt to both copiotrophic and oligotrophic life strategies. Genomic studies on enrichment cultures would provide key information to identify the life strategy of the dominant members, and potential auxotrophy status considering their symbiotic relationship with the hosts. Applying both regular plating and dilution to extinction, combined with customized methods for isolation of specific functional groups, will greatly increase our ability to cultivate novel microorganisms from sponges. Continuous effort to isolate microbial symbionts and obtain enrichment cultures is an important direction to complement the community diversity revealed by 16S rRNA based methods. Single cell genomic techniques are gaining popularity in environmental microbiology, and can be a powerful tool to reveal specific bacterial “dark matter” in the sponge microbiome community and provide guidelines for cultivation strategies [220].

Low $\delta^{15}\text{N}$ values found in some HMA sponges suggest that a strong biological fractionation must exist in these sponges that results in the enrichment of light N isotopes. Multiple N transformation processes in the sponges can contribute to these low $\delta^{15}\text{N}$ values. While N_2 fixation is known as a major driving force, other processes can also have a considerable impact. In bacteria, ammonia produced by N_2 fixation is incorporated into the biomass of the N fixers through the glutamine synthesis pathway. The organic N excreted from the microbes is subjected to ammonification and can be readily recycled in the mesohyl. It is known that AOA has a lower uptake threshold for ammonia than ammonia oxidizing bacteria (AOB) [221], giving them a competitive advantages in the ammonia depauperate oligotrophic ocean. This could explain the predominant presence of AOA in the outer pinacoderm of sponges [119], suggesting that ammonia might be effectively scavenged in the

pinacoderm. Therefore, ammonia oxidation might not contribute to the bio-fractionation of N isotope. Several N flux studies have confirmed that most sponges are a significant source of nitrate. A nitrate rich sponge mesohyl, combined with a possible hypoxic internal environment, would create a suitable niche for denitrifying bacteria. Excess nitrate, based on the fact that the sponge effluent is nitrate rich, suggests biological fractionation could happen in the denitrification process, in which nitrate with lighter N isotope could be preferentially consumed and returned back to N₂. In this case, the $\delta^{15}\text{N}$ in sponge tissues could be shifted toward higher values during either the denitrification or anammox processes, negating the effect of N₂ fixation, which lowers the $\delta^{15}\text{N}$ value. The relative contribution of N₂ fixation and denitrification could manipulate the N isotope fraction, accounting for the fact that $\delta^{15}\text{N}$ values are low in some HMA sponges but not in others. Basic energetic principles can be a useful tool to predict N cycle processes based on the possible chemical species and redox state inside sponge mesohyl [222].

The C and N cycles have received far more attention than the P cycle in the sponge microbiology research. One study in the 1990s showed that freshwater sponges can accumulate polyP under conditions of pollutant stress and suggested using sponges in wastewater and heavy metal treatment application [223]. My study showed that the sponge microbial community can be actively involved in redox sensitive P cycling. Unlike the animals that use calcium phosphate to form bones and teeth, sponge skeletons are composed of siliceous spicules, so the P quota of the sponges themselves is relatively low. Whether the accumulation of P by the sponge symbionts that I observed can directly benefit host physiology requires clarification in further work. The fact that the polyP synthesis gene is absent from the only available sponge genome [224] but the polyP hydrolysis gene exopolyphosphatase (*ppx*) is

present, confirmed by cloning and enzymatic characterization [225], suggests that the hosts have the ability to utilize polyP produced by bacteria in the sponge mesohyl to meet their P demand. From an ecological perspective, the process of P sequestration by polyP formation by sponge symbionts may have a major impact on the P cycle in the benthic environment and can be important in controlling the P availability in the coral reef water column. The key question is how important this polyP-mediated pathway is in coupling the pelagic-benthic P fluxes? To answer this question, in situ measurements of dissolved and particulate P fluxes are needed that cover diel patterns and seasonal variations. Metagenomic and transcriptomic studies are necessary to confirm the existence and expression of the *ppk* gene in the sponge symbionts and provide information on the genes diversity [38, 48, 146]. The results on the stability of microbial communities that carry this functional pathway and the transcriptional abundance of *ppk* genes over temporal scales can provide key information to understand how this process is functioned in the natural environment. Deep sequencing of the gene amplicon and quantitative PCR on cDNA template will be sufficient to accomplish this objective. The other important gene commonly found in the bacterial phosphate regulon is exopolyphosphatase (*ppx*), directly connected with polyP metabolism. Studies of *ppx* gene expression by microbial symbionts of sponges could provide useful information in calculating the sink of the polyP pool in the holobiont. More intriguing questions are still ahead: how do the symbiotic microbial communities regulate P availability through P sequestration? It remains to be seen the extent to which polyP and the potential formation of phosphorite deposits can contribute to the translocation of P from the water column to sediments and the overall influence on the marine P cycle.

Sponge pumping provides a mechanism to concentrate dissolved nutrients and bio-available particles from the surrounding oligotrophic seawater. Constant water flows through the sponge filter feeding also create environmental gradients like redox state, a continuous niche spectrum that can be occupied by diverse microbial symbionts, making sponges a “hot spot” for nutrient cycling in the benthic environment. Individual nutrient cycles can be dissected separately and are carried out by various microbial symbionts. Meanwhile, multiple anabolic and metabolic processes can also be conducted by the same microorganisms. For example, while conducting ammonia oxidation process in sponges, AOA are also capable of autotrophic C fixation using the energy gained from chemolithotrophy [75, 226]. Production of methane can be induced by phosphate starvation that results in utilization of methylphosphonic acid (MPn) in the cosmopolitan marine bacterium *Pelagibacter ubique* [164]. There is evidence demonstrating the interactions between elemental cycles and one cycle can exert constraints on the other through keystone microbes in the environment. The giant S bacteria *Beggiatoa* can accumulate intracellular polyP using energy generated by redox reactions, then the presence of sulfide can trigger the release of polyP, facilitating the formation of phosphorite mineral in the marine sediment [166, 227]. The N cycle can place an upper limit on the S cycle in the marine ecosystem [228]. Rich nitrate supply and an anoxic environment in the sponge mesohyl might promote sulfide oxidation activities by the symbionts. Similarly, the nutrient limitation means the imbalance between supply and demand. The accumulation of P can stimulate higher demand for other elements. Many oxidoreductase enzymes require transition metals (e.g. Fe, Cu, Mo) in their active sites [91], thus in many situations it could be the trace metals ultimately limiting biomass growth. Structural studies on phoX, a main P acquisition enzyme,

found that calcium and Fe are essential as cofactors in enzyme function [229]. In the oligotrophic environment where the growth can be co-limited by more than one nutrient element, the scarcity of Fe in the environment can constraint the organism's ability to acquire basic elements like P [230]. Subsequent studies should combine molecular markers and elemental isotope signatures to assign microbial identities with metabolic functions and relative strength in nutrient fluxes. Many scientific findings are driven by technology advances. With higher resolution of mass spectra and improving reference databases, recent research using target mass spectroscopy to characterize the meta-proteomic pattern confirmed that the microbial communities in open oceans face different nutrient limitation conditions [231]. Possibly similar studies can be done using sponge meta-proteomics. Nano-SIMS is another powerful tool that can be particularly helpful in linking the microbial community structure with quantitative spatial information on a nano-scale [232]. Elements like C, N, O and S all have different stable isotopes, which can be applied as tracers in incubation experiments to understand the element flows in the sponges. The deviation of the natural abundance of respective isotopes can reflect microbially mediated nutrient cycling. Although P has only one stable isotope ^{31}P , the bonding with four O in phosphate, which has three stable isotopes (^{16}O , ^{17}O and ^{18}O), theoretically can produce 15 phosphate isotopes, and the biological meaning of these phosphate isotopes is just beginning to be examined in various environments [233, 234]. These delicate measurements can be very challenging for environmental samples and complex biological tissues, but comparative studies on microbial cultures with phosphate rich granules could be a promising entry point. For example, comparison of C, N, O and S isotope fraction ratios in cyanobacterial filaments with and without

polyP granules by nano-SIMS could lead to clues connecting polyP metabolism with other nutrient cycle like C and N₂ fixation with convincing spatial resolution.

In the sponge holobiont, dynamic changes in environmental factors and the fact that hosting such a high microbial diversity, the number of nutrient cycling pathway and possible interactions is likely to be large and covering them all will be a formidable task. To untangle these interactions, sophisticated network analysis is necessary to generate insights with statistical support [235]. Use of a common model sponge system could be very important in pooling efforts and expertise from researchers in this field [236]. The following features are worth considering in selecting sponge model species: an abundant sponge with well characterized and persistent microbial community; consistent isotope signature indicating stability in life strategy; from coral reef research sites with rich chemical and physical background data; a well assembled and annotated sponge genome.

Although we can only get a peek at the whole picture for now, a comprehensive diagram to illustrate nutrient cycles in sponge microbiome based on existing findings can be very helpful in evaluating the potential ecological impacts of sponges in the coral reef ecosystem. The following conceptual diagram (Fig. 5.1) illustrates aerobic processes like photosynthesis and aerobic ammonia oxidation located in the epidermal layer, where the environmental requirements for these processes like light penetration and oxic state can be met. Anaerobic processes like N₂ fixation, anammox and denitrification are located in the inner mesohyl, where O₂ can be depleted by intense cell respiration and reduced water flows. The local redox state can be transient and very dynamic, therefore those facultative anaerobe may occupy a niche oscillating between aerobic and anaerobic conditions. The existence of anammox and denitrification removes the nitrate and completes the N cycle in the

holobiont. The interactions between nutrient cycles are also illustrated in the diagram. Carbohydrate fixed by unicellular and filamentous cyanobacteria can provide an energy source for N_2 fixation in the same organisms, and also supplement the regular host diet obtained by filter feeding on dissolved and particulate organic matter. Excess energy and P supply in the microenvironment can fuel the accumulation of intracellular polyP granules in cyanobacteria and heterotrophic bacteria. I speculate that these P storage granules may be discharged later under energy starvation conditions like dark or anaerobic situations, and produce ATP to sustain biosynthesis or N_2 fixation, serving as a back-up battery to maintain continuous cell metabolism. With future studies focused on the microbially mediated P cycle, the relevant metabolic pathways and the interactions with other nutrient cycles can be uncovered.

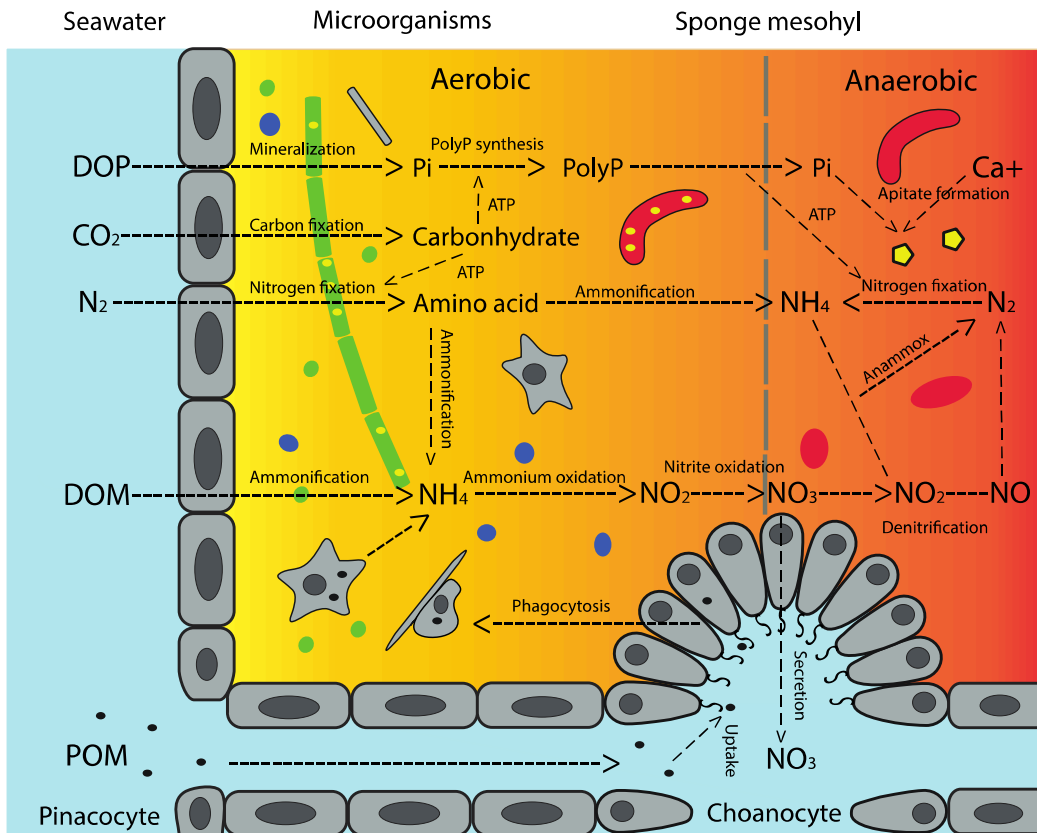


Figure 5.1. Conceptual diagram of microbially mediated nutrient cycles in sponge mesohyl. Color gradient (yellow to red) background indicates O_2 availability in sponge mesohyl, with yellow color represents the most oxic state and red color represents most anoxic state. Microbial symbionts are divided in three groups: cyanobacteria (green), archaea (blue) and heterotrophic bacteria (red). PolyP granules (yellow) accumulated in microbial cells can contribute to the formation of apatite minerals (yellow pentagon) inside sponges. Sponge cells (grey) involve in 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).

The oceans cover 70% of the Earth surface, and the majority of the oceans are low productivity areas. Large-scale Fe fertilization experiments tend to stimulate local primary production temporarily but leave much uncertainty in terms of the long-term C sequestration [237, 238]. Without a sustainable and stable community to anchor the biomass, the C captured and biomass production stimulated by nutrient addition will eventually dilute back to the external environment. Numerous cases in the past proved that, lacking a thorough understanding of the ecosystems and an appreciation of the intrinsic complexity, human intervention can drive the environment in unexpected directions that impair the stability of the ecosystem. Studies by the Effect of Nutrient Enrichment on Coral Reefs (ENCORE) project generated invaluable lessons in interpreting nutrient cycles in a systematic perspective [10]. In a complex ecosystem, the participation of organisms from all tropical levels increases the resilience of the ecosystem under constant environment stress. Imbalance of N and P input can stimulate N_2 fixation. Feedback mechanisms in the system can diverge the excessive nutrient loading by denitrification and polyP formation. However, like all systems that frequently exchange energy and matter with the outside, there is always a tipping point [239]. Studies have found that higher nutrients can increase the growth of coral, but make it easier to break. Most importantly, elevated nutrient levels in the water column can compromise close symbiotic relationships like the ones between dinoflagellates and corals, or cyanobacteria and sponges. The results from the ENCORE project show that a balanced input and output is critical to maintain the stability of the community. The increasing anthropogenic nutrient loading might represent the biggest threat to flip the tipping point, breaking the boundary of sustainability in this crucial ecosystem [10].

Coral reef ecosystems represent a system of sustainable high production with no need of continuous fertilizer input. Increasing evidence suggests that keystone species like corals and sponges are the leaders in creating this self-sustainable ecosystem.

Research on the nutrient cycles mediated by sponge holobionts, plus many other reef building organisms will undoubtedly untangle the intricacies of the symbioses and illustrate the broad ecological processes. A better understanding of the underlying mechanisms that govern the operation of ecosystems may represent the best chance to increase the presence of “oases” in the vast marine desert and provide a long-term solution to absorb elevated anthropogenic CO₂, nitrate and phosphate, alleviate pressing environmental issues such as global warming, ocean acidification and eutrophication.

Appendices

Table S2.1. *nifH* gene OTUs found in sponge sample and their closest BLAST sequence matches.

Sponge- derived 90%-OTUs	No. of reads in each OTU per sponge species				Closest BLAST match (accession no., %identity, source)	Closest cultivated microorganism (accession no., %identity, source)
	ISG	ISC	MLG	MLC		
OTU01	673	3580	1310	4320	EU594242.1 (100%) Sponge RTMLH02	KC256775.1 (88%) <i>Leptolyngbya minuta</i>
OTU02	173	1967	989	2315	HM601491.1 (92%) Florida key reef water	AB264111.1 (84%) <i>Cyanothece sp</i>
OTU03	338	2215	282	627	EU594072.1 (96%) Sponge IS15S	HQ906641.1 (99%) <i>Mastigocladus testaurum</i>
OTU04	122	855	1688	3152	KF657100.1 (88%) Coral associated	FR669148.1 (84%) <i>Klebsiella sp.</i>
OTU05	428	558	155	663	GU594006.1 (95%) Freshwater lake	DQ439648.1 (95%) <i>Anabaena sphaerica</i>
OTU06	305	1458	198	1130	EU594012.1 (93%) Sponge IS3H07	CP000781.1 (98%) <i>Xanthobacter autotrophicus</i>
OTU07	977	978	804	0	JF897530.1 (90%) Microbial mats	CP005095.1(86%) <i>Azotobacter vinelandii</i>
OTU08	0	0	195	1536	AF414668.1 (85%) Tropical sea grass	CP001614.2 (84%) <i>Teredinibacter turnerae</i>
OTU09	627	0	0	0	AF227929.1 (88%) Marine stromatolite	KC992986.1 (85%) <i>Okeania plumata</i>
OTU10	583	555	13	427	KF657052.1 (96%) Coral reef	GU238282.1 (95%) <i>Hydrocoleum lynghyaceum</i>
OTU11	426	1499	205	882	KF657112.1 (90%) Coral reef	FJ822999.1 (81%) <i>Stenotrophomonas sp.</i>
OTU12	0	0	67	1179	GU192789.1 (86%) Intertidal Microbial mats	EU622788.1 (83%) <i>Allochromatium minutissimum</i>

OTU13	573	1252	678	0	DQ177014.1 (81%) Mangrove roots	KF800047.1 (75%) <i>Desulfobulbus alkaliphilus</i>
OTU14	578	485	212	0	JQ514141.1 (89%) Mountain river	AF013025.1 (90%) <i>Microcoleus chthonoplastes</i>
OTU15	537	0	52	680	HM601491.1 (87%) Florida key seawater	EF397893.1(88%) <i>Lyngbya wollei</i>
OTU16	474	668	8	0	HQ634506.1 (94%) Red Sea	KC256774.1 (85%) <i>Hyella sp.</i>
OTU17	7	0	18	660	DQ077995.1 (85%) Marine sediment	KC256767.1 (85%) <i>Leptolyngbya saxicola</i>
OTU18	225	794	192	0	JN638712.1 (82%) Black Sea seawater	CP001649.1(84%) <i>Desulfovibrio salexigens</i>
OTU19	626	0	94	0	KF854571.1 (87%) Coral associated	AB189641.1 (86%) <i>Halorhodospira halophila</i>
OTU20	101	0	98	0	KF657141.1 (90%) Coral associated	AJ297529.2(84%) <i>Pseudomonas stutzeri</i>
OTU21	10	0	24	0	EU594006.1 (99%) Sponge IS3F08	KC243670.1 (99%) <i>Calothrix sp.</i>
OTU22	484	558	13	20	AF216914.1 (90%) Intertidal wetland	CDQ402935.1 (90%) <i>Sulfitobacter sp.</i>
OTU23	23	433	88	0	KF032146.1 (90%) Soil	FJ347433.1(88%) <i>Bradyrhizobium sp.</i>
OTU24	7	5	11	946	KF846589.1 (87%) Marine microbial mats	CP007053.1(85%) <i>Opitutaceae bacterium</i>
OTU25	0	0	0	0	KF854574.1 (81%) Coral associated	CP001998.1(79%) <i>Coraliomargarita akajimensis</i>
OTU26	0	0	0	0	EU693410.1 (92%) Coral associated	CP000143.2(88%) <i>Rhodobacter sphaeroides</i>

OTU27	0	0	0	0	AF216936.1 (82%) Cordgrass associated	AB189641.1 (80%) <i>Halorhodospira halophila</i>
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Table S3.1. Operational taxonomic units (OTUs) at 97% of sequence affiliation, isolation source and GenBank accession number for non-redundant archaeal 16S rRNA gene sequences derived from *Ircinia fasciculata*, *Ircinia oros*, *Ircinia strobilina*, *Mycale laxissima* and seawater (Key Largo) samples collected during this study.

OTU	Source	Clone	GenBank Acc	
001	<i>I. fasciculata</i>	IF33-C03	KJ504280	
		IF32-C03	KJ504282	
		IF33-C08	KJ504283	
		IF33-C06	KJ504284	
		IF93-C07	KJ504285	
		IF33-C09	KJ504294	
	<i>I. oros</i>	IO93-C02	KJ504281	
		IO91-C07	KJ504286	
		IO33-C01	KJ504287	
		IO93-C08	KJ504288	
		IO31-C11	KJ504289	
		IO92-C04	KJ504290	
		IO31-C02	KJ504291	
		IO31-C06	KJ504292	
		IO31-C09	KJ504293	
		IO92-C05	KJ504295	
		IO92-C02	KJ504296	
		IO31-C08	KJ504297	
		IO33-C08	KJ504298	
		IO32-C08	KJ504299	
		IO32-C03	KJ504300	
		<i>I. strobilina</i>	IR2ARH04	KJ504304
			IR3ARE01	KJ504305
			IR2ARB04	KJ504306

Table S3.1. (continued)

OTU	Source	Clone	GenBank Acc
001	<i>I. strobilina</i>	IR2ARF03	KJ504307
		IR3ARE06	KJ504308
		IR1ARF05	KJ504309
		IR3ARE02	KJ504310
		IR3ARC05	KJ504311
		IR1ARE01	KJ504312
		IR2ARH05	KJ504313
		IR2ARF01	KJ504314
		IR3ARA06	KJ504315
		IR2ARF05	KJ504316
		IR2ARG05	KJ504317
		IR3ARC06	KJ504318
		IR3ARA03	KJ504319
		IR2ARH02	KJ504320
		IR3ARE04	KJ504321
		IR3ARD03	KJ504322
002	<i>I. fasciculata</i>	IF31-C05	KJ504270
		IF92-C20	KJ504271
	<i>I. oros</i>	IO33-C03	KJ504301
		IO33-C02	KJ504302
		IO92-C03	KJ504303
	<i>M. laxissima</i>	MLAR-F12	KJ504342
		MLAR-D06	KJ504343
		MLAR-C12	KJ504344
		MLAR-B04	KJ504345
		MLAR-D03	KJ504346
Seawater		SWAR-G06	KJ504341
	SWAR-A04	KJ504333	
	SWAR-F03	KJ504334	
003	Seawater	SWAR-B06	KJ504335
		SWAR-G05	KJ504336

Table S3.1. (continued)

OTU	Source	Clone	GenBank Acc
003	Seawater	SWAR-E05	KJ504337
		SWAR-A05	KJ504338
007	<i>I. fasciculata</i>	IF32-C05	KJ504272
		IF92-C11	KJ504274
007	<i>I. fasciculata</i>	IF93-C06	KJ504276
		IF93-C08	KJ504277
		IF92-C13	KJ504278
		IF32-C02	KJ504279
	<i>I. oros</i>	IO33-C09	KJ504273
		IO91-C09	KJ504275
008	Seawater	SWAR-H08	KJ504326
		SWAR-D08	KJ504327
		SWAR-C08	KJ504328
		SWAR-F09	KJ504329
009	Seawater	SWAR-D09	KJ504330
		SWAR-F08	KJ504332
010	Seawater	SWAR-G03	KJ504324
		SWAR-D06	KJ504325
013	Seawater	SWAR-G08	KJ504352
016	<i>M. laxissima</i>	MLAR-F01	KJ504348
		MLAR-H03	KJ504351
	Seawater	SWAR-E06	KJ504347
		SWAR-C04	KJ504350
023	<i>M. laxissima</i>	MLAR-B12	KJ504340
024	Seawater	SWAR-A07	KJ504323
025	<i>M. laxissima</i>	MLAR-A12	KJ504349
026	Seawater	SWAR-A08	KJ504339
027	Seawater	SWAR-B09	KJ504331

Table S3.2. Operational taxonomic units (OTUs) at 97% of sequence affiliation, GenBank accession numbers and isolation source for non-redundant archaeal *amoA* gene sequences derived from *I. fasciculata*, *I. oros*, *I. strobilina*, *M. laxissima* and seawater (Key Largo) samples collected during this study.

OTU	Source	Clone	GenBank Acc
010	Seawater	SWAr-amoA-C10	KJ526740
009		SWAr-amoA-D12	KJ526741
008		SWAr-amoA-G12	KJ526742
		SWAr-amoA-B10	KJ526743
		SWAr-amoA-A12	KJ526744
		SWAr-amoA-G10	KJ526745
		SWAr-amoA-A10	KJ526746
		SWAr-amoA-B12	KJ526747
		SWAr-amoA-F11	KJ526748
011		SWAr-amoA-A11	KJ526749
		SWAr-amoA-B11	KJ526750
007	SWAr-amoA-C12	KM042426	
	SWAr-amoA-H10	KM042427	
006	<i>M. laxissima</i>	SWAr-amoA-H11	KM042428
		ML4A7	KJ526752
		ML4A2	KJ526753
		ML4A6	KJ526751
004	<i>I. strobilina</i>	IS1A7	KJ526754
		IS1A5	KJ526755
		IS1B1	KJ526756
		IS1A1	KJ526757
		IS1A12	KJ526758
005		ISmex	KJ526759
002	<i>I. fasciculata</i>	IF3g17	KJ526760
		IF3g19	KJ526761
		IF3g22	KJ526762
		IF3g24	KJ526763

Table S3.2. (continued)

OTU	Source	Clone	GenBank Acc
002	<i>I. fasciculata</i>	IF3g21	KJ526772
		IF3g23	KJ526764
001	<i>I. oros</i>	IO3g01	KJ526765
		IO3g04	KJ526766
		IO3g08	KJ526767
		IO3g03	KJ526768
		IO3g05	KJ526769
		IO3g06	KJ526770
		IO3g07	KJ526771

Glossary

AHL: Acylhomoserine lactone

ANAMMOX: Anaerobic ammonium oxidation

AMO: Ammonia monooxygenase

AOA: Ammonia oxidizing archaea

AOB: Ammonia oxidizing bacteria

cDNA: complementary DNA

DOC: Dissolved organic carbon

DOM: Dissolved organic matter

DON: Dissolved organic nitrogen

DOP: Dissolved organic phosphorus

EDS-SEM: Energy dispersive spectroscopy-scanning electron microscope

gDNA: genomic DNA

HMA: High Microbial Abundance

LMA: Low Microbial Abundance

NIF: Nitrogenase

OTU: Operational taxonomic unit

Pi: Phosphate

POC: Particulate organic carbon

POLYP: Polyphosphate

POM: Particulate organic matter

PPK: Polyphosphate kinase

PPX: Exopolyphosphatase

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