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# Investigation of Sulfur Cycling in Marine Sponge Cinachyrella Spp. from a South Florida Reef

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# Thesis of
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Submitted in Partial Fulfillment of the Requirements for the Degree of

## Master of Science
## Marine Science

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HALMOS COLLEGE OF NATURAL SCIENCES AND OCEANOGRAPHY

# Investigation of Sulfur Cycling in Marine Sponge *Cinachyrella* spp. from a South Florida Reef

By

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## I. ABSTRACT:

Symbionts within marine sponges are actively participating in the biogeochemical cycles. Among them, the role of symbiont microbes in the sulfur cycle remains a mystery. This study measured the abundance of microbes within the genus *Cinachyrella* before and after exposure to hydrogen sulfide. A four-part study was conducted: a) five-hour drop experiments, b) vertical distribution experiments, c) five-hour uptake experiments, and d) long-term exposure experiments. The five-hour drop experiment utilized a microsensor to measure sulfide levels, which was lowered 1.0 mm every thirty minutes for a total of 5 hours. Three trials were performed, each with one sponge and a control with no sponge. The vertical distribution experiments measured hydrogen sulfide levels throughout 9.0 mm. A five-hour uptake experiment measured hydrogen sulfide over five hours without the use of microsensors. The bacterial composition was detailed during long-term exposure experiments, where three sponges were exposed to 60 μmol/L for several weeks. Tissue samples collected from the long-term exposure experiment underwent microbial DNA extractions and high-throughput sequencing. Hydrogen sulfide concentrations from the five-hour drop, vertical-distribution, and five-hour experiments underwent various generalized additive models and generalized linear models. A significant relationship between time (depth for the vertical-distribution) and hydrogen sulfide concentration ($p$-value$<0.05$) resulted. A significant difference based on the type (sponge and control group) of sample ($p$-value$<0.05$) was also seen. Long-term exposure indicated that hydrogen sulfide affected the relative abundance of genus *Draconibacterium*, family Rhodobacteraceae, and genus *Halodesulfovibrio* within sponges. These data suggest that *Cinachyrella* spp. can filter and process hydrogen sulfide from the water column with help from its microbiome.

**Keywords:** *Cinachyrella* spp., sulfur cycle, symbionts, 16S rRNA, sulfur-reducing/oxidizing bacteria

## II. LIST OF FIGURES:

## III. LIST OF TABLES:

## IV. LIST OF ABBREVIATIONS:

ASVs- Amplicon Sequence Variants
CCA- Canonical Correspondence Analysis
cm- Centimeter
CVS- Comma-separated Values file
DMSP- Dimethylsulfoniopropionate
DNA- Deoxyribonucleic Acid
DOM- Dissolved Organic Matter
e.g.- exempli gratia (for example)
EMP- Earth Microbiome Project
et al.- et alia (and others)
Fig- Figure
FISH- Fluorescent in situ hybridization
GAM- Generalized Additive Model
GLM- Generalized Linear Model
$H_2S$- Hydrogen Sulfide
HMA- High Microbial Abundance
HMP-Human Microbiome Project
hr- Hour
Kg- Kilogram
L- Liter
LMA- Low Microbial Abundance
MAGs- Metagenomically Assembled Genomes
mL- Milliliter
N- Nitrogen
nmol- Nanomoles
PCR- Polymerase Chain Reaction
QIIME2- Quantitative Insights into Microbial Ecology (2nd version)
$R^2$ -Coefficient of Determination
rRNA- Ribosomal Ribonucleic Acid
s- Seconds
$S^o$- Elemental Sulfur
$SO_2^-$- Sulfur Dioxide
$SO_4^{-2}$- Sulfate
SOB- Sulfur-Oxidizing Bacteria
Sp- Species (singular)
Spp- Species (plural)
SRB- Sulfur-Reducing Bacteria
µm- Micrometer
µmol- Micromole

## V. INTRODUCTION:

Sponges are a vital part of the marine ecosystem, where they provide shelter for a variety of other organisms (Cuvelier et al., 2014). Sponge abundance and filter-feeding lifestyle allow sponges to fill a significant ecological niche by removing suspended matter (e.g., dissolved organic matter (DOM), picoplankton, and bacterioplankton) from the water column (Reiswig, 1971; Pile, Patterson & Witman, 1997; Peterson et al., 2006).

These sessile filter-feeders are involved in various marine biogeochemical cycles and are extremely important to the reef-ecosystem (De Goeij et al., 2013). Once microbes capable of sulfur metabolism were isolated from marine sponges, research began to focus on the sulfur cycle to understand the contribution from this host-symbiont interaction. These studies are limited to identifying various taxa that have demonstrated the ability to metabolize sulfur (Meyer & Kuever, 2008; Tian et al., 2014; Tian et al., 2016; Jensen et al., 2017; Tian et al., 2017). Recent data suggests the genus *Cinachyrella*, like many other marine sponge species, shows symbiosis with highly diverse microbes (Sharma et al., 2016). Some symbionts may play roles in the sulfur cycle (Cuvelier et al., 2014; Vijayan, 2015). Understanding the relationship between host and symbiont can reveal how the symbiosis occurs and persists. Symbiosis is not merely an interaction between organisms but an innovative mechanism of survival (Seckbach, 2006; Mcfall-Ngai, 2014).

### Marine Sponges:

Sponges (Porifera) are one of the most basal multicellular organisms. There was much debate if Porifera or comb jellies (Ctenophora) was the sister phylum to all other animals. Recent genomic data suggest that Porifera is the actual sister group to all other organisms (Pisani et al., 2015). Marine sponges are benthic organisms that occur in every ocean in various shapes, sizes, and colors (Bergquist, 2004). They possess an active aquiferous system, i.e., incurrent openings, channels, chambers, and excurrent openings. Poriferans uses this system to obtain food from the surrounding environment. The internal space (mesophyll) is filled with flagellated and ameboid cells, collagen, and skeletal elements (Müller, 2003). The flagellated cells, known as choanocytes, are responsible for the water current in and out of the sponge. Choanocytes achieve this by the whip-like motion of the flagella. Incurrent channels move water toward the

spongocoel to exit through excurrent openings called an osculum (plural: oscula). This is the site of release for all waste products (Bergquist, 2004).

A kilogram sponge can filter up to 24,000 L of seawater per day (Vogel, 1977), making poriferans highly efficient at removing particulate organic matter. For example, other organisms consume discarded choanocytes. For example, other organisms consume discarded choanocytes, which allows dissolved organic carbon to be accessible to various marine organisms. Thus, linking pelagic and benthic systems (Webster et al., 2011). In addition, marine sponges are metazoans that harbor many symbiotic relationships with bacteria, archaea, and microeukaryotes. The most critical factors for symbiosis are temperature and microbial abundance (Taylor et al., 2007; Lurgi et al., 2019). The symbionts are phylogenetically diverse, comprising of 48 bacterial phyla, 3 archaeal phyla, 3 fungal phyla, and phylogenetically diverse algae (Webster et al., 2004; Pape et al., 2006; Holmes & Blanch, 2007; Lee et al., 2011; He et al., 2014; Li et al., 2016; Thomas et al., 2016). Symbionts, totaling up to 50% of sponge biomass, appear to be species-specific, varying between host, geological location, and season (Santavy & Colwell, 1990; Cuvelier et al., 2014). Species with high bacterial biomass are known as 'high microbial abundance' (HMA) sponges containing $10^8$–$10^{10}$ microbes per gram of sponge tissue. This is 2-4 orders of magnitude higher than the water column's microbial concentration (Hentschel et al., 2003). HMA sponges possess a denser mesophyll and a more complex aquiferous system (Weisz, Lindquist & Martens, 2008). There are also 'low microbial abundance' (LMA) sponges, with an abundance of $10^6$ microbes per gram of sponge tissue (Hentschel et al., 2003).

**Location of Microsymbionts:**

Sponges house symbionts within the mesophyll. This tissue is an extracellular matrix mostly populated by sponge cells (**Fig. 1**). However, symbionts have also been found intracellularly. Bergquist (2004) was the first to investigate sponge cells' capacity to distinguish between food and symbionts. Feeding studies have demonstrated that the host does not ingest its symbionts but allows them to pass through unharmed. Other non-symbiotic bacteria will be consumed (Hentschel et al., 2012). Other metatranscriptomics of sponge holobiont indicates tetratricopeptide repeats allows symbionts to secrete an extracellular protein to avoid digestion (Nguyen, Liu & Thomas, 2014). Even bacterial-cell recognition has been displayed in various compounds within poriferans (Müller, 2003; Steindler et al., 2007).

Researchers believe that microbial symbionts require a stable nutrient supply. Dominant phyla are Proteobacteria (especially the classes Alpha-, Gamma- and Deltaproteobacteria), Chloroflexi, Actinobacteria, Acidobacteria, Nitrospirae, and the candidate phylum Poribacteria (Hentschel et al., 2012). These phyla are always sequenced with the sponge regardless of the detection technique or geological location, suggesting the groups represent true symbionts within sponges.

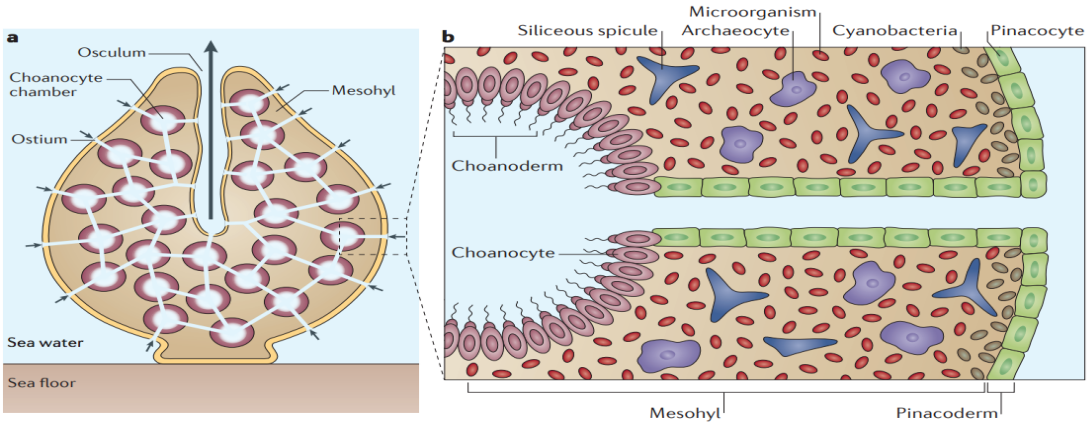


**Figure 1: Organization of tissues and symbionts of marine sponges.** The figure illustrates the organization of marine sponges adapted from Hentschel et al. (2003).

**Microbiomes within Marine Sponges:**

The microbiome is a collection of all microbial symbiont genes that provide traits not evolved by the host (Turnbaugh et al., 2007). It is estimated that less than 2% of microbes are culturable (Wilson, Weightman & Wade, 1997). Even with this low number of culturable microorganisms, Sfanos et al. (2005) cultured and characterized over 2,000 bacterial isolates from Porifera species. Using gene markers, more extensive surveys can be done. For example, 11,000 16S rRNA sequences from bacterial symbionts were reported within the mesophyll (Webster & Taylor, 2012).

A novel *Vibrio* sp. was also seen within the marine sponge *Scleritoderma cyanea* (Hoffmann et al., 2012). Marine sponges provide a large attachment substrate for microbial symbionts (Hoffmann et al., 2010). The holobiont, host and all microsymbionts, has been

thought to supply B12 to the sponge (Thomas et al., 2010; Fiore et al., 2015). Microbes also assist in ammonium assimilation and generate reductive energy (Schippers, 2013). Fiore et al. (2015) found key enzymes for thiamin synthesis in the holobiont metatranscriptome of *Xestospongia muta.* They also identified genes within the sponge transcriptome that activate the pathway for key enzymes within thiamine synthesis. However, the exact functions of many associated symbionts within marine sponges are still unknown (Fiore et al., 2015).

Microbial communities of sponges with similar evolutionary lineages are more alike than sponges that do not share evolutionary lines (Thomas et al., 2016; Lopez, 2019). There are cases where sponges maintain a stable bacterial community across temporal and spatial scales (Erwin et al., 2012; Björk et al., 2013). However, marine sponges can be affected by many different factors, including environmental changes, geography (Friedrich et al., 2001), pollution (Taylor et al., 2005), temperature (Webster et al., 2001), transfer into aquaculture (Webster & Blackall, 2009), or disease-related physiological changes (Webster et al., 2001).

**Symbionts Appear to be Species-Specific:**

*Aplysina aerophoba* and *Theonella swinhoei* show highly similar bacterial communities which are distinct from the ambient seawater even at geographically separated regions (Hentschel et al., 2002). *Cymbastela concentrica*, *Callyspongia* sp., and *Stylinos* sp. show substantial differences between genera but little between species (Taylor et al., 2004). Both results do not support the species-specific characterization. However, various sponges collected from the Indian Ocean, Pacific Ocean, Mediterranean Sea, Caribbean Sea, and the Red Sea found that common Amplicon Sequence Variant (ASVs) were specific to the species found in different locations (Schmitt et al., 2012). Taylor et al. (2013) found that bacteria, exclusive to low abundance sponges (e.g., *Poribacteria*), are detectable in seawater. The holobiont could actively maintain these rare symbiotic bacteria to respond quickly to environmental perturbations (Lopez, 2019). However, other researchers propose that species-specific microbes are demonstrated within HMA sponges (Hentschel et al., 2003). The sponge-microbe interaction complexities can provide clues of origin, evolution, and maintenance of sponge-microbe interaction.

**Relation to Biogeochemical Cycles:**

The high concentration of microbes found in many marine sponges suggests an active functional interaction between microbial communities and surrounding environments, which can be viewed as a platform for biogeochemical cycles (Taylor et al., 2007; Mohamed et al., 2008). These microbes undergo diverse metabolic processes such as nitrogen fixation, nitrification, sulfate reduction, and photosynthesis (Wilkinson, 1979; Hoffmann, Rapp & Reitner, 2006; Bayer, Schmitt & Hentschel, 2008; Hoffmann et al., 2009; Mohamed et al., 2010). These contribute to the sponges overall nutrition (Weisz, Lindquist & Martens, 2008). An example can be seen with *Geodia barretti*, which has an estimated nitrification rate of 566 nmol N $cm^{-3}$ per sponge per day. This rate is higher than that in the surrounding sediment (Hoffmann et al., 2005; Hoffmann et al., 2009). Sulfate reduction rates from *G. barretti* are among the highest recorded in natural systems, up to 1,200 nmol $SO_4^{2-}$ $cm^{-3}$ per sponge per day (Hoffmann et al., 2005). Thus, the understanding of biogeochemical functions of sponges and the harboring of microbial consortia is essential to nutrient cycling in coral reef ecosystems.

**Bacteria in the Sulfur Cycle:**

Some researchers have explored biogeochemical cycles within various sponge species, but there is still much that is unknown about these processes. Sulfate-reducing bacteria (SRB) have been found in several sponge species (Mohamed et al., 2008; Tian et al., 2014; Jensen et al., 2017; Tian et al., 2017), along with sulfur-oxidizing bacteria (SOB) (Taylor et al., 2007; White et al., 2012; Pawlik et al., 2013; Tian et al., 2016). SRB are a group of anaerobic bacteria that can obtain energy by oxidizing molecular hydrogen or organic compounds while reducing sulfate to hydrogen sulfide ($H_2S$). SOB receives energy by oxidizing $H_2S$ into forms of sulfur, which includes elemental sulfur ($S^o$), sulfate ($SO_4^{2-}$), and more (Tian et al., 2014) (**Fig. 3**). SRB and SOB play significant roles within biological ecosystems because sulfur is essential for proteins and vitamins. A main reservoir of sulfur is the oceans, where phytoplankton participate in the sulfur cycle (**Fig. 2**) by producing dimethylsulfoniopropionate (DMSP) (Sievert, Kiene & Schulz-Vogt, 2007). Due to this ecological importance, researchers have intensely studied SRB, which can act as primary mediators for various processes in marine biogeochemical cycles, including the mercury cycle (Yoch, 2002; Han et al., 2010) and anaerobic methane oxidation

(Kim & Zoh, 2012). As dissimilatory sulfate reducers, the bacteria can be found in marine sediment where they perform nearly half of all organic mineralization (Orphan et al., 2001). In this context, SRB can establish different metabolisms, such as sulfidogenic, acetogenic, and hydrogenogenic. The generated sulfides are toxic to the host sponge, which need to be balanced by SOB (Plugge et al., 2011).

Sulfur metabolism is complex and mediated by various microbes (**Fig. 3**). Dissimilated sulfur compounds can be the energy sources in various prokaryotes, generally serving as the electron donor and electron acceptors for SOB and SRB, respectively (Vavourakis et al., 2019). Sulfate and sulfide cannot be oxidized or reduced further, thus are the final products of most pathways. Most of the $H_2S$ is dioxide in $SO_4^{2-}$, although some precipitates within sediments (**Fig. 2**). Desulfurylation is by many aerobic and anaerobic prokaryotes, where assimilatory sulfate reduction is performed by many aerobic and anaerobic microorganisms (Barton, Fardeau & Fauque, 2014). Genes of these microbes are not fully understood and many have not been identified (Vavourakis et al., 2019). A list of sulfur reducers, sulfide reducers and sulfur oxidizers are listed (**Table 1**).

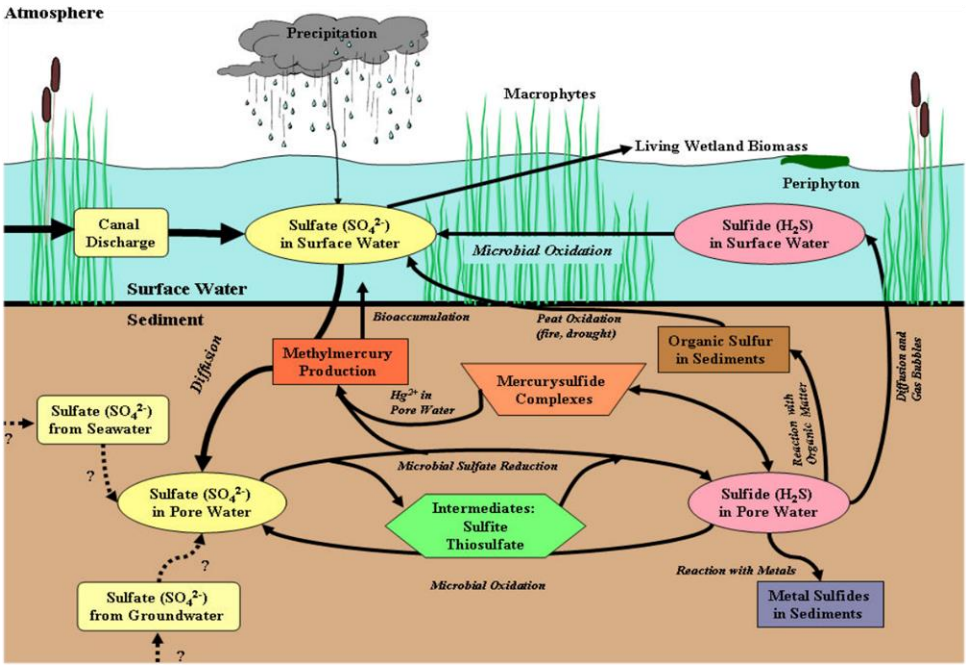


**Figure 2: Simplified sulfur cycle within a seawater environment.** The simplified version of the sulfur cycle is seen above. It is unknown if sulfate is imported to the environment via seawater, pore water, or groundwater. However, it is known to be imported from precipitation. Sulfate is then reduced into sulfide, which is oxidized back into sulfate. Both reduction and oxidation are mediated through environmental microbes. The image was adapted from Orem (2007).

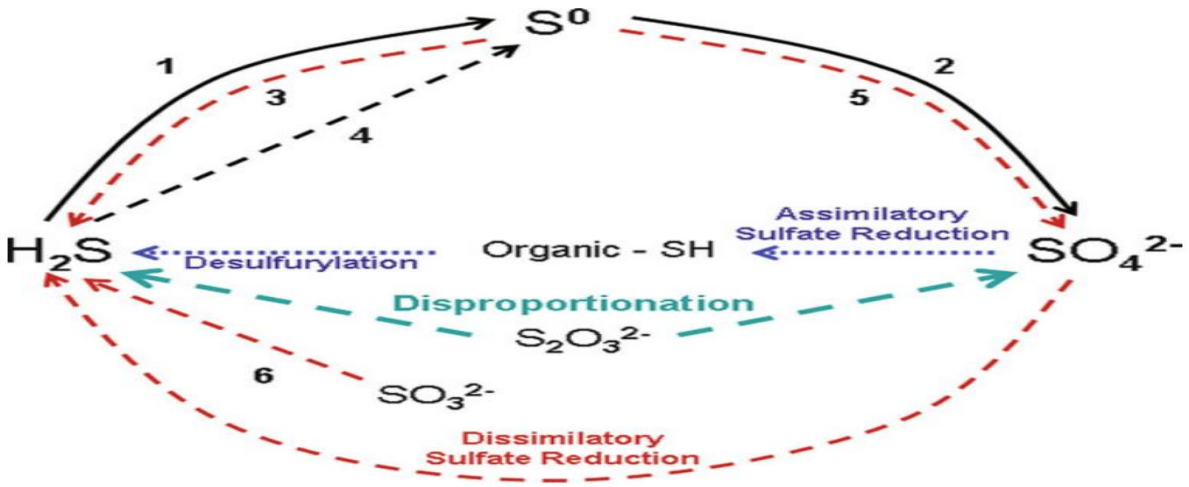


**Figure 3: Molecular change of the sulfur cycle.** The biological sulfur cycle with roles of bacteria are seen in the above cycle. 1 and 2: Sulfide and sulfur oxidation by colorless sulfur bacteria. 3: Sulfur reduction by the anaerobic microorganisms. 4 and 5: Anaerobic sulfide and sulfur oxidation by purple sulfur bacteria and green sulfur bacteria. 6: Sulfite-reducing bacteria. (Barton, Fardeau & Fauque, 2014).

**Table 1: Sulfur-reducing, sulfate-reducing, and sulfur-oxidizing bacteria.** A detailed list of main sulfur-reducing, sulfate-reducing, and sulfur-oxidizing taxonomic groups are below. All sulfate reducers and sulfur reducers were taken from Barton, Fardeau & Fauque (2014).

| Sulfate Reducers | Sulfur Reducers | Sulfur Oxidizers |
|---|---|---|
| *Ammonifex* | *Campylobacter* | *Thiobacilliaceae (Fike, Bradley & Leavitt, 2016)* |
| *Candidatus desulforudis* | *Desulfomicrobium* | *Beggiatoaceae (Fike, Bradley & Leavitt, 2016)* |
| *Desulfacinum* | *Desulfotomaculum* | *Acidithiobacillus (Kelly & Wood, 2000)* |
| *Desulfobacter* | *Desulfovibrio* | *Aquaspirillum (Friedrich & Mitrenga, 1981)* |
| *Desulfobacterium autotrophicum* | *Desulfurella* | *Aquifex (Huber & Eder, 2006)* |
| *Desulfobulbus* | *Desulfurobacterium* | *Bacillus (Aragno, 1992)* |
| *Desulfocapsa* | *Desulfuromonas acetoxidans* | *Methylobacterium (Kelly & Smith, 1990)* |
| *Desulfococcus* | *Salmonella* | *Paracoccus (Friedrich & Mitrenga, 1981)* |
| *Desulfocurvus* | *Sulfurospirillum deleyianum* | *Pseudomonas (Friedrich & Mitrenga, 1981)* |
| *Desulfofustis* | | *Starkeya (Kelly, Mcdonald & Wood, 2000)* |
| *Desulfohalobium* | | *Thermithiobacillus (Kelly & Wood, 2000)* |
| *Desulfoluna* | | *Xanthobacter (Friedrich & Mitrenga, 1981)* |
| *Desulfomicrobium norvegicum* | | *Candidatus Electronema (Trojan et al., 2016)* |
| *Desulfonatronovibrio* | | *Candidatus Electrothrix (Trojan et al., 2016)* |
| *Desulfosarcina* | | *Chromatiaceae (Imhoff, Süling & Petri, 1998)* |
| *Desulfosporosinus* | | *Chlorobiaceae (Brune, 1989)* |
| *Desulfovibrio vulgaris H* | | *Rhodospirillaceae (Brune, 1989)* |
| *Desulfovirga* | | *Cyanobacteria (Fike, Bradley & Leavitt, 2016)* |
| *Syntrophobacter* | | *Oscillatoria (Cohen, Padan & Shilo, 1975)* |
| *Thermodesulfatator* | | *Lyngbya (Cohen, Padan & Shilo, 1975)* |
| *Thermodesulfobacterium commune* | | *Aphanotece (Cohen, Padan & Shilo, 1975)* |
| *Thermodesulfobium* | | *Microcoleus (Cohen, Padan & Shilo, 1975)* |
| *Thermodesulfovibrio* | | *Phormidium (Cohen, Padan & Shilo, 1975)* |
| | | *Chloroflexaceae (Fike, Bradley & Leavitt, 2016)* |
| | | *Thiobacilli (Fike, Bradley & Leavitt, 2016)* |

**Other Invertebrates within the Sulfur Cycle:**

Studies have focused very little on sulfur-reducing and sulfur-oxidizing symbionts of sponges but instead focused on various other invertebrates from hydrothermal vents. Just over the past 40 years, many symbionts have been discovered, such as *Riftia*, *Lamellibrachia*, *Escarpia* (Gauthier, Watson & Degnan, 2016), *Thyasira* (Bright & Giere, 2005), *Bathymodiolus* (Dufour, 2005), and *Tubificoides* (Suzuki et al., 2005; Gauthier, Watson & Degnan, 2016). Recently this type of symbiosis is seen within *Kuphus polythalamius*, the giant shipworm (Dubilier, Bergin & Lott, 2008), and within the Enteropneusta, *Saccoglossus bromophenolosus* (Altamia et al., 2019). The candidate genus *Kentron*, symbionts hosted by *Kentrophoros*, a diverse genus of ciliates, has been found to be completely heterotrophic and possess either the Calvin-Benson-Bassham or reverse tricarboxylic acid cycles for autotrophy (King, 2018). Numerous examples demonstrate the widespread symbiosis of SOB and SRB. Each exact relationship can be different. *Riftia* collects compounds, including sulfide, from the water (Stewart & Cavanaugh, 2005). The symbiont will utilize these compounds to provide nourishment for *Riftia*, who lacks a digestive system (Felbeck, 1981).

**Marine Sponges and the Sulfur Cycle:**

The role and interplay of SRB with and within biological systems, such as eukaryotic marine symbiotic hosts, like sponges, are less known. Tian et al. (2014) proposed that the SOB symbionts coevolved with the sponge hosts. However, the sponge-specific SOB are closely related to the free-living SOB (Tian et al., 2014), indicating symbionts first began through increased accumulation by the filtration of marine sponges. It was also observed that sponge-specific SRB are more closely related to other various sponge-specific SRB rather than terrestrial SRB (Pawlik et al., 2013). Sipkema et al. (2015) advocate that the sponge holobiont undergoes both vertical and horizontal transfer. Still, detailed characteristics, evolutionary processes (underlying the symbiosis), and physiology remain mostly unknown because of enrichment and cultivation difficulties.

Several species of sponges have been determined to have symbionts containing genes involved in the sulfur cycle. Tian et al. (2014) found genes within sulfur oxidation pathways (sox complex and reverse dissimilatory sulfate reduction pathway) within *Haliclona* (*Gellius*) *cymaeformis*. These pathways can also be seen in several other marine sponges: *Theonella*

*swinhoei* (Lenk et al., 2012), *Suberites* sp. (Tian et al., 2017), *Amphimedon queenslandica* (Lavy et al., 2018), and *Lophophysema eversa* (Tian et al., 2016). Jensen et al. (2017) isolated Gammaproteobacteria in *G. barretti* and found the reverse dissimilatory sulfate reduction gene *aprA*. Meyer & Kuever (2008) sequenced similar Gammaproteobacteria using *aprA* gene as a marker.

The most intensive research for sulfur metabolism within sponges has been done on *G. barretti* (Hoffmann et al., 2005). The authors reported the presence of sulfate-reducing bacteria belonging to *Desulfoarculus*/*Desulfomonile*/*Syntrophus* cluster. Two genera, *Desulfomonile* and *Syntrophus* were also found in *Axinella corrugata* with an unexpectedly wide variety of SOB (Mohamed et al., 2008; White et al., 2012). In *Lophophysema eversa*, using genetic analysis, SOB (Tian et al., 2016) and SRB (Mohamed et al., 2008) were seen within the species. In other sponges, common *Roseobacter* is present (Taylor et al., 2004) and might have a role in sulfide/sulfur-oxidation in sponges (Conway, Esiobu & Lopez, 2012). The most well-known bacterial sulfide oxidizers from the order Chromatiales (Muyzer et al., 2011; Hardoim et al., 2012; Kennedy et al., 2014; Tian et al., 2016) and Family Chlorobiaceae (Eimhjellen, 1967) has been documented in sponges.

*Cinachyrella* spp. has even been documented to have many possible microbes performing sulfur-reduction and oxidation, such as Acidobacteria, Cellvibrionaceae, Colwelliaceae, Rhodobacteraceae, and Gammaproteobacteria (Vijayan, 2015). Within *Cinachyrella* spp., Shmakova recently described sulfur metabolism in five metagenomically assembled genomes (MAGs): *Opitutaceae bacterium, Thioalkalivibrio paradoxus, Desulfobacterium autotrophicum, Thioalkalivibrio sulfidiphilus, Sulfurifustis variabilis*. This study also identified 27 other MAGS with sulfide reducing genes (**Fig. 4**) (Shmakova, 2020). Sulfatase hydrolase/transferase, along with other genes, has been found in the *Cinachyrella* spp. holobiont (Desplat, 2020).

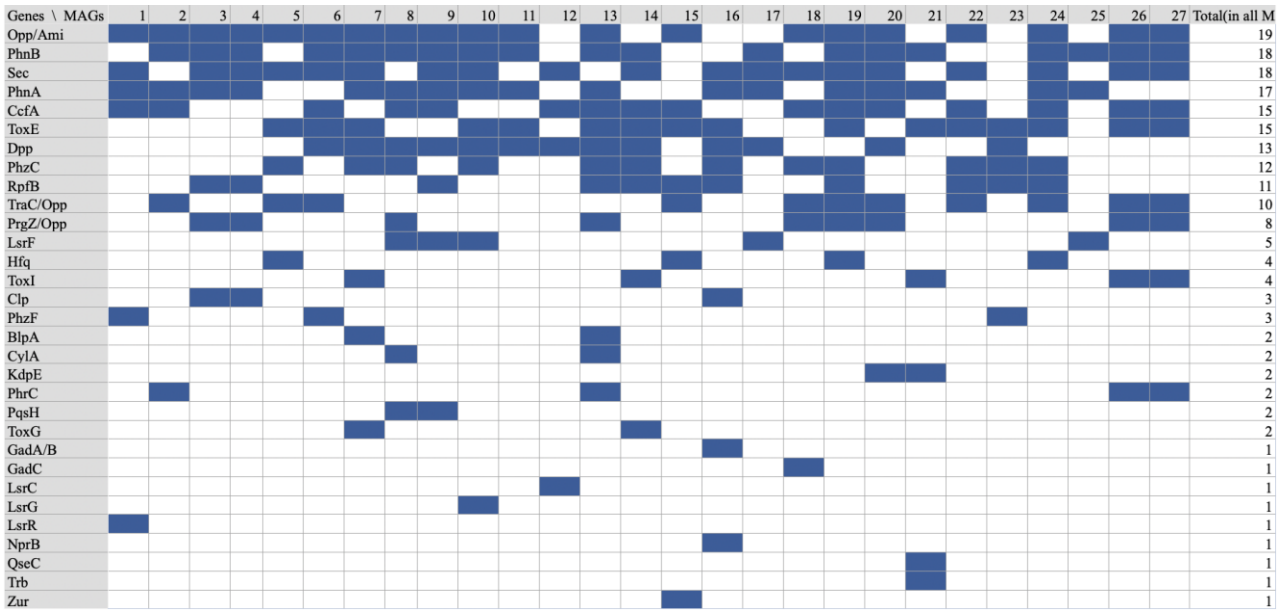


**Figure 4: Metagenomically assembled genomes with sulfur metabolism.** Quorum sensing functional potential of 27 metagomically assembled genomes (MAGs) from Shmakova (2020) can be seen above.

***Cinachyrella* spp.:**

The *Cinachyrella* genus is within the family Tetillidae of the order Tetractinellida and class Demospongiae (Rützler & Smith, 1992). Porocalices, concave depressions of the globed shaped sponges, contain aggregations of microscopic incoming pores. *Cinachyrella* sp. is commonly called “gold ball sponge”, which is yellow to orange-red externally. However, internally the sponge is yellow-orange (Morrow & Cárdenas, 2015). This genus ranges from the shallow coastal waters of North Carolina to the South Atlantic waters of Brazil. Within South Florida, there are three common species, *C. kuekenthali*, *C. alloclada,* and *C. apion* (Rützler & Smith, 1992). These species are laborious to distinguish due to structural similarities (**Table 2**). The optimal identification method is sequencing analysis and an intron amplification method described by Steindler et al. (2007). *Cinachyrella* has been chosen as a model sponge for the Lopez laboratory due to many positive features (extended survival in aquaculture, natural along nearby reefs, the possibility of reproduction, etc.) (Barton, Fardeau & Fauque, 2014; Vijayan,

2015). Dominate microbial groups seen in this genus are Proteobacteria (especially the classes Alpha-, Gamma- and Deltaproteobacteria), Chloroflexi, Actinobacteria, Acidobacteria, Nitrospirae, and the candidate phylum Poribacteria (Hentschel et al., 2012; Cuvelier et al., 2014; Vijayan, 2015).

**Table 2: Morphological characterization of common *Cinachyrella* species.** Morphological description of the three common *Cinachyrella* species within South Florida (Smith, 2013). Pictures were provided by Porifera Tree of Life Project (http://porifera.myspecies.info/).

| ***Cinachyrella alloclada*** | ***Cinachyrella apion*** | ***Cinachyrella kuekenthali*** |
|---|---|---|
| Orange to yellow, shallow reef sponge (5-20 m) | Yellow to light grey, mangrove, and lagoon water habitats (0.3-60 m) | Orange, may appear grey-red, found on reef and coral rubble (4-100 m) |
| Grow to 10 cm diameter | Grow up to 7 cm in diameter | Massive subglobular with growth up to 15 cm diameter |
| Strongly hispid surface with small to large porocalices (3-15 mm) | Strongly hispid surface with evenly distributed porocalices (2 mm) on the sides. Oscula are rare. | moderately hispid with unevenly distributed porocalices (0.3-0.5 cm) and one or few oscula (1 cm) |
| Spicules are smooth oxeas with two/three size classes, pro- and anatriaenes of one size class, and spiny sigmaspires of variable size | Spicules with oxeas in two size classes with few subtylostyles and strongyles | Spicules with large oxeas of one size class, spiny microxeas, straight/slightly, protriaenes, anatriaenes commonly distributed, spiny sigmaspires |
| Photographer: Klaus Ruetzler  | Photographer: Charles Messing  | Photographer: Klaus Ruetzler  |

**Use of 16S rRNA for Bacterial Identification:**

The traditional method to identify bacterial symbionts is cultivation. However, 2% of microbes are culturable (Wilson, Weightman & Wade, 1997). Thus, to get a complete survey, gene markers can be used. This method can identify taxa without live samples. Since 1977, 16S rRNA has been used as a gene marker to identify the taxonomy and phylogeny characteristics of various microbes (Seah et al., 2019). This marker is found in all bacteria and has a low mutation rate, making it ideal for taxonomic and phylogenetic studies (Woese & Fox, 1977; Woese et al., 1980; Woese, 1982; Woese et al., 1984). The rRNA molecule is composed of two subunits. In prokaryotes, the smallest rRNA subunit is coded within the 16S rRNA gene (Janda & Abbott, 2007). This gene allows microbes to be distinguished at the genus level but gives low phylogenetic power at the species level (Woese, 1982; Woese, 1987).

16S rRNA marker can be used in combination with Illumina MiSeq, which is now the common sequencing platform. This machine allows the identification to the family level at a lower cost per sequence (Sogin et al., 2006; Caporaso et al., 2010). This platform ligates the adapters to target DNA fragments, then binds them to a glass flow cell containing one or more channels (Tremblay et al., 2015). Enzymes and nucleotides are added to the chambers to begin bridge amplification of DNA fragments. Sequencing occurs through a single base extension, then completes by adding fluorescently labeled reversible terminator nucleotides, primers, and DNA polymerase. The label is recorded by a camera allowing the nucleotide base to be determined. The fluorescent tag is then removed, and a new cycle starts (Mardis, 2011; Tremblay et al., 2015).

High throughput DNA sequencing is being used for large community studies, such as the Human Microbiome Project (HMP) and the Earth Microbiome Project (EMP) (Mardis, 2008). The Molecular Microbiology and Genetics Laboratory of Halmos College of Nova Southeastern University routinely apply this sequencing technique to characterize diverse marine microbiomes (Lopez et al., 2002; Lopez et al., 2008; Wang & Qian, 2009; Hoffmann et al., 2012; Cuvelier et al., 2014; Thomas et al., 2016; Thompson et al., 2017; Easson & Lopez, 2019).

**Preliminary data:**

The preliminary experiment was conducted at Florida Gulf Coast University within the laboratory of Dr. Hidetoshi Urakawa within the Department of Ecology and Environmental

Studies. $H_2S$ was concentrated in DI water utilizing a hydrogen sulfide salt. This solution had all oxygen removed from solution such that the solution could be stored. If the solution was stored with oxygen the $H_2S$ would react with the oxygen lowering the overall concentration of $H_2S$. A volume equaling ≈50 µmol/L was exposed to three sponges over a period of several weeks. The consumption of $H_2S$ was observed and recorded over four hours (**Fig. 5)**.

A decrease in $H_2S$ in both experimental samples can be seen. The test (slope= -0.0088) had almost doubled the slope of the control (slope= -0.0046). This difference indicated some $H_2S$ is dissolved within the water, but the majority is taken up by symbionts present in the host sponge. However, it is possible to be absorbed in the tissue of the sponge. This difference was seen on three different sizes of *Cinachyrella* sp. individuals (**Fig**. 6). It was seen that the slope of the small and medium sponge had a slightly similar slope as the control, while the large sponge does have a slope that is larger than the control's slope. The difference in slopes could be due to the different sizes of experimental sponge. A larger sponge would have a higher abundance of microbes. A larger population of SOB could explain the larger decrease compared to the other two sponges. It is also possible that the amount of SOB present does range within individuals of the same species; thus, it is important to have more trials to determine if this is an individual change. The sponge and the control were seen to start at different concentrations. The experiment did not utilize the same concentration for the sponge and control. The majority of the limited prepared solution was utilized in the experimental sponge treatment.

Later during the same experiment, a microbial mat was observed on the sponge's surface (**Fig. 10**). This mat was isolated and exposed to $H_2S$ just as the marine sponges had been before (**Fig. 7**). A control with no microbial mat was utilized to negate the differences in diffusion rate. $H_2S$ could be seen to be consumed readily, indicating this mat was probably composed of sulfide oxidizers. The oxidizers were enriched when the sponges were regularly exposed to $H_2S$. The sulfur cycle occurs in both anaerobic and aerobic environments depending on the species (Whittaker, 1972; Huber et al., 1992; Schönheit & Schäfer, 1995; Klenk et al., 1998; Friedrich et al., 2001; Friedrich et al., 2005; Kletzin, 2007; Kletzin, 2008). One sponge was seen to float above the aquarium's bed due to internal gas formation (**Fig. 11**). The release of various gases can explain this. The oxygen levels were recorded (**Fig. 8**). Oxygen levels were normoxic, except for the small sponge Ostia 4, most likely due to an issue with the sensor or possibly due to a misplacement of the probe. It should be noted that the large sponge died before any oxygen data

was seen. When the sponge was flipped upside down, air escaped. After some time, the sponges showed evidence of decay, such as a sulfur smell. At this time, production of $H_2S$ was documented, attributed to the decline of the sponge (**Fig. 9)** (Heidelberg et al., 2004). It is also possible the production of $H_2S$ was due to sulfur-reducing bacteria, as they can convert $S^o$ back to $H_2S$. Sulfur oxidation can be seen to convert $S^o$ to $SO_2^{4-}$, where SRB can convert to $H_2S$ (**Fig. 3**). The microbes could have been enriched after the consumption of $H_2S$. If this was the case, then a complex sulfur cycle is occurring within this species.

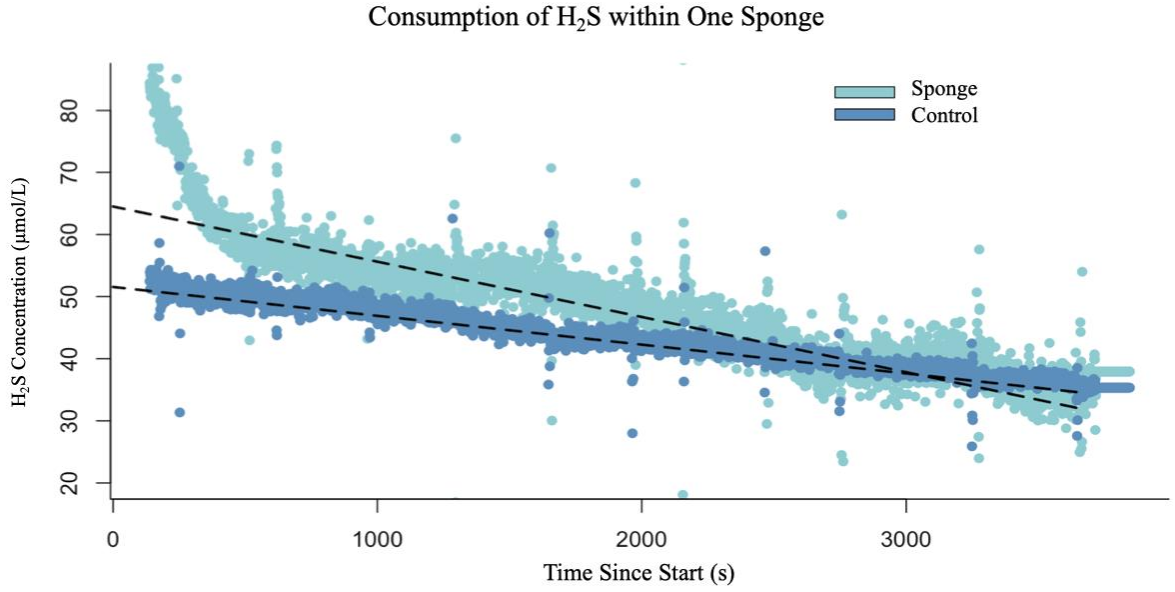


**Figure 5: Consumption of $H_2S$ in a marine sponge.** The figure above shows the consumption of $H_2S$ over 4500 seconds.

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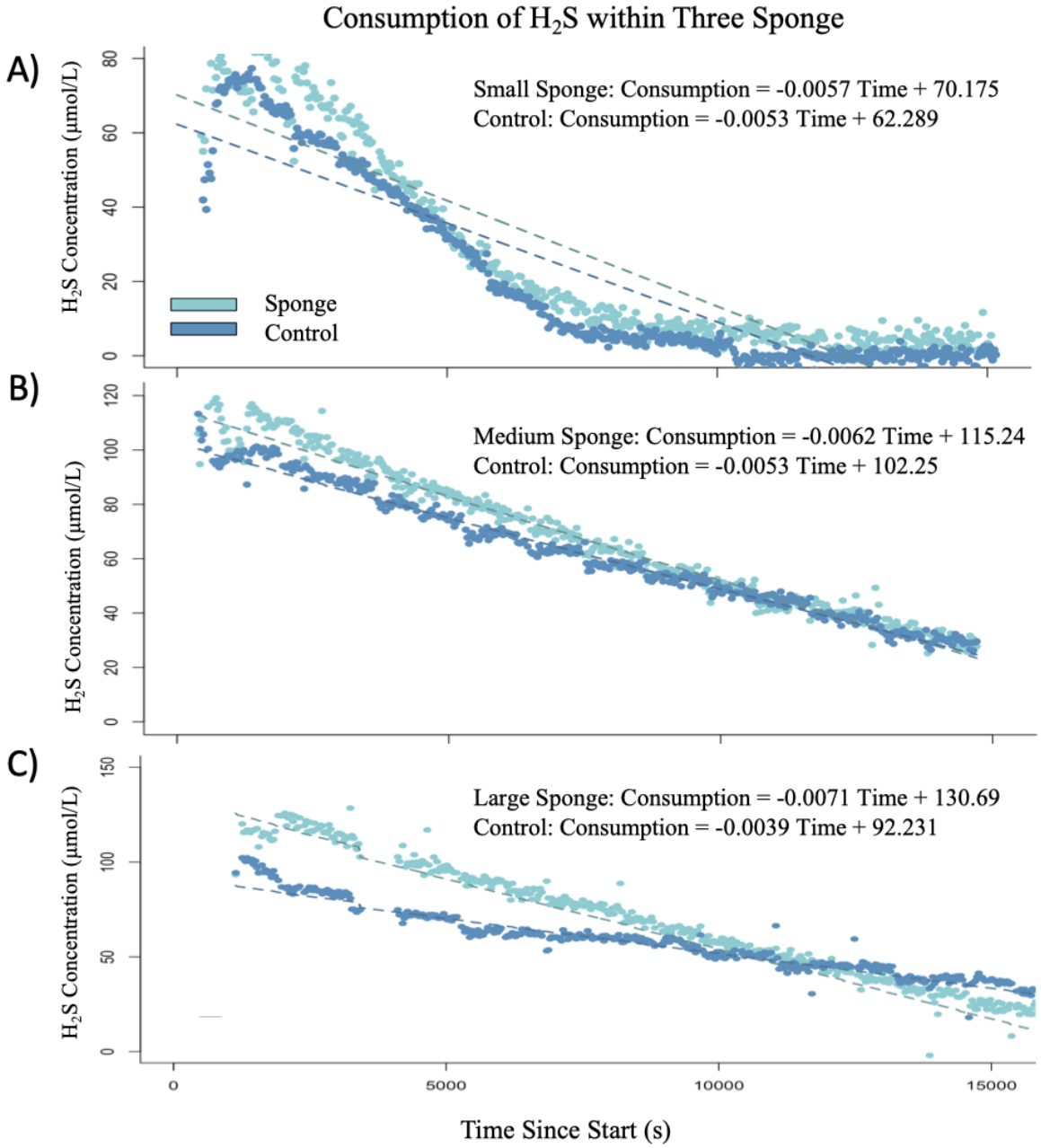


**Figure 6: Consumption of $H_2S$ in a marine sponge of three sizes.** The figure above shows the consumption of $H_2S$ over 4500 seconds, within three different size sponges. Note that the biggest sponge had the largest consumption of $H_2S$.

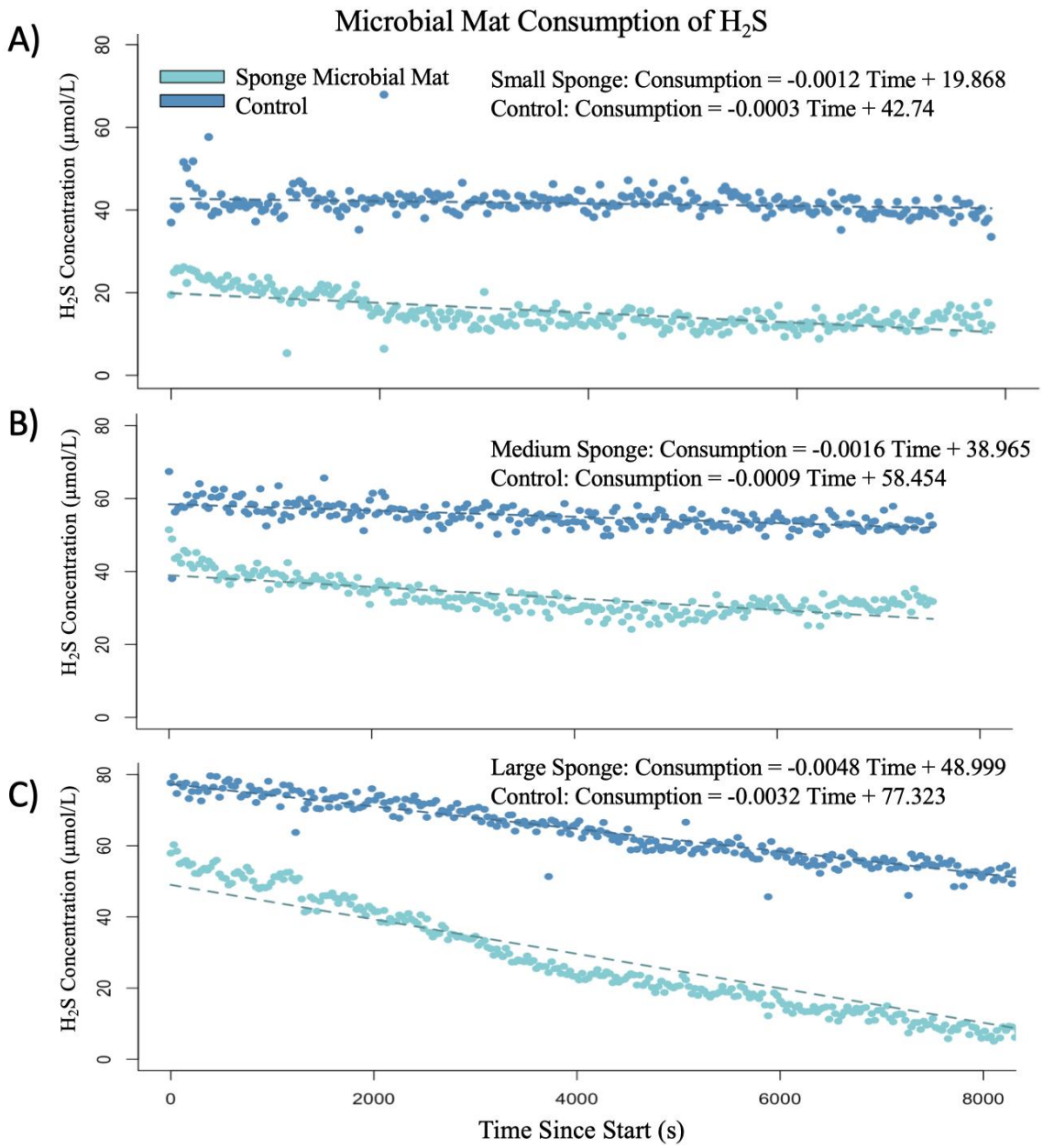


**Figure 7: Consumption of $H_2S$ within the microbial mat.** Part A, B, and C show the $H_2S$ consumption of the microbial matt associated with the small, medium, and largest sponge. Note the dramatic decrease in $H_2S$ for all sponges.

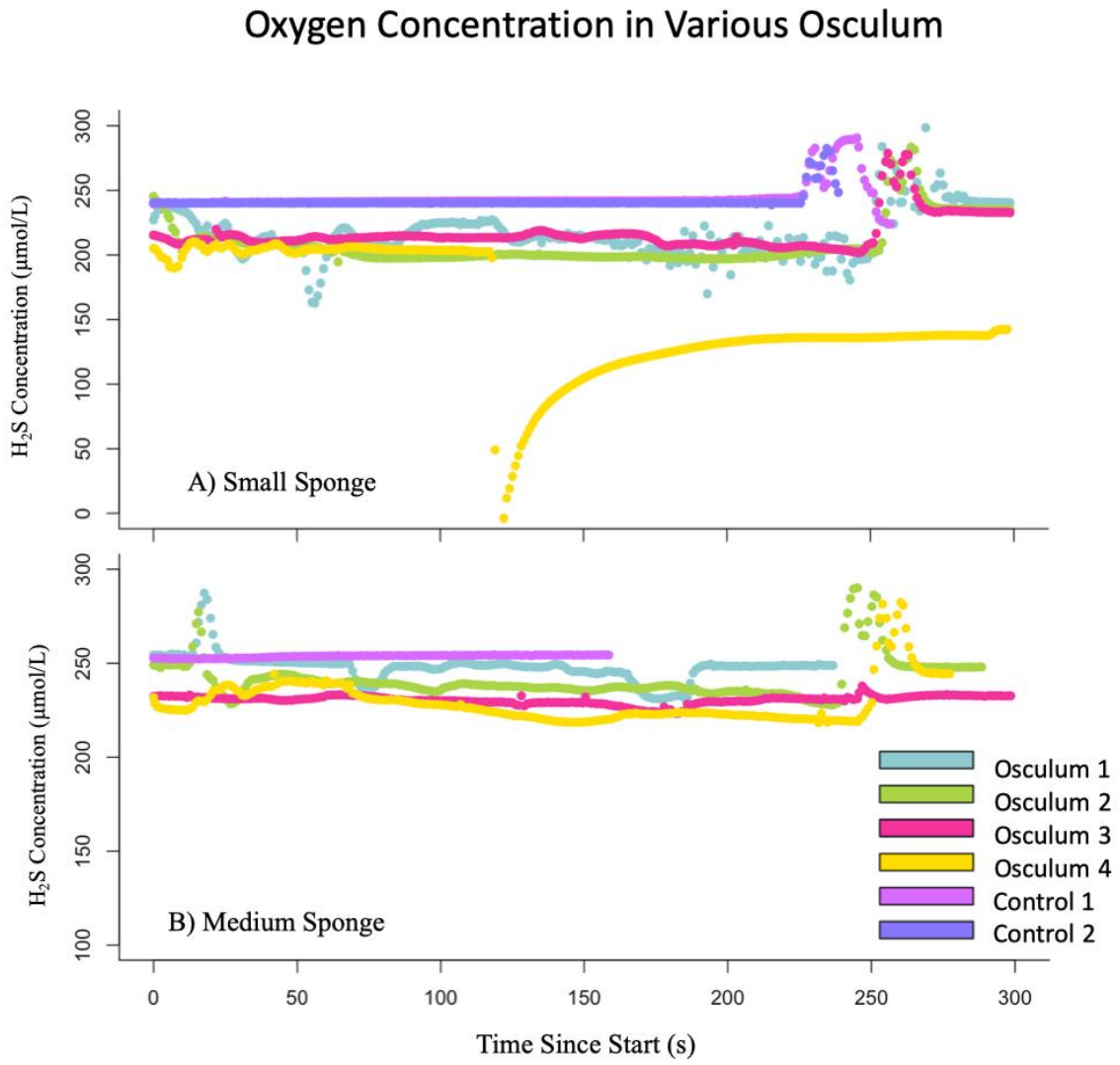


**Figure 8: Oxygen levels within two sized sponges.** Part A and B demonstrate the oxygen within the Ostia of the small and medium sponge. These individuals were not in an anoxic condition.

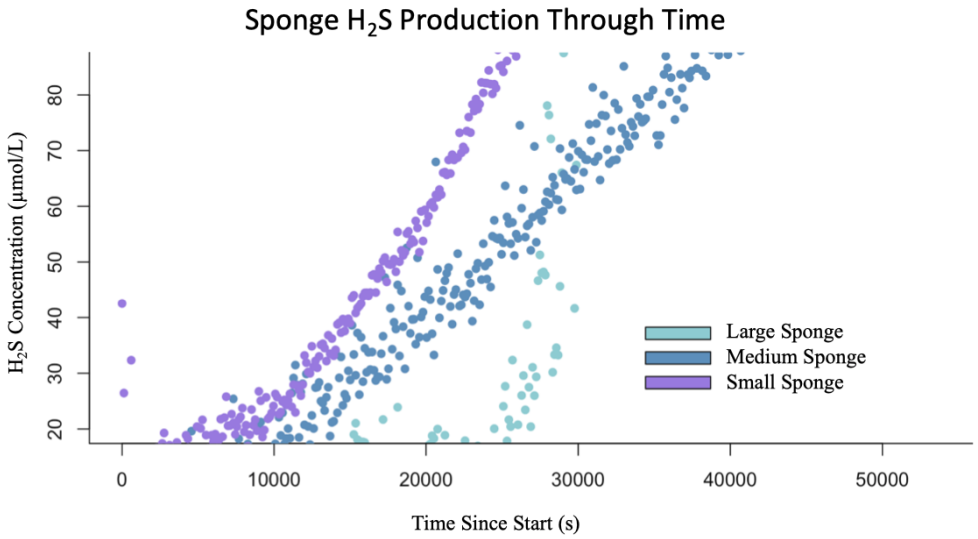


**Figure 9: Sponge production of $H_2S$ through time.** The production of $H_2S$ over 55,000 seconds. Note this was measured after the sponge had formed an unknown microbial mat.

**Figure 10: Growth of white microbial mat.** The pictures above demonstrate the white microbial mat. This mat appeared after the exposure to $H_2S$ (Pictures courtesy of Megan Feeney).

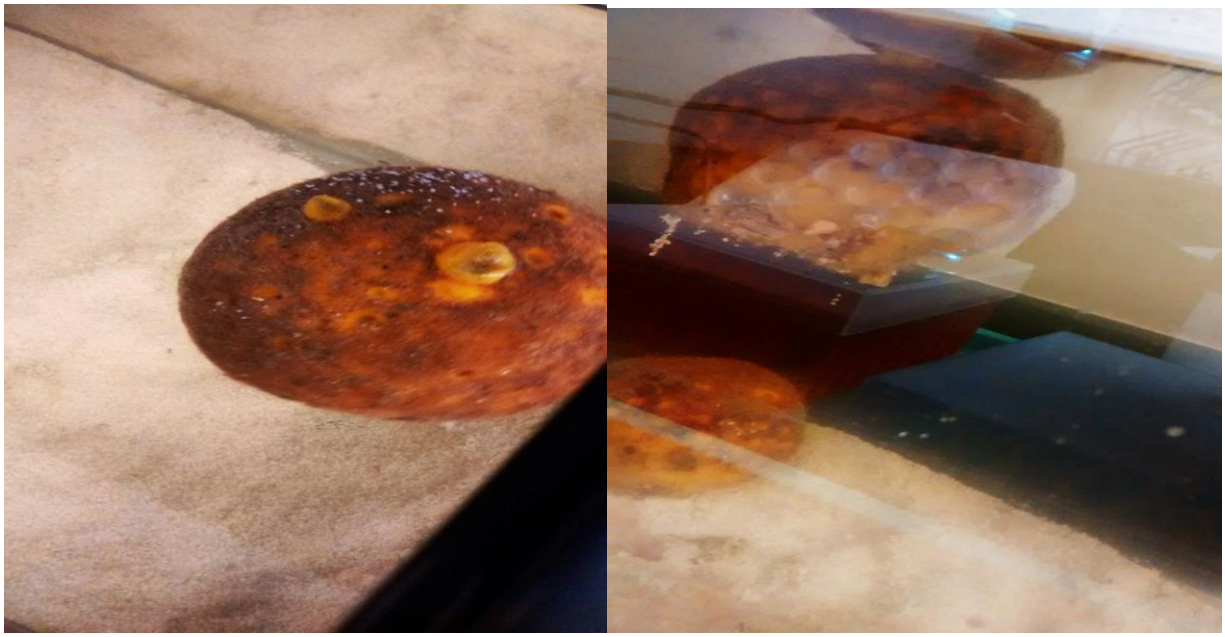

**Figure 11: Flotation of a large sponge.** The above image displays the flotation of the largest sponge that was exposed to $H_2S$. The image to the left was taken proximal to the sponge, while the image to the right was taken laterally (Pictures courtesy of Megan Feeney).

**In This Study:**

This study's objective was to investigate the role of bacteria in the sulfur cycle within *Cinachyrella* spp. of the Florida reefs. This genus is readily available from local waters and hardy within aquaculture (Cuvelier et al., 2014), which allowed the investigation within this cycle to answer many questions. During this project, I investigated the abundance of SRB and SOB using 16S rRNA sequences within sponges under the stress of increased sulfur concentration and the most abundant bacteria's identity. It is important to note that no study on *Cinachyrella* spp. has actively attempted to identify SRB or SOB. Vijayan (2015) has found ASVs of known sulfur taxa, and Shmakova (2020) found the presence of MAGs related to sulfur metabolism. Sulfatase hydrolase/transferase, along with other genes, has been found in the *Cinachyrella* spp. holobiont (Desplat, 2020). Much of the background information on other species only considered SRB's presence and did not investigate the magnitude. Our pilot study noted the formation of a microbial mat (a multilayered sheet of microorganisms) after the experimental exposure to $H_2S$, but no data is currently known about this phenomenon. I recreated

this phenomenon and determined the microbes associated with this mat. Currently, no data is available for SRB and SOB stability in our model sponge species in aquarium environments.

## VI. HYPOTHESIS AND OBJECTIVES:

The primary purpose of this study was to characterize SRB and SOB within a Florida sponge species, *Cinachyrella* spp. after exposure to hydrogen sulfide ($H_2S$) in a controlled environment. This was based on the following hypotheses:

- Hypothesis 1: A significant relationship between time and uptake of $H_2S$ (by a natural and sponge environment) would be seen and be modeled (refer to five-hour uptake)
- Hypothesis 2: A significant relationship between depth and uptake of $H_2S$ (by a natural and sponge environment) would be seen and be modeled (refer to vertical-distribution)
- Hypothesis 3: A significant relationship between time (with the interaction of depth) and uptake of $H_2S$ (by a natural and sponge environment) would be seen and be modeled (refer to five-hour uptake experiments)
- Hypothesis 4: There would be statistical differences in the control (used to represent the diffusion rate of $H_2S$) and sponge (refer to five-hour uptake, vertical-distribution, and five-hour uptake experiments)
- Hypothesis 5: *Cinachyrella* spp. host SRB and SOB (refer to long-term experiments)
- Hypothesis 6: There would be a change of relative bacterial abundance after the $H_2S$ exposure (refer to long-term experiments)
- Hypothesis 7: The bacterial mat seen on the sponges in the preliminary data are composed of SRB/SOB (refer to long-term experiments)
- Hypothesis 8: SRB and SOB in the sponge tissue functionally play roles in the sulfur cycle (refer to five-hour uptake, vertical-distribution, five-hour uptake experiments, and long-term experiments)

## VII. MATERIALS AND METHODOLOGY:

### Collection:

A total of 9 sponge specimens were collected off Halmos College of Nova Southeastern University on the Florida Reef Tract. Samples were collected while diving; careful measures

were taken such that specimens did not have air exposure. Sponges were cut from the substrate at the base of the organism. The Molecular Microbiology and Genomics Laboratory of Halmos College of Nova Southeastern University obtained Florida permits for sponge collection. The specimens were then taken back to Halmos College of Nova Southeastern University and placed within an aquarium system. Marine sponges can be affected by transfer into aquaculture (Webster & Blackall, 2009); thus, sponges were used within 24 hours of collection. Sponges chosen for each experiment are seen in **Table 3**.

**Table 3: Experimental test for each sponge tested.** The Sample number and experimental test are detailed below. Note Sponges 4 and 5 underwent two experimental tests: five-hour drop and vertical distribution experiments.

| Experimental Test | *Sample Number* |
|---|---|
| ***Five-Hour*** | Sponge 2 |
| | Sponge 6 |
| | Sponge 9 |
| ***Five-Hour Drop*** | Sponge 3 |
| | Sponge 4 |
| | Sponge 5 |
| ***Vertical Distribution*** | Sponge 4 |
| | Sponge 5 |
| ***Long-Term Exposure*** | Sponge 1 |
| | Sponge 7 |
| | Sponge 8 |

**Five-Hour Drop Experiments:**

To determine the relationship between the interaction of time and depth and uptake of $H_2S$ (by a natural and sponge environment), fresh sponges (n= 3, Sponges 3, 4, and 5) were collected, maintained under normal aquarium conditions. They were then exposed to hydrogen sulfide experimental conditions to test hypotheses 3. Two experimental beakers (500 mL) were used with normal aquarium seawater and 60 μmol/L of $H_2S$. The concentration of hydrogen sulfide was chosen due to previous research done at Florida Gulf Coast University. $H_2S$ was

concentrated in DI water utilizing a hydrogen sulfide salt. This solution had all oxygen removed from the solution such that the solution could be stored. If the solution were stored with oxygen, the $H_2S$ would react with the oxygen lowering the overall concentration of $H_2S$. Note only one of the two 500L-beakers contained a *Cinachyrella* spp., which was in a smaller beaker to prevent movement. Sponges were allowed to acclimate to the experimental beaker condition for 30 minutes before $H_2S$ exposure. There were three tests per environment, i.e., a total of 6 trials, with only 3 sponges (**Fig. 12)**. Natural microbial populations are known to shift within sponges after separation from natural environments and culture in aquaria (Cardenas et al., 2009; Webster & Blackall, 2009). Due to this, sponge samples were used within 24 hours. Sulfur was routinely monitored for 5 hours in intervals of 30 seconds by microsensors. Unisense microsensors recorded a gradual change but moving the sensor by 1000 μm every 30 minutes. Oxygen levels of each trial were measured before and after experimentation. The microelectrode measurements were taken using a glass microelectrode of 100 μm diameter, which was manipulated using a motorized micromanipulator. There was only one arm to hold the sensor; thus, the control did not move every 30 minutes, and a microsensor was placed at the bottom of the beaker.

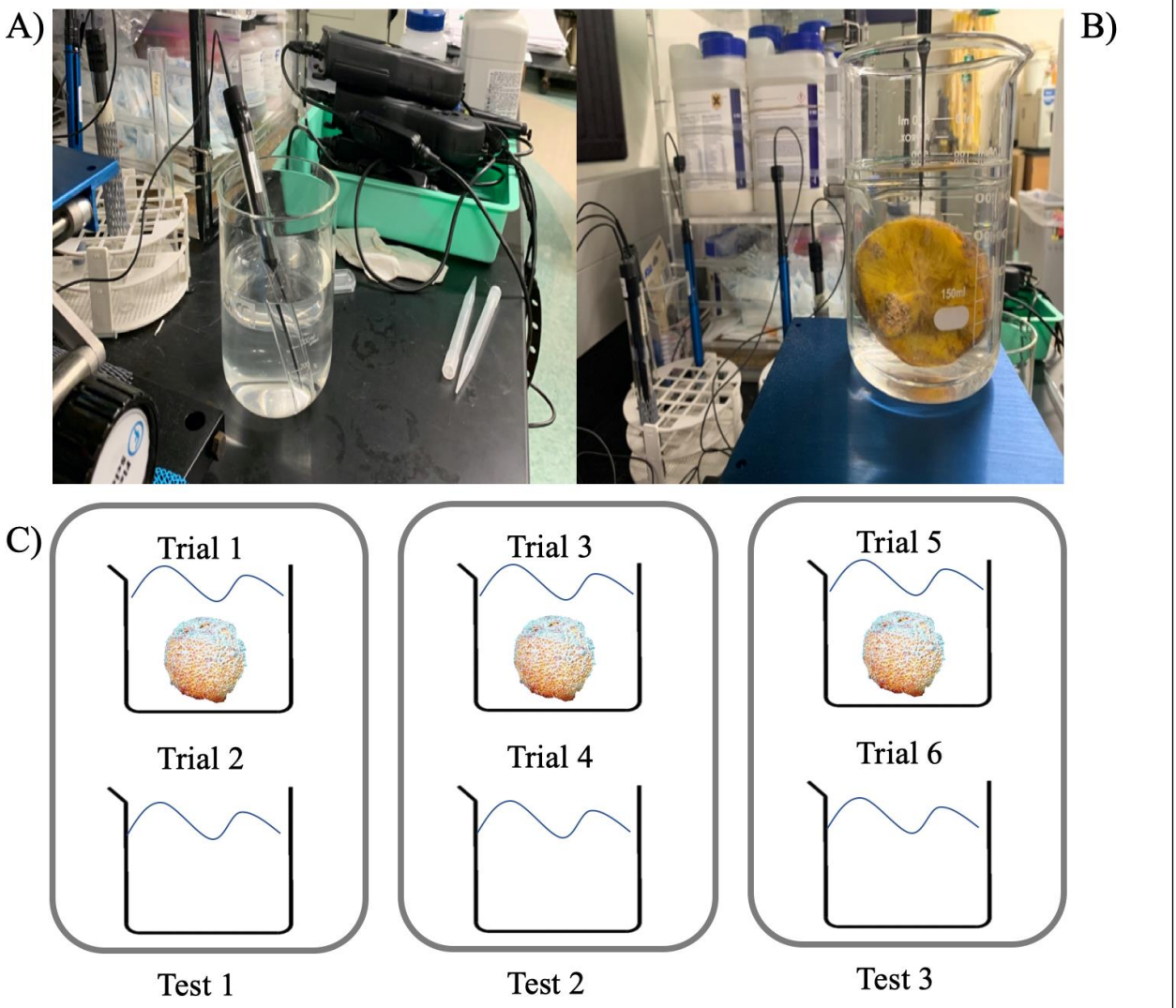


**Figure 12: Organization of 5-hour experiments.** The organization for the five-hour drop and vertical distribution experiments A) shows the control, with no sponge, while B) is the experimental beaker. Panel C demonstrates each trial of the five-hour drop and five-hour experiments. Three fresh, experimental sponges (Sponges 3,4, and 5 see **Table 3**) were placed in

**Vertical distribution Experiments:**

During the five-hour drop experiments, two sponges (Sponge 4 and 5) had the vertical distribution profiles of $H_2S$ measured to test hypotheses 2; a significant relationship between depth and uptake of $H_2S$ (by a natural and sponge environment) would be seen. A Unisense microsensor measured the concentration of $H_2S$ continuously over a 9 mm depth. A Sponge 4

was measured before the five-hour drop experiment, while the other was measured after the five-hour drop experiment.

**Five-Hour Uptake Experiments:**

Fresh sponges were collected (n=3, Sponges 2, 6, and 9), maintained under normal conditions, then placed under experimental conditions to determine the relationship between time and uptake of $H_2S$ (hypothesis 1). Two experimental beakers (2 L) were set up with normal aquarium water and 60 µmol/L of $H_2S$. This concentrated solution using hydrogen sulfide salt was made with DI water immediately before experimentation. The solution was slowly and gently, to reduce oxygenation, drained into the beaker immediately before the first measurement. Only one of the two beakers contained a *Cinachyrella* sponge. Sponges were allowed to acclimate to the experimental beaker condition for 30 minutes before $H_2S$ exposure. There were three tests per environment, i.e. a total of 6 trials, with only 3 sponges. A GENESYS 20 without printer spectrometer was utilized to measure absorbance. Absorbance (at 690 nm) was measured using the Sulfide Reagent Set, Methylene Blue (Hach product number 181732; methodology DOC316.53.01136) every 30 minutes for five hours. This kit allows the absorbance to be converted to $H_2S$ µmol/L utilizing a standard curve (**Fig. 13**). The curve was made before experimentation to determine the relationship between $H_2S$ and absorbance. Although very similar to five-hour drop experiments, the five-hour uptake experiments do not utilize microsensors and only had concentrations measured from the beakers' top.

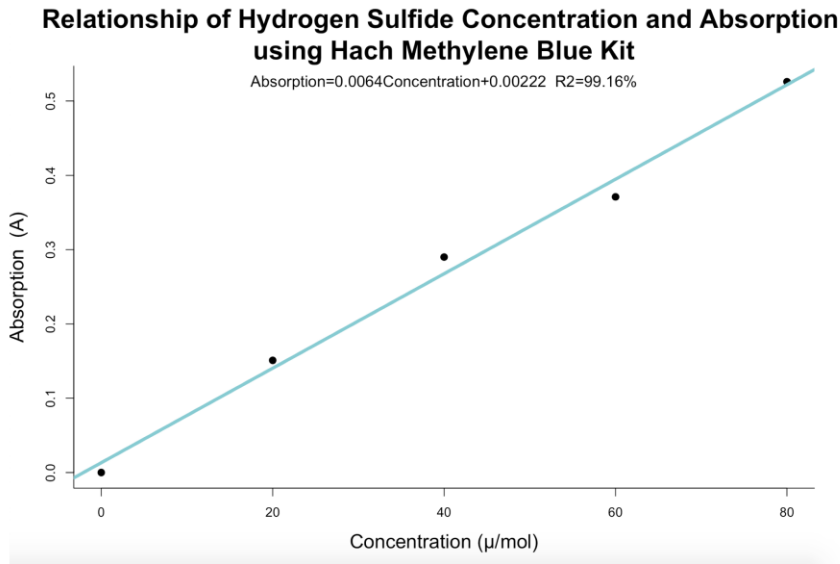


**Figure 13: Calibration curve for the concentration of $H_2S$.** The calibration curve above was used to determine all $H_2S$ concentrations for the five-hour uptake experiments.

**Statistical Analysis and Modeling Techniques of Five-Hour Drop, Vertical-Distribution, and Five-Hour Uptake Experiments:**

A modeling technique using Generalized Additive Model (GAM) allowed the determination of significant relationships between concentration and time (depth for all vertical distribution experiments). All data collected was recorded in Excel, under a comma-separated value file format (CSV). Sponge 4 was tested with a five-hour drop experiment, then vertical distribution was performed. Sponge 5 had a vertical distribution performed, then underwent a five-hour drop experiment. Both data sets were treated as independent events.

Statistical analysis was used to determine if the natural-uptake was significantly different from sponge uptake. A GAM was performed on the average values of the five-hour drop experiments to determine if the type of sample (control or sponge) and hour influenced the $H_2S$ concentration (hypothesis 4). A Generalized Linear Model (Poisson Distribution) (GLM) was performed on the accumulation of data from five-hour uptake experiments to determine if the type of sample (control or sponge) and hour influenced the $H_2S$ concentration (hypothesis 4). Long term exposure experiments were excluded because no chemical measurements were acquired during that test. All statistical tests were performed at a 95% confidence interval.

**Long-Term Exposure to Hydrogen Sulfide:**

An aquarium was maintained to house *Cinachyrella* spp. (Sponges 1, 7, and 8). Different sponges were utilized than those used in the five-hour drop experiments and five-hour uptake experiments to reduce the sponge's stress. Sponges were given 60 μmol/L of $H_2S$ twice weekly until the sponge appeared to decay. This concentrated solution using sodium hydrosulfide was made with DI water immediately before experimentation. The solution was slowly and gently, to reduce oxygenation, drained into the beaker immediately before the first measurement. Water was routinely monitored visually; temperature and salinity remained constant. The five-hour drop experiments sponge samples were taken before and after experimentation. Any changes in the sponge's appearance were recorded. A microbial mat formed was collected and stored at -80$^{o}$C without any solution. Water was collected and filtered using a 0.2 μm filter before and after experimentation. Triangle tissue samples from the bottom of the organism were taken before and after exposure to $H_2S$. These samples were then stored at -80$^{o}$C. The Long-Term Exposure experiments allowed hypotheses 5, 6, and 7 to be tested.

**DNA Extraction and Sequencing Methods for Long-Term Exposure:**

Tissue samples from long term exposed sponges then underwent DNA extraction using the Qiagen Powersoil PowerLyzer protocol. A 1% agarose gel was used to confirm a successful extraction. After confirmation, samples underwent polymerase chain reaction (PCR) using universal primers (MIDf-515F and 806rc) and Platinum 2X polymerase (Illumina) (Lopez et al., 2008). The PCR thermocycler followed an initial denaturation at 94°C for 3 minutes (one cycle). Then, denaturation at 94$^{o}$C for 45 seconds followed by annealing at 50°C for 1 minute, and finally, extension at 72°C for 1 minute and 30 seconds. This step was repeated for 29 cycles. There was a final extension at 72°C for 10 minutes, with the reactions held at 4°C indefinitely. Confirmation on 1% agarose gel was performed to ensure the presence of DNA.

The 16S rRNA gene's amplicon was sequenced per the EMP sequencing protocol for the Illumina MiSeq platform. This sequencing was completed using Illumina barcoded primers for the 16S rRNA region (MIDf-515F and 806rc) with Platinum 2X polymerase (Illumina) (Promega). PCR was performed using the same procedure within the previous paragraph. Unique barcodes provide samples with an Id, which allows samples to be traced through data analysis. PCR was then checked on a 1% agarose gel for proper amplification with clean bands.

Samples were purified using AMPure bead as outlined in the 16S metagenomic library prep guide (Illumina, 2013). Final DNA concentrations were determined using a Qubit 2.0 fluorometer for normalization (Life Technologies), then underwent library pooling. Sample's quality was checked by Agilent Bioanalyzer tape station 2200 as outlined in the Agilent High Sensitivity D1000 ScreenTape System Quick Guide (Agilent Technologies, 2013). A high-throughput Illumina MiSeq sequencing approach targeting the 16S rRNA gene V4 regions was applied to verify specific microbial groups' presence and abundances. Upon sequencing completion, two FASTQ files, a forward and a reverse read, were used for downstream analysis.

**Data Analysis of 16S rRNA data for Long-Term Exposure:**

Sponge 1, 7 and 8 16S rRNA FASTQ DNA sequence files were run through Quantitative Insights into Microbial Ecology (QIIME2) for demultiplexing, quality filtering, ASV picking, taxonomic assignment, phylogenetic reconstruction, diversity analysis, and all visuals. Mapping files were compared for errors using "validate_mapping_file.py", before demultiplexing and

quality filtering with “split_libraries_fastq.py”. Sequences were filtered to remove chimeras and any score under 25 (1 error in 10,000 base pairs based on the PHRED system). The sequences were then sorted into ASVs with a 99% or more significant similarity for the Silva database using the “pick_open_reference_otus.py”. All reads (forward and reverse) were combined into one "qza" file using the "demuc" command, then imported into QIIME2 with the "emp-import" command. Then filtered and trimmed using the "dada2 denoise" command creating a feature-table, which was used to generate phylogenetic reconstruction using the "phylogeny fasttree" command.

Alpha and beta diversity community metrics were determined in R Studio. Alpha diversity describes the number of taxa and abundance within communities or habitats (species richness and species evenness), while beta diversity is variation in community composition (Knight et al., 2012). The phyloseq package with R was used to assess alpha diversity. Beta diversity was measured with VEGAN. Bray-Curtis values, quantifying dissimilarities between the type of experiment (Sponge: Before, Sponge: After, Water: Before, Water: After, Microbial Mat, and Algae) were used. In both packages, Shannon's index and Inverse Simpson's index calculated alpha and beta diversity. Two t-tests were performed at a 95% interval to determine if sponge samples, before vs. after exposure, had a significantly different beta and alpha diversity.

Within primer, a non-metric multidimensional scaling (NMDS) plot was constructed using relative abundance. Analysis of similarities (ANOSIM) was performed in the following groups: sponge and water; before and after; before: sponge and before: water; after: sponge and after: water; after: algae, after: microbial mat, after: sponge, and after: water; before: sponge and after: sponge; before: water and after: water. A shaded plot was constructed in PRIMER to show the differences in classes, orders, and families within all samples. The topmost 30 abundant taxa were displayed. A Simper analysis was performed in PRIMER to determine the top similar and dissimilar ASVs. Any abundant taxa with a percent contribution under 1% were discarded. A Simper analysis was also performed in R Studio to identify the significant contributions of taxa differing between water and sponges before and after exposure, independently.

## VIII. RESULTS:

**Five-Hour Drop Experiments:**

Sponges 3, 4, and 5 were placed in beakers with a sufficient amount of water and 60 μmol/L of $H_2S$. Using microsensors, $H_2S$ was measure over five hours. All five-hour drop experiments had the best model with a GAM. All models indicate that time significantly affects the concentration of $H_2S$ (p-value<$2e^{-16}$and $R^2$>92%). A GAM also demonstrated that the type of sample (control or sponge) had a significant impact on the rate of uptake (p-value<$2e^{-16}$and $R^2$>92%) (**Figs. 14,15, 16, and 17**).

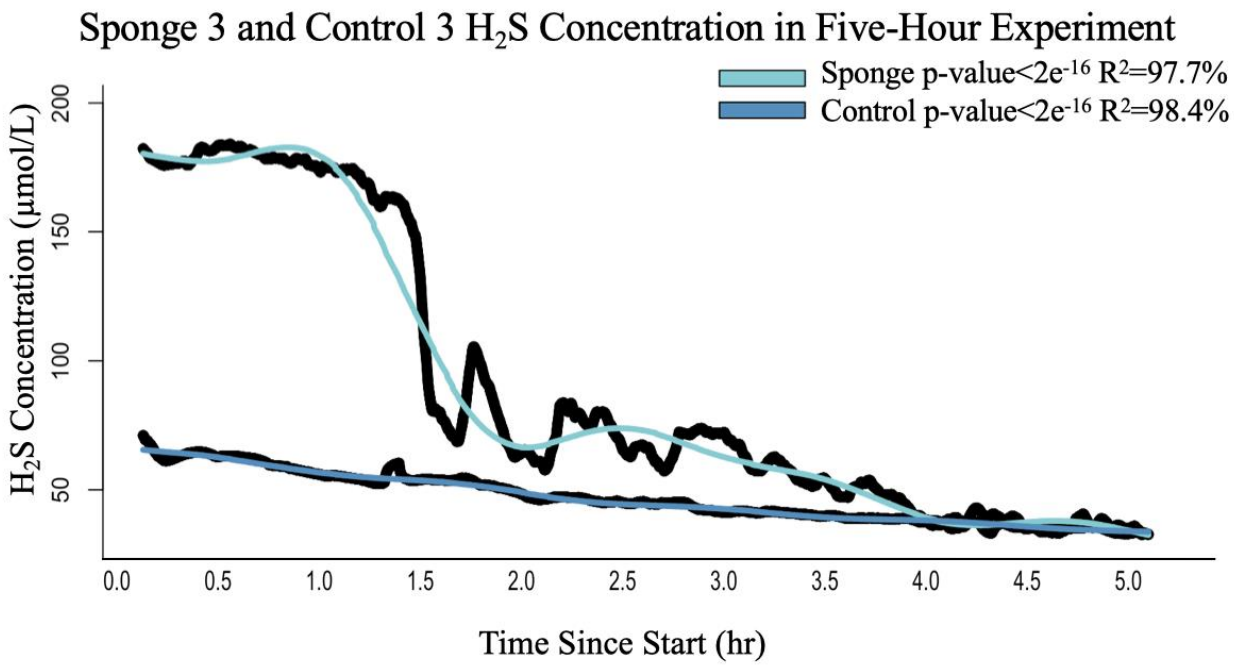


**Figure 14: $H_2S$ measurement of Sponge 3 and Control during 5 hour-drop.** Above is the model constructed for Sponge 3 and the corresponding control. Recall every 30 minutes the microsensor was dropped 1000 μm. A GAM was most appropriate at describing both sets of data, with an $R^2$ value of > 97%. Both models demonstrated that the time since start had a significant effect on the $H_2S$ concentration (p-value<$2e^{-16}$).

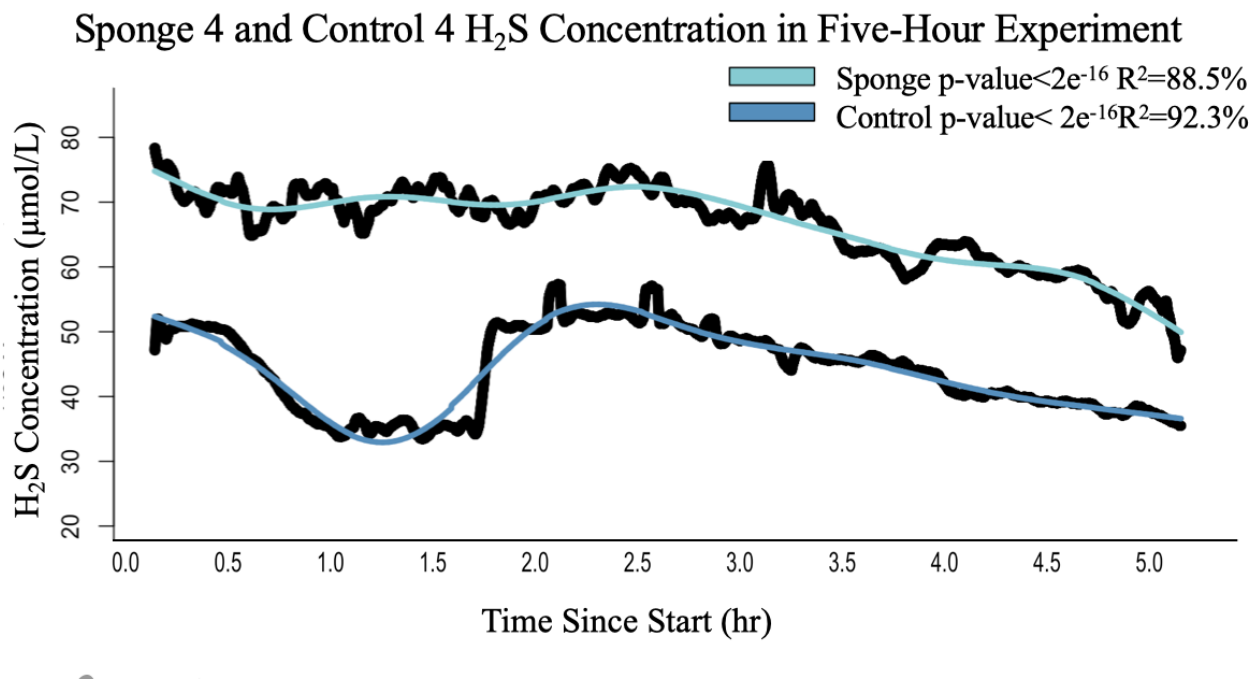


**Figure 15: $H_2S$ measurement of Sponge 4 and Control during 5 hour-drop.** The model for Sponge 4 and corresponding control is demonstrated above. Recall every 30 minutes microsensor dropped 1000 µm. A GAM described both sets of data the best, with an $R^2$value of > 88%. Both models demonstrated that the time since start had a significant effect on the $H_2S$ concentration (p-value<$2e^{-16}$).

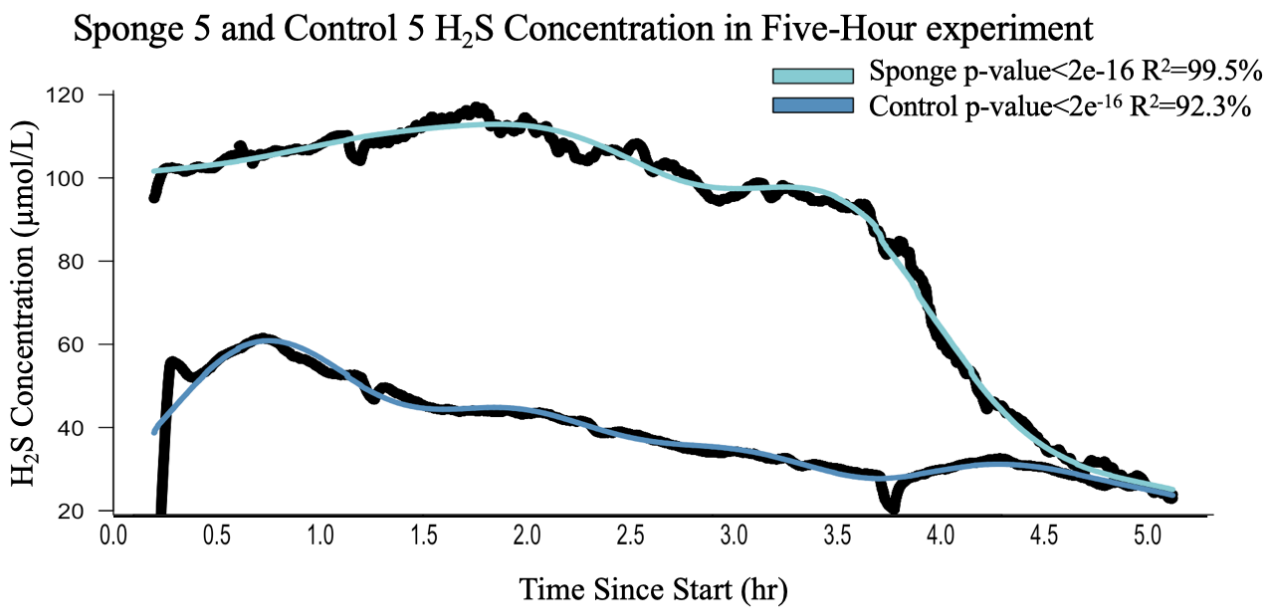


**Figure 16: $H_2S$ measurement of Sponge 5 and Control during 5 hour-drop.** Above demonstrates the model constructed for Sponge 5 and the corresponding control. Every 30 minutes microsensor dropped 1000 µm. A GAM described both sets of data the best, with an $R^2$ value of > 92%. Both models demonstrated that the time since start had a significant effect on $H_2S$ concentration (p-value<$2e^{-16}$).

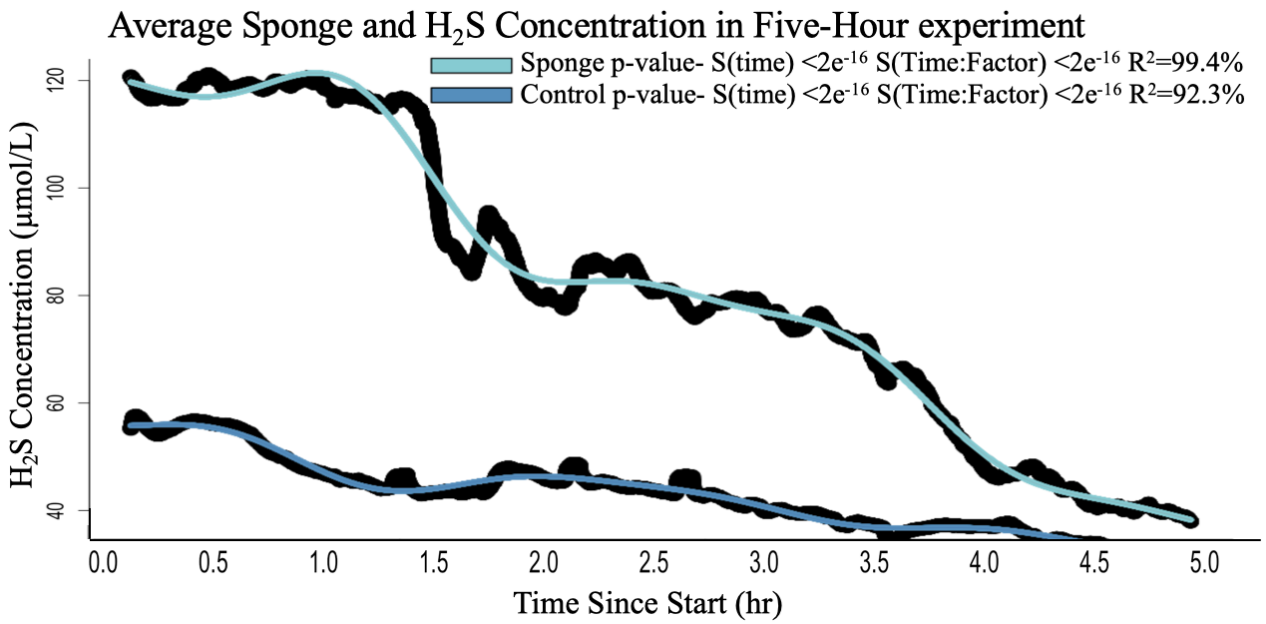


**Figure 17: Average H2S measurement of the Sponge and Control during 5 hour-drop.** Above is the model constructed for the average five-hour drop experiments. Every 30 microsensor dropped 1000 µm. A GAM demonstrated significance relationship between time and concentration (p-value<$2e^{-16}$and $R^2$=99.4). It also indicated significance between the type of sample (sponge or control) (p-value<$2e^{-16}$and $R^2$=92.3). Sponge samples are indicated in light blue, and control samples are indicated in dark blue.

**Vertical Distribution Experiments:**

Vertical distribution profiles of $H_2S$, using microsensors, were taken of Sponge 4 and Sponge 5. The profile of Sponge 4 was taken before the five-hour drop experiments, while the vertical distribution of Sponge 5 was taken after the five-hour drop experiments. This measurement was taken with the microsensor, moving a total of 9000 µm. The depth significantly affected the $H_2S$ measurement for sponge 4 ($F=86.91$, p-value$<2e^{-16}$), explaining 93.2% variation ($R^2 = 0.932$) (**Fig. 18**). Sponge 5 data indicated that depth significantly affected the $H_2S$ measurement ($F=38.61$, p-value$<2e^{-16}$). Depth explains 87.2% $H_2S$ measurement for sponge 5 ($R^2 = 0.872$) (**Fig. 18**).

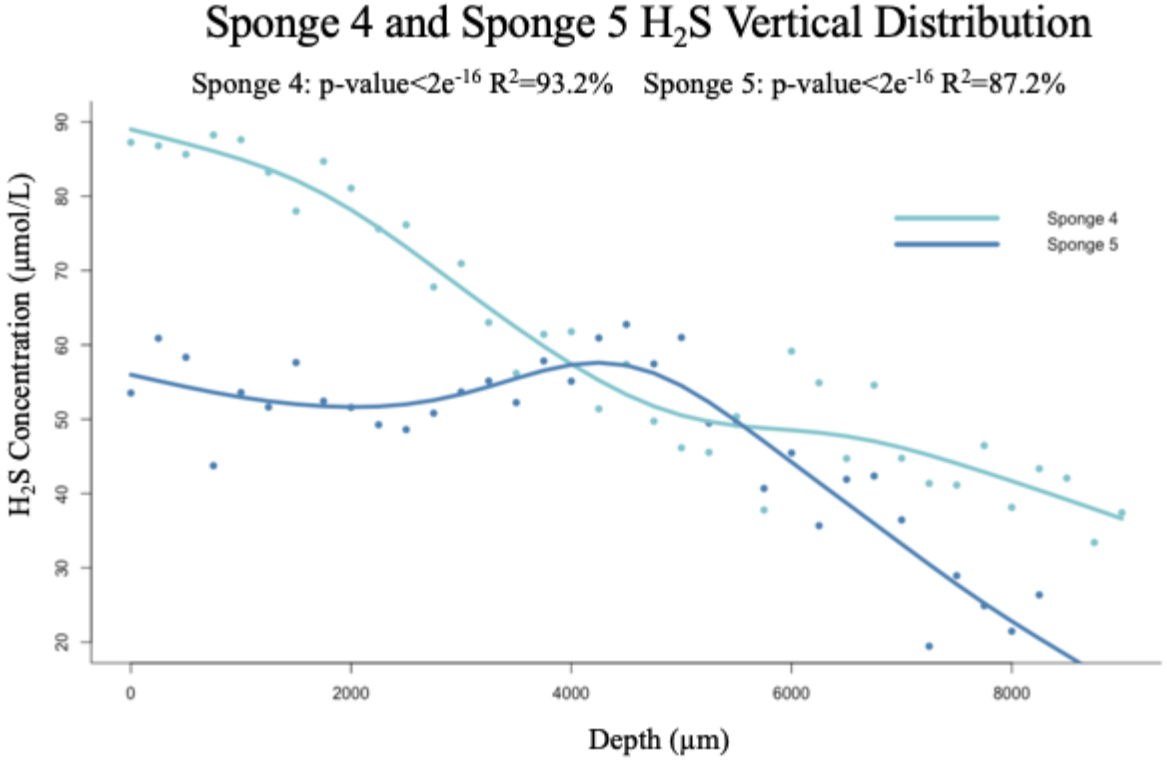


**Figure 18: Vertical distribution of $H_2S$ measurement of Sponge 4 and Sponge 5.** The vertical distribution of Sponge 4 and Sponge 5 is seen above. The light blue represents Sponge 4, and the dark blue represents Sponge 5. A GAM was the leading model, with an $R^2$ value of > 87 %. Both models demonstrated that the depth had a significant effect on $H_2S$ concentration (p-value$<2e^{-16}$). Note the vertical distribution of Sponge 4 and Sponge 5 was performed before and after the five-hour drop experiments, respectively.

**Five-Hour Uptake Experiments:**

Sponges 2, 6, and 9 $H_2S$ consumption rate was measured every 30 minutes using the Sulfide Reagent Set, Methylene Blue (Hach product number 181732; methodology DOC316.53.01136). GAM modeling techniques were utilized to model the relationship and determine if time significantly affected $H_2S$ consumption. Sponge 2, Sponge 6, and Sponge 9 have a p-value $<2e^{-16}$; thus, it is concluded that hours significantly affected the $H_2S$ consumption (**Fig. 19**). Note that nine knots were used for Sponge 9.

Significant consumption within the controls was also tested. Control 2 (p-value $<2e^{-16}$), Control 6 (p-value= $1.18e^{-6}$), and Control 9 (p-value=0.00744) indicate hour significantly affected the natural loss of $H_2S$ consumption (**Fig. 19**). Note that six knots were used for control 6 and control 9. The first value of control 9 was dropped as it was an outlier of the data.

A GLM determined that time significantly influenced the consumption of $H_2S$ (p-value $<2e^{-16}$). Additionally, the type of sample does significantly influence the consumption of $H_2S$ (p-value=$5.019e^{-12}$ There is a significant interaction between the type of sample and hour (p-value =$6.793e^{-11}$). The GLM explains 80.61% of deviations within the data (**Fig. 20**).

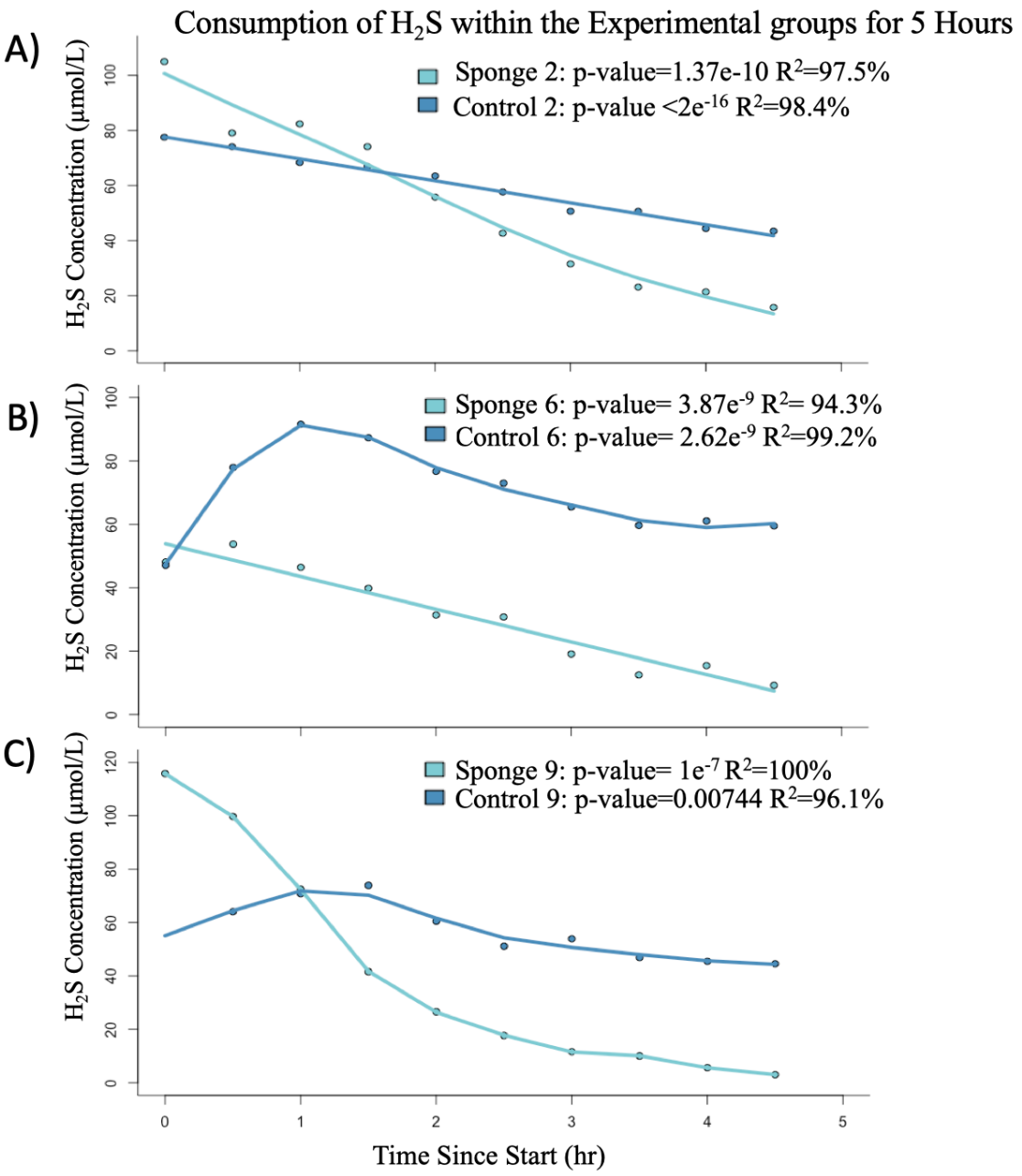


**Figure 19: $H_2S$ measurement of Sponge 2 with Control 2, Sponge 6 with Control 6, and Sponge 9 with Control 9during 5 hours.** Part A, B, and C demonstrated the model constructed for Sponge 2 with Control 2, Sponge 6 with Control 6, and Sponge 9 with Control 9, respectively. All models demonstrated that time since start had a significant effect on $H_2S$ concentration (p-value<0.05), with an $R^2$>94%.

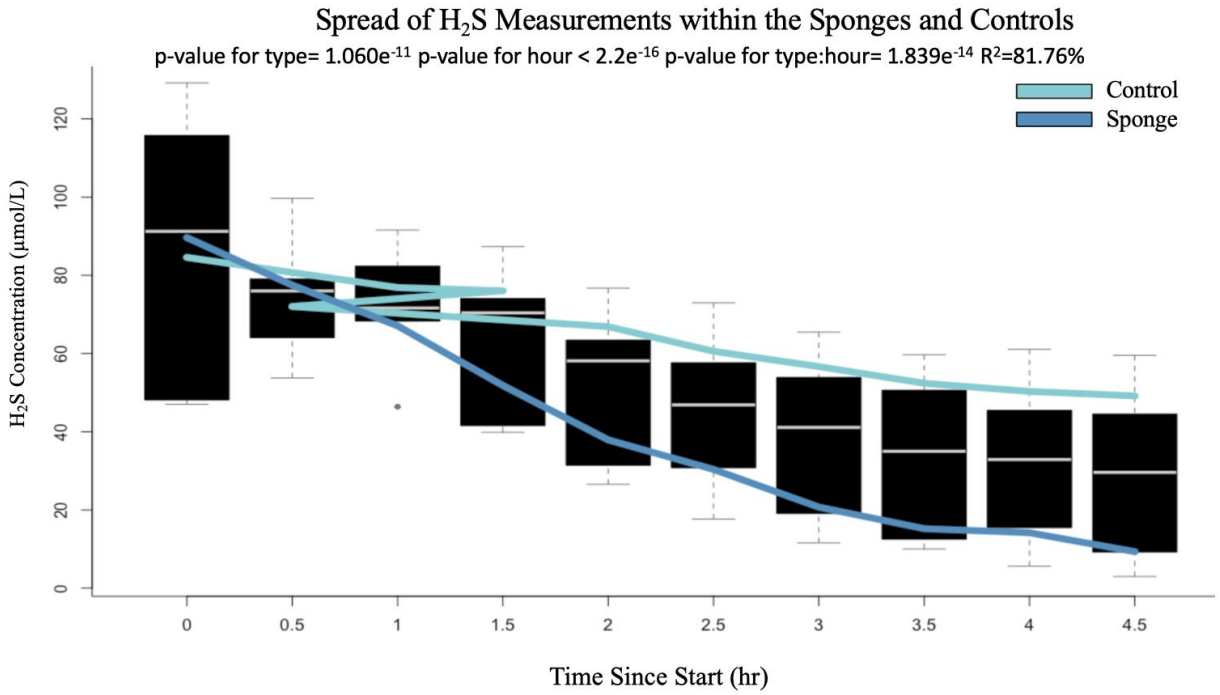


**Figure 20: Average $H_2S$ model for sponge and control.** All possible $H_2S$ measurements for sponge and control samples are displayed in the above boxplot. A GLM fit the data best, with an r-sq of 81.76%, creating two types of linear equations, displaying the control samples and another demonstrating the sponge samples. The model demonstrated that time since start, type of sample, and interaction significantly affected $H_2S$ concentration (p-value<0.05).

**Microbiome Analyses of Long-Term Exposure to Hydrogen Sulfide:**

Sponges 1, 7, and 8 were kept over several weeks and were exposed to 60 µmol/L of $H_2S$ twice weekly. Samples of each sponge were taken before exposure (S#B) and after (S#A). Water samples were also taken before exposure (W#B) and after (W#A). Note water from the tank of Sponge 7 was mistakenly not taken after. Algae formed on all long-term sponges, and samples were taken (A#). A microbial mat formed on Sponge 7 before the first exposure of $H_2S$. Thus, a sample was taken when initially forming (MM7B) and when the experimentation was complete (MM7A). Sponge 8 was also seen to have a microbial mat form (MM8).

Seventeen samples were sequenced using a MiSeq sequencer (**Table 4**). A total of 1,100,167 raw 16S rRNA amplicon sequences were obtained. After filtration with dada2, 824,409 reads were generated. The average number of reads in each sample was 48,495, with a 41,926 standard deviation. The cut off for quality scores was 25, as default in QIME2. The average length of the samples was about 251 base pairs.

The alpha and beta diversity metrics were determined for Long-term Exposure experiments. Alpha diversity describes the number of taxa and abundance within communities or habitats (species richness and species evenness), while beta diversity is variation in community composition (Knight et al. 2012). The phyloseq package with R was used to assess alpha diversity. Beta diversity was measured with the vegan package. Bray-Curtis values, a method for quantifying dissimilarities between different types, were used. The types used here were Sponge: Before, Sponge: After, Water: Before, Water: After, Microbial Mat, and Algae. In both packages, Shannon's index and Inverse Simpson's index were used. Alpha diversity appears to be in two groups. One group appears to contain sponge samples after exposure, microbial mat, and algae. The second group contains sponge samples before exposure, water samples before exposure, and water samples after exposure. This separation is seen in Shannon's Index and Inverse Simpson's Index (**Fig. 21**). The same trend is seen with beta diversity (**Fig. 22).** A t-test was performed at a 95% interval to determine if sponge samples (after and before exposure) had a significantly different beta diversity and alpha diversity (beta: $t = 2.5749$, $df = 3.9593$, p-value = 0.06228 alpha: $t = 2.5789$, $df = 3.9604$, p-value = 0.062).

An NMDS was plotted in PRIMER utilizing relative abundance (**Fig. 23**). Using the ANOSIM (Analysis of similarities) function, no significance was seen between the relative abundance and the type of experiment (p-value=0.073). The same trend was seen when comparing the following: sponge and water (p-value=0.054), After samples (p-value=0.567), Before and After (p-value=0.14), sponge samples after exposure and water samples after exposure (p-value>0.05), sponge samples before exposure and water samples before exposure (p-value= 0.20), sponge samples before exposure and sponge samples after exposure (p-value>0.05). An ANOSIM was also performed to determine if individual sponge (Sponge 1, 7, and 8) influenced relative abundance. A significant relationship between the individual sponge and relative abundance was seen (p-value=0.01, R=55.8%).

A shaded plot with clustering was constructed to dominate 30 classes within the Long-Term samples (**Fig. 24**). Clostridia was seen to be high in Sponge 7 before exposure, which decreased in abundance after exposure. Bacteroides was seen to increase in relative abundance within all sponge samples after exposure compared to before exposure. Gammaproteobacteria was seen to decrease in all samples after exposure compared to before exposure. Deltaproteobacteria increased in Sponge 7 and 8 after exposure compared to before. However, Deltaproteobacteria

decreased in Sponge 1 after exposure compared to before exposure. Alphaproteobacteria increased after exposure compared to before exposure in Sponge 1 and 7. Alphaproteobacteria decreased after exposure compared to before exposure in Sponge 8. Water from the tank of sponge 7 showed a high abundance of Alphaproteobacteria, but the sample was not taken after exposure. Water from the tank of sponge 1 decreased in Alphaproteobacteria, decreased in Bacteroidia, increased in Oxyprotobacteria after exposure compared to before exposure. Water from the tank of sponge 8 increased in Deltaproteobacteria after exposure compared to before exposure. Water from the tank of Sponge 8 decreased in Gammaproteobacteria, Alphaproteobacteria, and Verrucomicrobiae after exposure compared to before exposure. The microbial mat consisted of Alphaproteobacteria, Clostridia, Deltaproteobacteria, and Bacteroides. Algae had a high amount of Alphaproteobacteria, Gammaproteobacteria, Bacteroidia, and Oxyphotobacteria.

A shaded plot with clustering was constructed to dominate 30 orders and families within the long-Term samples (**Fig. 25** and **Fig. 26**). Within sponge 1 and 7, there were abundant Rhodobacterales before exposure, but the relative abundance still increased after exposure. This trend was not seen for Sponge 8, who decreased in relative abundance after exposure. The majority of this abundance can be attributed to the family Rhodobacteraceae (Genera *Rhodobacter, Paracoccus, Desulfovibrio, Loktanella, and Oceanicella*). Sponge 1 samples of Flavobacteriales and Cytophagales increased after exposure, while decreased after exposure in Sponges 7 and 8. Sponges 8 and 7 both increased Desulfovibrionales (Family Desulfovibrionaceae) and Desulfuromonadales (Family Desulfuromonadaceae). Sponge 8 and 7 also increased in the order Bacteroidetes. Sponge 7 had an abundant Clostridia (Family XII), which was seen to decrease after exposure.

Within water samples, Rhodobacterales (family Rhodobacteraceae) were seen to be abundant. This family increased in the water from Sponge 8 and decreased from Sponge 1. In water from the tank of Sponge 1, a high abundance of Flavobacteriales (Family Flavobacteriuaceae) slightly increased after exposure. In Sponge 8, these taxa decreased. Recall the water from the tank of Sponge 7 did not get collected after exposure. Sponge 8 decreased in Oceanospirillales (From Saccharospirillaceae and Nitrinoclaceae) and Verrucomirobiales (From the Family Rubritaleaceae). An increase of Bacteriodales and Clostridiales (families of XII, family XIII, and Lachnospiraceae) within Sponge 8 after exposure.

Microbial mat samples contained an abundance of the order Rhodobacterales (family Rhodobacteraceae), Oceanspirillales (Nitrincolaceae, and Oceanospirillales), Clostridiales (Lechnospiraceae, families of XII, and family XIII), Desuldovibrionales (Family Desulfovibrionacaea), and Desulfuromonadales (Family Desulfuromonadaceae), Bacteriodales, Campylobacterales, Flavobacteriales. Algae showed an abundant of Rhodobacterales (Family Hyphomonadaceae and Rhodobacteraceae), Oceanospirillales (Family Nitrincolaceae), Flavobacteriales (Family Crymorphoraceae), Rickettsiales, Alteromonadales (Family Alteromonadaceae, Colwelliaceae), Caulobacterales (Family Parvularculaceae), Chitinophagales (Family Saprospiraceae), Cytophagales (Family Cyclobacteriaceae), Nostocales, and Phormidesmiales (Family Nodosilineaceae).

Simper analysis was implemented on all samples. ASVs that contribute to the overall similarity of sample type (sponge, algae, microbial mat, and water) can be seen in Appendix A (**Table 12,13,14,15,16,17**). Similarly, ASVs contributing to the overall dissimilarity of types (sponge vs. algae, sponge vs. microbial mat, water vs. algae, microbial mat vs. water) can be seen in Appendix A **(Table 18,19,20,21)**. Sponge samples, taken before exposure, had an overall similarity of 11.83% (**Table 5**). Sponge samples, taken after exposure, had an overall similarity of 11.3% (**Table 6**). The sponge samples before vs. after was different 89.66%, with major contributing taxa include ASVs from Nitroopumilaceae, *Draconibacterium*, Rhodobacteraceae, Clostridials, Betaproteobacteriales, Deltaproteobacteria, *Halodesuflovibrio*, and Phycisphaerales (**Table 7**). A similarity of 6.14% and 1.32% was seen for water samples before and after exposure, respectively (**Table 8 and Table 9**). Major contributing ASVs were the taxa Flavobacteriaceae, *Cylindrotheca*, Oxyphotobacteria, *Marinifilum*, Rhodobacteraceae *Phaeodactylibacter*. Simper showed a dissimilar rate of 89.41% between water samples before and after exposure (**Table 10**). Water and sponge samples had a different relative abundance between the same ASVs 92.57% of the time (**Table 11**) with major contributing ASVs of the taxa Nitrosopumilaceae, Rhodobacteraceae, Flavobacteriaceae, and *Draconibacterium*. The percent of taxa with sulfur metabolism was seen to be 69% for sponge samples after exposure (**Table 5**), 77% for sponge samples before exposure (**Table 6**), 62.5% for sponge samples before compared to after exposure (**Table 7**), 35% for water samples before exposure (**Table 8**), 100% for water samples after exposure (**Table 9)**, 28% for tank water

before exposure compared to after exposure (**Table 10**), and 50% for sponge samples compared to water samples (**Table 11**).

**Table 4: MiSeq sequencing read statistics.** The sequencing reads per sample are detailed below. Filtered reads are the reads that were kept after the filtration with dada2. The date at which the experiment was started and ended can be seen on the right-hand side. Average and standard deviation reads can be seen in the last two rows.

| ***Sequencing Reads per Samples*** | | | | | | |
|---|---|---|---|---|---|---|
| ***Sample ID*** | **Raw Reads** | **Filtered Reads** | **Percentage of filtered reads in raw reads** | **Date Experiment Started** | **Date Experiment Ended** | **Total Experimental Time** |
| ***W1B*** | 166987 | 145070 | 86.88 | Sept 24, 2019 | Oct 22, 2019 | 28 days |
| ***S1B*** | 60933 | 21022 | 34.5 | | | |
| ***A1*** | 49921 | 23301 | 46.68 | | | |
| ***S1A*** | 23940 | 19968 | 83.41 | | | |
| ***W1A*** | 66931 | 36766 | 54.93 | | | |
| ***W7B*** | 50099 | 44451 | 88.73 | Feb 4, 2019 | Feb 22, 2019 | 18 days |
| ***MM7B*** | 28948 | 25103 | 86.72 | | | |
| ***S7B*** | 34602 | 22990 | 66.44 | | | |
| ***A7*** | 150992 | 131440 | 87.05 | | | |
| ***S7A*** | 38523 | 34562 | 89.72 | | | |
| ***MM7A*** | 68949 | 57706 | 83.69 | | | |
| ***W8B*** | 48819 | 22443 | 45.97 | Feb 4, 2019 | Feb 29, 2019 | 25 days |
| ***S8B*** | 168651 | 123540 | 73.25 | | | |
| ***A8*** | 36469 | 31509 | 86.4 | | | |
| ***MM8*** | 27108 | 20297 | 74.87 | | | |
| ***S8A*** | 29681 | 23363 | 78.71 | | | |
| ***W8A*** | 48614 | 40878 | 84.09 | | | |
| ***Average*** | **64715.7059** | **48494.6471** | - | - | - | |
| ***SD*** | **48516.574** | **41925.6992** | - | - | - | |

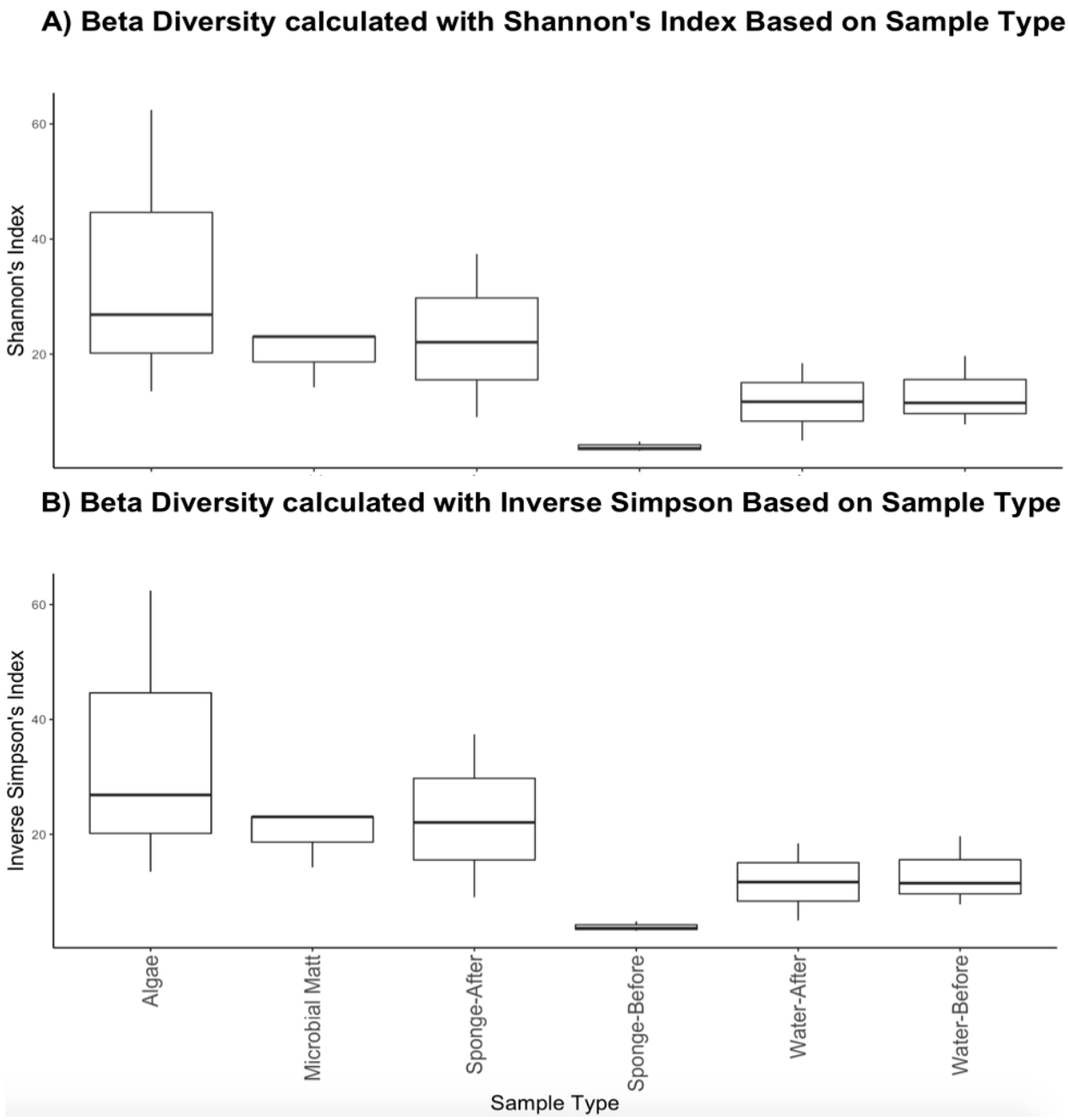


**Figure 21: Beta diversity within long term samples.** Beta diversity was calculated using Shannon's index (part A) and Inverse Simpson's index (part B).

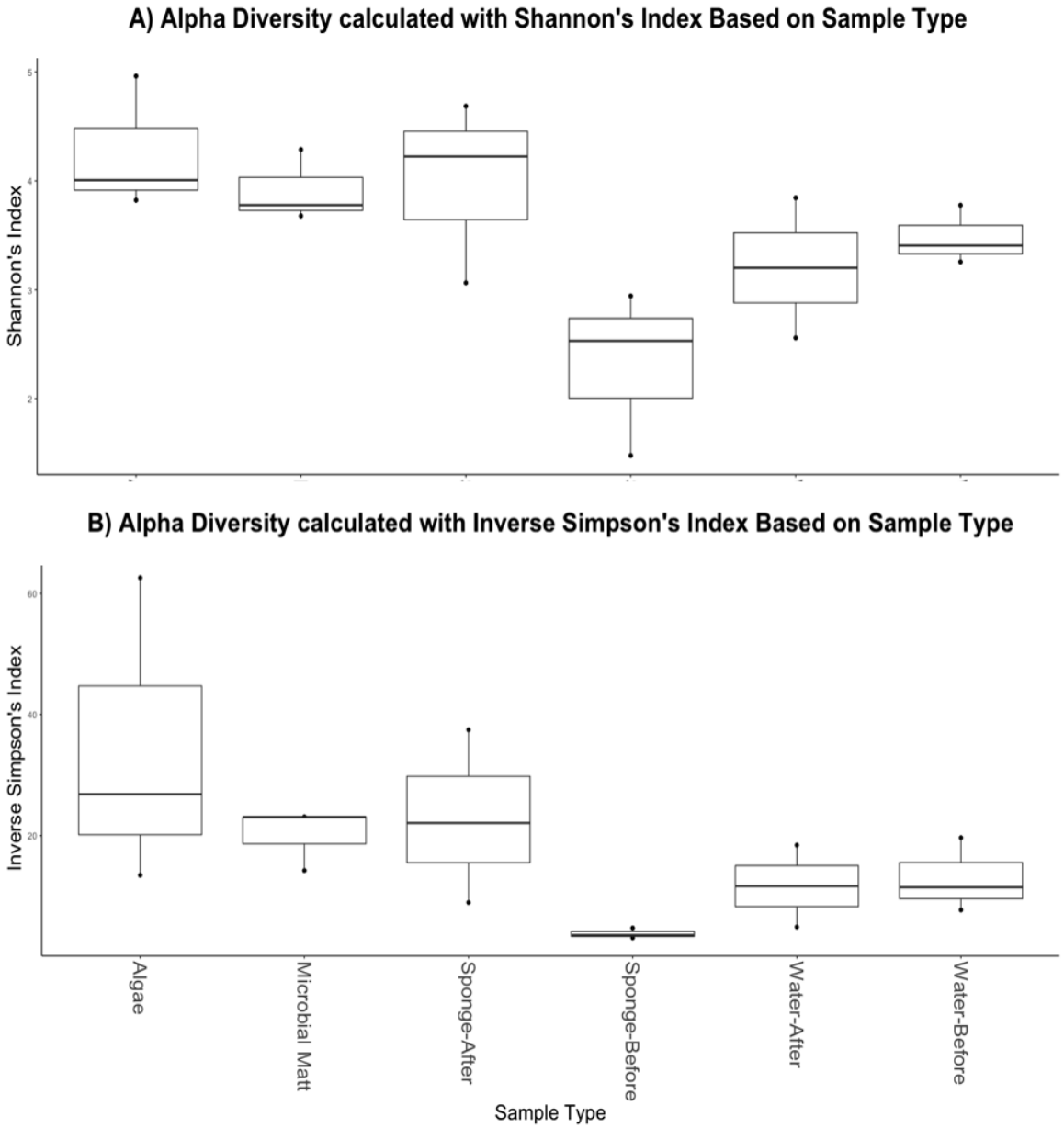


**Figure 22: Alpha diversity within long term samples.** Alpha diversity was calculated using Shannon's index (part A) and Inverse Simpson's index (part B).


**Figure 23: NMDS plot based on various samples and exposures.** The above illustration shows the non-metric multidimensional scaling ran on multiple groups. The analysis is shown for all samples based on the type of sample (part A), for all sponge and water samples (part B), all samples based on time (part C), and based on sponge number (part D).

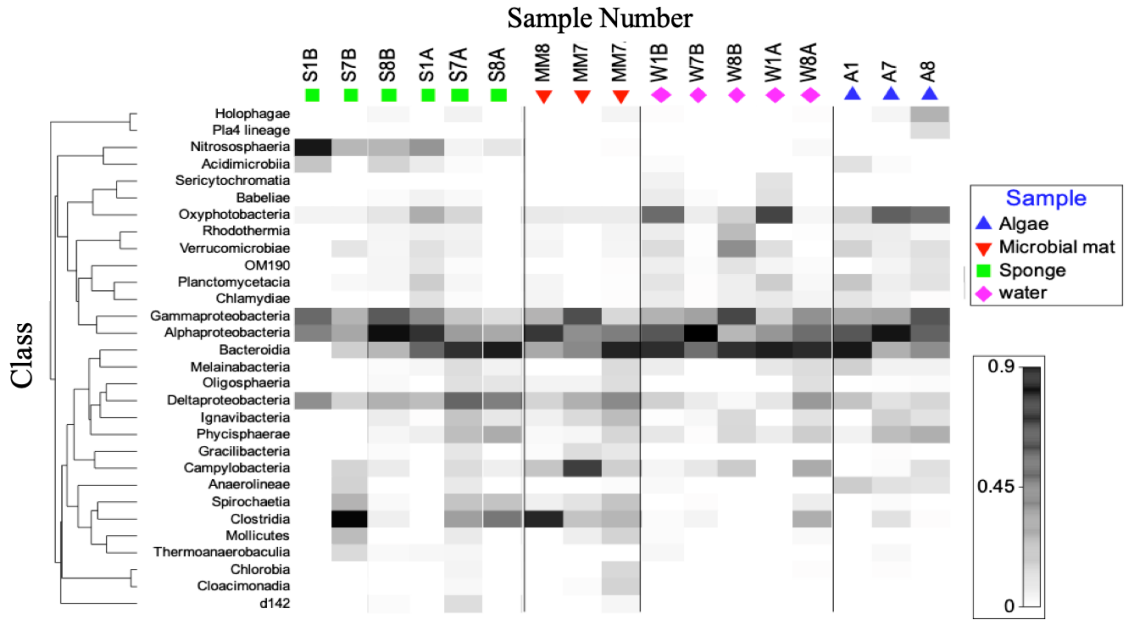


**Figure 24: Shaded plot based on the relative abundance of classes.** The shaded plot and clustering based on the relative abundance of classes in all samples tested. The sample legend is seen on the right. Recall only the top 30 abundant classes are graphed.

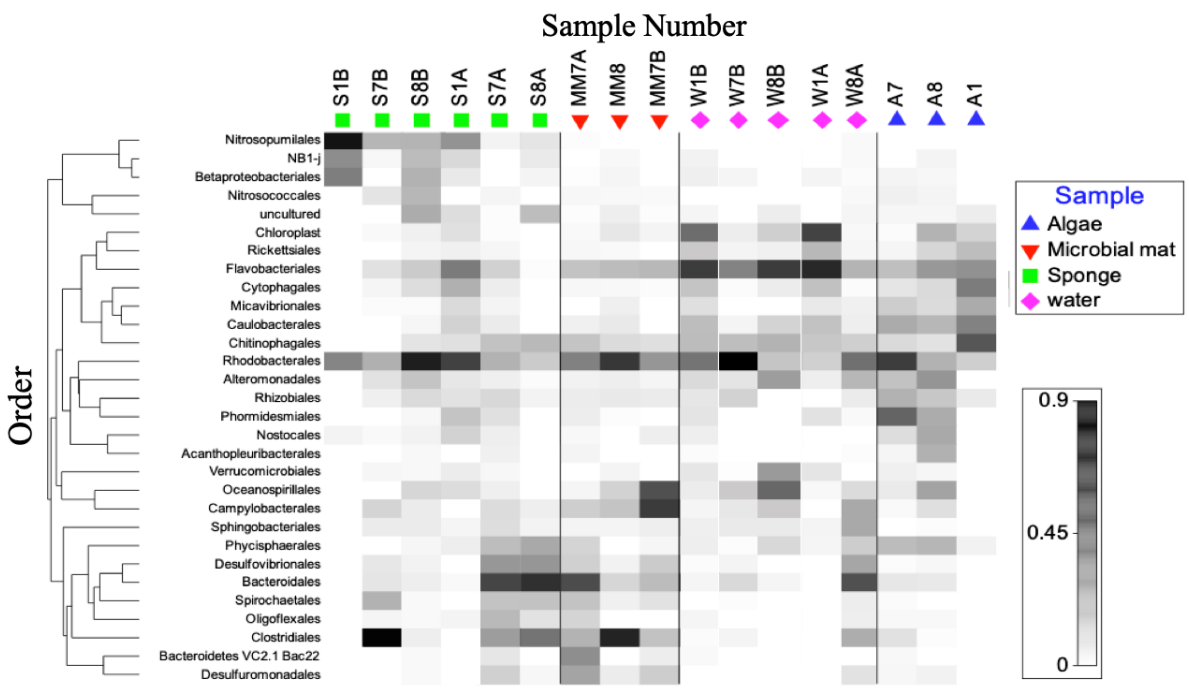


**Figure 25: Shaded plot based on the relative abundance of orders.** The shaded plot and clustering based on the relative abundance of order in all samples tested. The sample legend is seen on the right. Recall only the top 30 abundant orders are graphed.

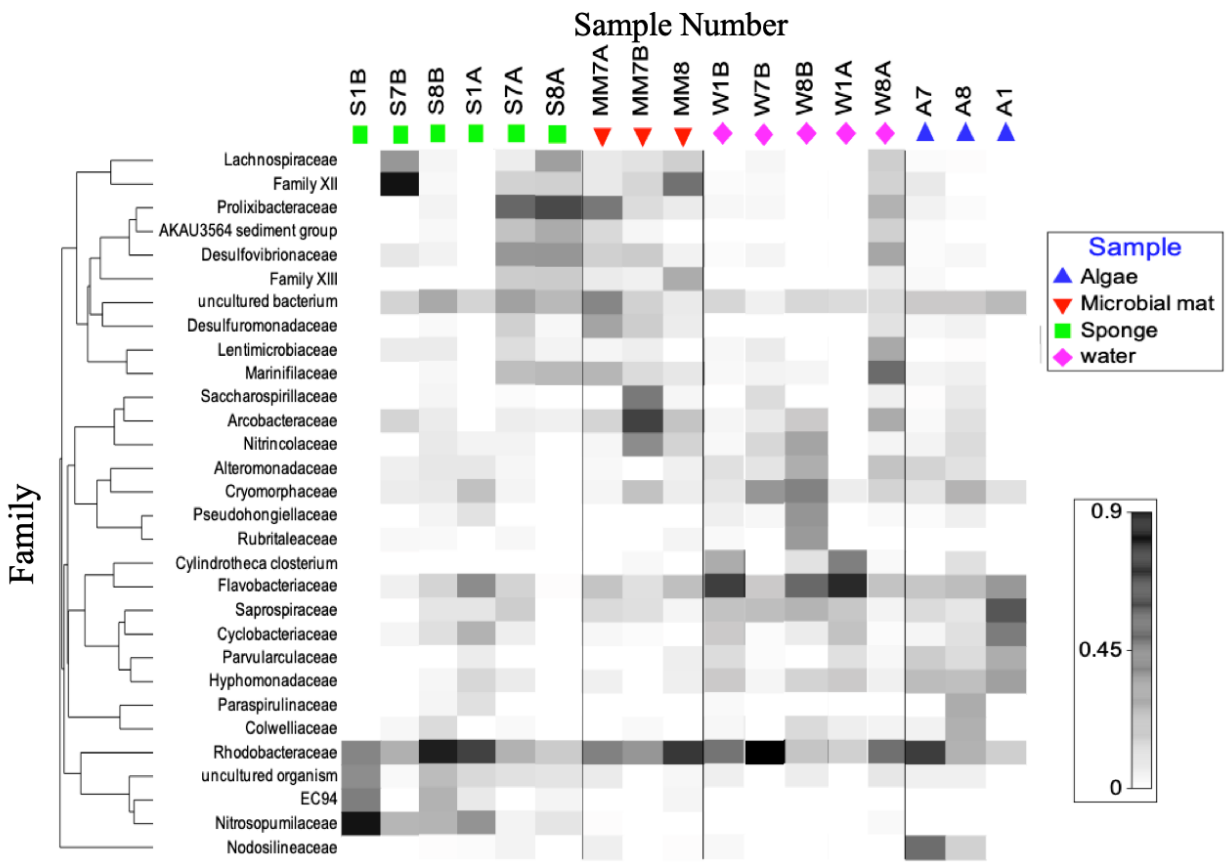


**Figure 26: Shaded plot based on the relative abundance of families.** The shaded plot and clustering based on the relative abundance of the family in all samples tested. The sample legend is seen on the right. Recall only the top 30 abundant families are graphed.

**Table 5: Similar taxa within sponges before exposure using simper.** The above was the results of a Simper test to determine major contributing ASVs to the similarity of sponge samples before the exposure to $H_2S$. Overall, similarly of 11.83% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Similar Taxa within Sponges Before Exposure using Simper***

| *ASVs* | Taxonomy | Average Abundance | Average Similarity | Percent Contribution | Sulfur Metabolism |
|---|---|---|---|---|---|
| *52* | Family Rhodobacteraceae - Uncultured | 0.35 | 2.2 | 18.6 | Possible Thiosulfate oxidation/ sulfur reduction |
| *54* | Class Gammaproteobacteria - Unknown | 0.16 | 1.22 | 10.29 | Possible sulfur reduction |
| *55* | Class Deltaproteobacteria - NB1-j | 0.18 | 1.2 | 10.13 | Possible sulfur Reduction |
| *53* | Order Nitrosopumilaceae | 0.31 | 1.11 | 9.38 | - |
| *56* | Order Betaproteobacteriales - EC94 | 0.17 | 0.75 | 6.35 | sulfur reduction |
| *62* | Class Actinomarinales | 0.1 | 0.66 | 5.62 | - |
| *59* | Class Alphaproteobacteria | 0.07 | 0.5 | 4.21 | Possible sulfur reduction |
| *12* | Genus *Ruegeria* | 0.06 | 0.34 | 2.84 | Possible Thiosulfate oxidation |
| *83* | Genus *Vibrio* | 0.07 | 0.33 | 2.75 | Possible sulfite reduction |

**Table 6: Similar taxa within sponges after exposure using simper.** The above was the Simper test results to determine major contributing ASVs to sponges' similarity after exposure to $H_2S$. Overall, similarly of 11.3% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Similar Taxa within Sponges After Exposure using Simper***

| *ASVs* | Lowest Taxonomy | Average Abundance | Average Similarity | Percent Contribution | Sulfur Metabolism |
|---|---|---|---|---|---|
| *58* | Genus *Draconibacterium* | 0.29 | 1.06 | 9.39 | Possible Sulfate Reduction |
| *28* | Genus *Halodesulfovibrio* | 0.16 | 0.71 | 6.25 | Known Sulfur Reduction |
| *12* | Genus *Ruegeria* | 0.11 | 0.62 | 5.49 | Possible Sulfur Oxidation |
| *52* | Family Rhodobacteraceae - Uncultured | 0.18 | 0.57 | 5.07 | Possible Sulfur Oxidation/Sulfur Reduction |
| *63* | Order Phycisphaerales - AKAU3564 | 0.13 | 0.48 | 4.26 | - |
| *43* | Genus *Desulfovibrio* | 0.09 | 0.36 | 3.18 | Known Sulfate Reduction |
| *57* | Class Alphaproteobacteria - Unculutred | 0.1 | 0.34 | 3.02 | Possible Sulfur Oxidation/Sulfur Reduction |
| *54* | Class Gammaproteobacteria - Unkown | 0.08 | 0.32 | 2.87 | Possible Sulfur reduction |
| *34* | Order Chitinophagales - Uncultured | 0.1 | 0.31 | 2.77 | sulfur reduction |
| *88* | Order Oligoflexales - Uncultured | 0.06 | 0.27 | 2.37 | - |
| *20* | Family Rhodobacteraceae - Unknown | 0.03 | 0.26 | 2.29 | Possible Sulfur Oxidation/Sulfur Reduction |
| *53* | Family Nitrosopumilaceae | 0.13 | 0.26 | 2.28 | - |
| *55* | Class Deltaproteobacteria - NB1-j | 0.06 | 0.22 | 1.91 | Possible sulfur reduction |
| *60* | Genus *Halodesulfovibrio* | 0.05 | 0.2 | 1.77 | Known Sulfur Reduction |
| *89* | Genus Sediminispirochaeta | 0.05 | 0.2 | 1.75 | Known Sulfur Reduction |
| *90* | Class Phycisphaerales - AKAU3564 | 0.05 | 0.19 | 1.7 | - |
| *82* | Order Clostridiales - Family XII | 0.07 | 0.19 | 1.7 | - |
| *91* | Genus *Desulfobacter* | 0.05 | 0.17 | 1.54 | Known Sulfur Reduction |
| *92* | Family Spirochaetaceae | 0.04 | 0.17 | 1.5 | - |
| *93* | Genus *Halodesulfovibrio* | 0.05 | 0.17 | 1.47 | Known Sulfur Reduction |
| *94* | Class Clostridiales - Family XII | 0.04 | 0.17 | 1.47 | - |
| *49* | Class Bacteroidales | 0.04 | 0.16 | 1.42 | Sulfate Reduction |
| *95* | Order Ruminococcaceae | 0.04 | 0.16 | 1.39 | - |
| *96* | Class Bacteroidia | 0.04 | 0.14 | 1.27 | Anaerobic organosulfonate |
| *97* | Genus *Sediminispirochaeta* | 0.03 | 0.14 | 1.22 | Known Sulfur Reduction |
| *98* | Class Bacteroidales | 0.05 | 0.13 | 1.19 | Possible Sulfate Reduction |

**Table 7: Dissimilar taxa within the sponge sample before and after exposure using simper percent contribution.** The above was the results of a Simper test to determine major dissimilar ASVs within sponges before and after the exposure to $H_2S$. Overall, a dissimilarly of 89.66% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Dissimilar Taxa within before Sponges Sample before and After Exposure using Simper***

| *ASVs* | Taxonomy | After Average Abundance | Before Average Abundance | Average Dissimilarity | Percent Contribution | Sulfur Metabolism | P-value |
|---|---|---|---|---|---|---|---|
| *53* | Family Nitrosopumilaceae | 0.13 | 0.31 | 2.04 | 2.28 | - | 0.03 |
| *58* | Genus *Draconibacterium* | 0.29 | 0.01 | 2.02 | 2.25 | Possible Sulfate Reduction | 0.45 |
| *52* | Family Rhodobacteraceae - Unculutred | 0.18 | 0.35 | 1.57 | 1.76 | Possible Thiosulfate oxidation | 0.03 |
| *50* | Order Clostridiales - Family XII | 0.03 | 0.25 | 1.48 | 1.65 | - | 0.08 |
| *56* | Order Betaproteobacteriales - EC94 | 0.03 | 0.17 | 1.15 | 1.29 | Possible sulfur reduction | 0.02 |
| *55* | Class Deltaproteobacteria - NB1-j | 0.06 | 0.18 | 1.04 | 1.16 | Possible sulfur Reduction | 0.02 |
| *28* | Genus *Halodesulfovibrio* | 0.16 | 0.02 | 1.01 | 1.13 | Known sulfur Reduction | 0.22 |
| *63* | Order Phycisphaerales - AKAU3564 | 0.13 | 0.01 | 0.89 | 1 | - | 0.1 |

**Table 8: Similar taxa within water before exposure using simper.** The above was the Simper test results to determine major contributing ASVs to water samples before the exposure to $H_2S$. Overall, a similar of 6.14% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Similar Taxa within Water Before Exposure using Simper***

| *ASVs* | Taxonomy | Average Abundance | Average Similarity | Percent Contribution | Sulfur Metabolism |
|---|---|---|---|---|---|
| *40* | Genus *Phaeodactylibacter* | 0.14 | 0.89 | 14.42 | |
| *1* | Genus *Hyphomonas* | 0.09 | 0.54 | 8.79 | Possible Sulfur metabolism |
| *11* | Family Cryomorphaceae - Uncultured | 0.07 | 0.46 | 7.46 | Possible Thiosulfate oxidation |
| *72* | Genus *Mesoflavibacter* | 0.08 | 0.43 | 6.98 | Possible Sulfate Reduction |
| *75* | Genus *Francisella* | 0.05 | 0.31 | 5.1 | Possible Sulfur metabolism |
| *76* | Family Flavobacteriaceae - Uncultured | 0.06 | 0.27 | 4.42 | Possible Sulfate Reduction |
| *67* | Genus *Thalassobius* | 0.06 | 0.26 | 4.2 | Possible Thiosulfate oxidation |
| *45* | Genus *Minutocellus* | 0.05 | 0.25 | 4.02 | - |
| *77* | Genus *Pseudofulvibacter* | 0.11 | 0.24 | 3.98 | |
| *70* | Family Alteromonadaceae - Uncltured | 0.05 | 0.17 | 2.8 | |
| *10* | Class Ignavibacteria - OPB56 | 0.05 | 0.15 | 2.38 | |
| *21* | Genus *Arcobacter* | 0.06 | 0.14 | 2.34 | Possible Thiosulfate oxidation |
| *78* | Genus *Aquibacter* | 0.06 | 0.14 | 2.31 | |
| *79* | Genus *Aquibacter* | 0.05 | 0.13 | 2.07 | |

**Table 9: Similar taxa within the water after exposure using simper.** The above was the Simper test results to determine major contributing ASVs to water samples after the exposure to $H_2S$. Overall, similarly of 1.32% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Similar Taxa within Water After Exposure using Simper***

| *ASVs* | Taxonomy | Average Abundance | Average Similarity | Percent Contribution | Sulfur Metabolism |
|---|---|---|---|---|---|
| *71* | Genus *Francisella* | 0.07 | 0.71 | 53.43 | Possible Sulfur metabolism |
| *1* | Genus *Hyphomonas* | 0.09 | 0.35 | 26.63 | Possible Sulfur metabolism |

**Table 10: Dissimilar taxa within before sponges water before and after exposure using simper.** The above was the results of a Simper test to determine major dissimilar ASVs within water samples before and after the exposure to $H_2S$. Overall, a dissimilarly of 89.41% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Dissimilar Taxa within before Sponges Water before and After Exposure using Simper***

| *ASVs* | Taxonomy | After Average Abundance | Before Average Abundance | Average Dissimilarity | Percent Contribution | Sulfur Metabolism | P-value |
|---|---|---|---|---|---|---|---|
| *65* | Family Flavobacteriaceae | 0.19 | 0.32 | 2.02 | 2.25 | Possible Sulfate Reduction | 0.13 |
| *80* | Genus *Cylindrotheca* | 0.08 | 0.18 | 1.15 | 1.29 | | 0.06 |
| *68* | Class Oxyphotobacteria | 0.11 | 0.17 | 1.1 | 1.23 | | 0.12 |
| *81* | Genus *Marinifilum* | 0.01 | 0.21 | 1.08 | 1.21 | | 0.46 |
| *14* | Family Rhodobacteraceae | 0.16 | 0 | 1.02 | 1.14 | Possible sulfur oxidation | 0.72 |
| *31* | Family Rhodobacteraceae | 0.13 | 0.14 | 0.98 | 1.09 | Possible sulfur oxidation | - |
| *40* | Genus *Phaeodactylibacter* | 0.14 | 0 | 0.91 | 1.01 | | 0.16 |

**Table 11: Dissimilar taxa within sponges and water samples using simper.** The above was the results of a Simper test to determine major dissimilar ASVs within water and Sponge samples. Overall, a dissimilarly of 92.75% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Dissimilar Taxa within Sponges and Water Samples using Simper***

| *ASVs* | Taxonomy | Sponge Average Abundance | Water Average Abundance | Average Dissimilarity | Percent Contribution | Sulfur Metabolism |
|---|---|---|---|---|---|---|
| *53* | Family Nitrosopumilaceae | 0.22 | 0 | 1.61 | 1.73 | - |
| *52* | Family Rhodobacteraceae | 0.27 | 0.01 | 1.56 | 1.68 | Possible sulfur oxidation |
| *65* | Family Flavobacteriaceae | 0.05 | 0.24 | 1.44 | 1.55 | Possible Sulfate Reduction |
| *58* | Genus *Draconibacterium* | 0.15 | 0.05 | 0.94 | 1.01 | Possible sulfate reduction |

## IX. DISCUSSION:

### Five-Hour Drop Experiments:

The consumption rate of hydrogen sulfide ($H_2S$) within both sponge and the control (non-sponge) samples had a significant relationship with time (p-value<0.05). It should be noted that the sponge 3 and 5 had a dramatic drop in concentration (estimated 5000 seconds for Sponge 3 and 15000 seconds for Sponge 5). This extreme drop could result from a change in sponge pumping, directly correlated to the amount of water flow through the sponge (Massaro et al., 2012; Ludeman et al., 2014; Ludeman, Reidenbach & Leys, 2017). Water and other molecules move at a faster speed when near the sponge. Molecules farther from the oscula will move slowly, potentially taking hours to reach the sponge (**Fig. 25**). This change in current could create a dramatic decrease in $H_2S$, creating a pumping threshold. It was noted but not measured that the osculum would become larger throughout the experiment. Current research has suggested sponges have a sensory ability termed 'inflation-contraction response'. This response suggests that the sponge increase pumping to eliminate unwanted waste (Ludeman et al., 2014). No water flow was within the experiment, which could influence the rate of sulfur exposure and

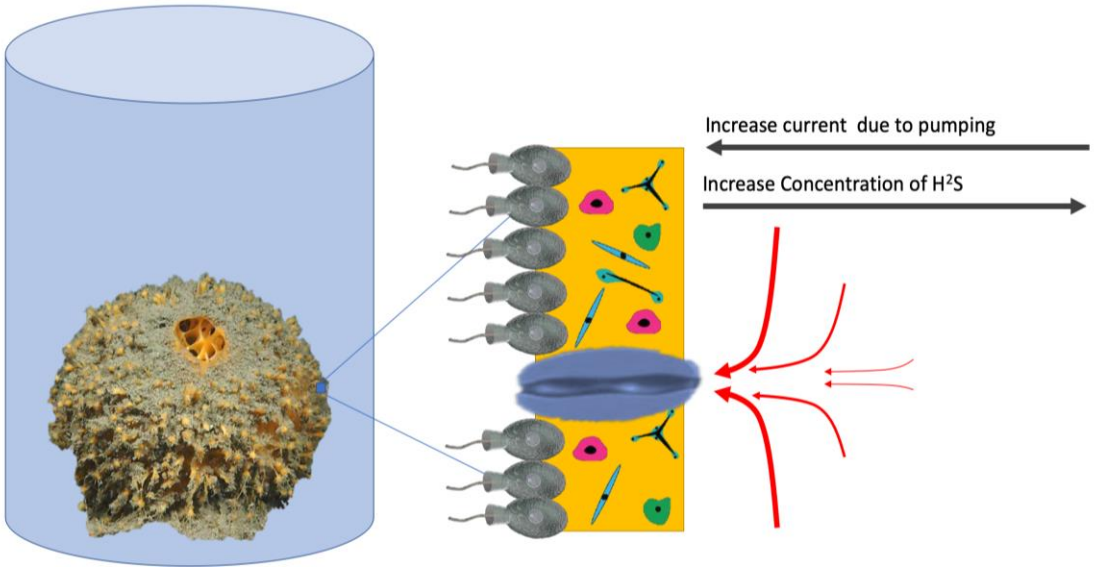


**Figure 27: Pumping action moving molecules through the sponge.** The pumping action created by the choanocytes (shown in gray) causes the water to enter the Ostia of the sponge. Water and other molecules close to the Ostia will be quickly taken into the system of channels. Molecules farther from the Ostia have a lower speed than those closer. The increasing speed is represented by the red arrows, which become thicker, the faster the molecules move. This increase in current, as the molecules are closer to the Ostia, causes an increase in the concentration of $H_2S$. This increases in concentration near the Ostia allow the sponge to filter $H_2S$ quickly, causing a dramatic drop

sulfur processing rate. No drop was seen in the measurements of Sponge 4. This individual could be a different species than Sponge 5 and 3. Different species have different pumping rates. For example, *Neogombata magnifica* has a specific filtration rate of 10.5 ml per min (Hadas, Ilan & Shpigel, 2008), while *Geodia barretti* has a specific filtration rate of 0.26 ml per min (Leys et al., 2018). Pumping rates should be determined for each individual, and molecular analysis should be determined.

GAM models indicated a significance based on the type of sample (control or sponge) **Fig. 13,14, 15, and 16**). The control sample indicates the natural diffusion of $H_2S$ into the atmosphere. The significance supports that the sponge does have an impact on the uptake of $H_2S$. An impact on the uptake of $H_2S$ suggests that sponges have an active role in the sulfur cycle.

There are several oscillations that the GAM does not explain. They may be due to the improper handling of the probe. If the lab bench was bumped or disturbed, the probe could have varying measurements. The probe is extremely sensitive. Thus, these varying measurements could be the movement of water and $H_2S$ molecules.

Additionally, the solutions were not mixed because it would cause increased oxidation. Hence, the solution may not have been homogeneous. The probes are extremely precise, down to the µmol. If there is a change, the probe will detect it. All experiments had noticeable increases of $H_2S$ within the sponge compared to the Control. This could be due to the hydrogen sulfide previously present in the tissues of the sponges. If the sponge is already producing $H_2S$, it could be transferred into the experiment. More experimentation should be performed with a non-tissue-based object to determine this. Overall, the rate is what was being compared, not the starting concentration. It should be noted that Control 4 has a dramatic decrease then increased between 2500 and 7500. Probes within the control were placed on the bottom of the beaker. This could have created the drop then increase seen in Control 4, as more freshwater would create a decrease.

**Vertical Distribution Experiments:**

Both Sponge 4 and Sponge 5 uptake of $H_2S$ concentration have a significant relationship with respect to depth (p-value>0.5). Sponge 4 showed more of a linear decrease, while Sponge 5 showed a plateau from depth 0 to about 400 µm followed by a linear decrease. Sponge 4 had its vertical distribution measure before the five-hour drop measurements were taken, while Sponge

5 had its vertical distribution take after the five-hour drop measurements. The solution was not mixed because it would cause increased oxidation. Therefore, the plateau seen in Sponge 5 was most-likely caused by the threshold of pumping action seen in the five-hour drop experiments. Recall the solutions were not mixed. Thus, the increased starting concentration of sponge 4 could be due to a non-homologous mixture or just having the sponge present in the treatment beakers.

**Five-Hour Uptake Experiments:**

All samples showed a significant relationship between $H_2S$ consumption and time ($p<0.05$). All functions were of the Gaussian Family and Identify link function with a formula of $H_2S$_measurment ~ s(Hour). The GLM shows a significant difference for the average sponge samples and average control samples ($p<0.05$), meaning they do not have the same uptake rate. This difference did have significant interaction between type (sponge or control) and hour. The boxplot demonstrates that the control and sponge values begin around the sample value; the control then consistently stays above the sponge values, indicating that the sponge has an increased uptake rate compared to the control.

**Microbiome Analyses to Characterize of Long-Term Exposure to Hydrogen Sulfide:**

The alpha and beta diversity metrics were determined for long-term exposure experiments. Alpha diversity appears, by studying the boxplot, to be separated into two groups (one containing Sponge: After, Microbial Mat, Algae and Sponge: Before, another containing Water: Before, Water: After) (**Fig. 21**). This trend was also seen for beta diversity (**Fig. 22**). A t-test did show light insignificance in alpha and beta diversity of sponge samples before compared to after at a 95% interval (p-value≈0.06). This difference was expected as a change in nutrients should, over time, change the bacterial composition, suggesting that the species composition and abundance changes in sponges before and after the exposure. The significance in alpha and beta diversity suggests that bacterial composition did change after exposure. However, relative abundance did not show a significant difference in any groups (p-value>0.05). It should be noted that the relative abundance of families was slightly insignificant (p-value≈0.06). The slight insignificance suggests that there are not large community differences, but there are differences seen on the microscale.

The NMDS plots did not show any specific trends when looking at all samples in sample type (**Fig. 23, part A**). No trend was seen when comparing all samples based on time (**Fig. 23, part C**). The NMDS show the water samples clustered more closely together than throughout sponge samples (**Fig. 23, part B**). This clustering was not significant but noticeable. *Cinachyrella* sponges and surrounding water have been found to have a significantly different microbiome (Cuvelier et al., 2014). Data compiled here may not have enough replicates to see a significant difference.

The NMDS did demonstrate samples taken from the same sponge are more similar than samples from the same type (sponge, microbial matt, algae, and water) or the same exposure (before and after) (**Fig. 23, part D)**. With this seen, ANOSIM was performed to determine if the samples taken from the same sponge influenced relative abundance. A significant relationship between sponge number and relative abundance was seen (p-value>0.05). This relationship could be due to a difference in species. Cuvelier et al. (2014) demonstrated that different species of *Cinachyrella* have distinct microbial communities. However, species were unable to be determined in this study.

A shaded plot was constructed and allowed the determination of enrichment of specific samples (**Figs. 24**, **25**, and **26**). Within microbial mat samples, only one group is knowns to undergo sulfur metabolism, Rhodobacteraceae (Pujalte et al., 2014a). This group is highly abundant in the microbial mat formed on Sponge 8. Rhodobacteraceae is considered one of the most diverse bacterial lineages in the marine habitat (Giovannoni & Rappé, 2000; Garrity et al., 2005; Pohlner et al., 2019). Rhodobacteraceae is found readily in the waters of Ft. Lauderdale (Campbell et al., 2015) and *Cinachyrella* (Cuvelier et al., 2014). This lineage undergoes sulfur metabolism, aerobic anoxygenic photosynthesis, carbon monoxide oxidation, and the use of organic or inorganic compounds (Pujalte et al., 2014a). The ASVs found in this study did not indicate a particular species or genus. All were listed as uncultured. Thus, it is debatable that these isolates engage in sulfur metabolism.

Samples from Sponge 1 showed elevated counts of ASVs in the order Rhodobacterales and the class Deltaproteobacteria. Both of these groups play active roles in the sulfur cycle (Garrity, 2005; Muyzer & Stams, 2008), with Deltaproteobacteria engages in sulfur reduction while Rhodobacterales engages in sulfur oxidation. These taxa's presence indicates that SRB and SOB in the sponge tissue perform a functional role in the sulfur cycle. However, after being enriched

with $H_2S$, these bacterial counts were depleted. Sponge 7 and Sponge 8 samples also showed ASVs in the order Rhodobacterales. After enrichment, this bacterial order was depleted, but Desulfvibrionacaea and Prolixibacteraceae (genus *Draconibacterium*), a well-known family of sulfur metabolites, was enriched. Order Rhodobacterales was seen in the water samples taken from the tank of Sponge 7 before exposure. The water from Sponge 8 after exposure showed enrichment of Rhodobacteraceae and Desulfvibrionacaea. This particular sponge appeared to disintegrate towards the end of the exposure. This desecration could have easily mixed spongy tissue into the water column, meaning transfer from sponge symbionts to the water column. It is also possible the disintegration of sponge tissue trapped water, meaning the transfer of microbes from the water to the sponge. It can be concluded that enrichment did occur. Because Sponge 1 also contains both taxa within sponge tissue, it is more likely the enrichment was initiated by the sponge, then transferred to the water column.

Simper files were constructed using PRIMER. A variety of sulfur metabolizing microbes, including genus *Desulfuromusa* (ASV 24), family Rhodobacteraceae (ASV 25, 27, 20,31, 33, 35, 16, 48, and 51), genus *Halodesulfovibrio* (ASV 28), and genus *Desulfovibrio* (ASV 43), was seen to contribute up to 22% of the microbial mat samples (**Appendix A, Table 13**). This high abundance of sulfur cycle engaging microbes suggests that the microbial mat was formed by SRB and SOB bacteria due to the addition of $H_2S$.

Before exposure, sponge samples had the highest abundance of microbes from Rhodobacteraceae (ASV 52), Gammaproteobacteria (ASV 54), Deltaproteobacteria (ASV 55), Nitrosopumilaceae (ASV 53), totaling more than 48% combined (**Table 5**). The major contributing taxa within sponge samples after exposure included *Desulfovibrio* (ASV 43), Halodesulfovibrio (ASV 28), and *Desulfobacter* (ASV 91) (**Table 6**). These taxa are known to be sulfate reducers (**Table 1**). These taxa showed a percent contribution of 6.49% together. Overall the number of sulfur metabolites was 18 out of the 24 top contributors. Taxa that contributed to the sponge's most different composition before exposure compared to after was Nitrosopumilaceae (ASV 53) at 2.28%. This particular family was seen to have a higher abundance before exposure. On the other hand, Genus *Draconibacterium* (ASV 58) was not in sponge samples before exposure but increased to 0.29 count after exposure (**Table 7**). *Draconibacterium* is a relatively new bacterial taxa, only proposed in 2014 (Du et al., 2014). NCBI taxonomy browser recognizes three species: *Draconibacterium filum*, *Draconibacterium*

*orientale*, and *Draconibacterium sediminis*. Kegg currently only recognizes *D. orienta* as a sulfate reducer.

The highest abundance of taxa in tank water before exposure stemmed from *Phaeodactylibacter* (ASV 40), *Hyphomonas* (ASV 1), *Cryomorphaceae* (ASV 11), *Mesoflavibacter* (ASV 72), and *Francisella* (ASV 75), totaling 42% (**Table 8**). After exposure, tank water increased in *Francisella* (ASV 71) and *Hyphomonas* (ASV 1) to 80% contribution (**Table 9**). *Francisella* is of order Thiotrichales. Individuals of this genus strictly aerobic and contain the species *Francisella tularensis*, which causes tularemia in animals and humans (Slack, 2010). It is not unusual for this group to be isolated from the marine habitat (Petersen et al., 2009). No sulfur metabolism was found in the literature for *Francisella* (ASV 71). *Hyphomonas* is a genus within the order Rhodobacterles. This group is mainly found in the seawater (Lee et al., 2005) and is known to undergo sulfur oxidation (Moore, Weiner & Gebers, 1984). Flavobacteriaceae (ASV 65) and Cylindrotheca (ASV 80) drove key differences between tank water before and after exposure. Both taxa have a higher abundance before exposure compared to after exposure. It should be noted that Rhodobacteraceae (ASV 14) and Phaeodactylibacter (ASV 40) increased from a zero abundance before exposure to 0.15 relative abundance after exposure (**Table 10**). It is not abnormal to see an increase in Rhodobacteraceae because it is known to have members undergo sulfur oxidation (Pujalte et al., 2014b). No sulfur metabolism was identified for Phaeodactylibacter. The influential taxa contributing to the differences in all sponge and water samples were Nitrosopumilaceae (ASV 53) and Rhodobacteraceae (ASV 52) (**Table 11**). Neither group was present in water, but rather in sponge samples.

It should be noted that sulfur metabolism was inferred through both microbial profiles and a literature search. I would have applied functional analysis, such as using PICRUSt2 analyses of KEGG pathways, but I ran out of time. As in Vijayan (2015) Acidobacteria, Cellvibrionaceae, Colwelliaceae, Rhodobacteraceae, and Gammaproteobacteria were documented in the host species. A small abundance of Chromatiales, purple sulfur bacteria, and family Chlorobiaceae, green sulfur bacteria, was seen. Dominant microbial phyla associated with marine sponges are Proteobacteria (especially the classes Alpha-, Gamma- and Deltaproteobacteria), Chloroflexi, Actinobacteria, Acidobacteria, Nitrospirae, and the candidate phylum Poribacteria (Hentschel et al., 2012). Various Proteobacteria, Chloroflexi, actinobacteria, Acidobacteria, and Nitrospirae,

were observed in the data. However, Poribacteria was curiously not seen even though Cuvelier et al. (2014) determined that this was an abundant phylum in genus *Cinachyrella*.

## X. CONCLUSION:

Five-hour drop and five-hour uptake experiments showed a significant relationship between time and $H_2S$ consumption, while vertical distribution showed a significant relationship between depth and $H_2S$ consumption. A GAM was the best model for all experiments. These experiments show over time and depth that $H_2S$ is consumed readily in a sponge environment. In each of these instances, the sponge always increased consumption compared to the control, representing the natural diffusion rate. When a GLM and GAM compared the natural diffusion rate to the uptake rate caused by a sponge, there was a significant difference; meaning the $H_2S$ consumption rate was significantly affected when a marine sponge was introduced. All of these support Hypotheses 1, 2, 3, and 4.

Long-Term exposures did not show a significant difference in relative abundance on a community scale, not supporting hypothesis 6. There was a significant difference in beta and alpha diversity. Sponge samples were seen to host SRB and SOB before exposure supporting hypothesis 5 and was seen to be enriched when introducing $H_2S$ supporting hypothesis 8. Using 16S rRNA data, the microbial mat appeared to host SRB and SOB bacterial taxa, specifically genus *Desulfuromusa,* family Rhodobacteraceae, genus *Halodesulfovibrio,* and genus *Desulfovibrio,* supporting hypothesis 7. This abounding data indicates that SRB and SOB within *Cinachyrella* spp. play a functional role in the sulfur cycle.

Sponges evolved in prevalent sulfur oceans (Balter, 2015; Fike, Bradley & Rose, 2015). A high amount of sulfide is extremely toxic to many animals. By partnering with an organism that can remove toxins from an environment, individuals can continue to live. This relationship may have begun this way, a way for both parties to survive, the microbe getting housing and protection, while the sponge was getting toxins removed from its tissues. The inflation-contraction response seen was the sponge's attempt to remove the toxin faster. Over time the ocean became less sulfur concentrated, possibly influencing the sulfur metabolites by shrink in number but not disappearing. The sponge still needed to remove harmful sulfur toxins but did not necessarily need a high abundance, leading to a lower abundance of sulfur metabolites than other

metabolites. Studies on inverts, such as oligochaete worms (Dubilier et al., 2001), have similar SOB and SRB relationships. The host receives carbohydrates, while the microbes receive protection, housing, and nutrients. Thus, it is thought that sponges also receive a carbohydrate benefit (Tian et al., 2016). This benefit could be one reason this relation continues to survive in a less sulfur-concentrated ocean.

Sulfate-reducing bacteria use sulfate as the electron acceptor producing sulfide. Sulfide-oxidizing bacteria utilize sulfide to produce biological sulfur and sulfate. This study saw taxa such as *Desulfobacter* producing $H_2S$ and *Ruegeria* removing $H_2S$. Thus, SRB may produce sulfide for SOB, which produces sulfate for SOB and continue in a cycle, utilizing the same sulfur molecules. Thus, isotopic tracing of sulfur should be conducted to determine the converted molecules produced, helping determine what carbohydrates are being produced. A more functional-based study should be done to determine what genes and pathways produce the carbohydrates or other molecules produced. Targeted sequencing of sulfur metabolite microbes should be completed to get a complete look at the sulfur cycle of sponges. To date, only one study has identified sulfur metabolite genes in genus *Cinachyrella*. Shmakova (2020) identified characterize five sulfur related metagenomically assembled genomes (MAGs) (Shmakova 2020): *Opitutaceae bacterium, Thioalkalivibrio paradoxus, Desulfobacterium autotrophicum, Thioalkalivibrio sulfidiphilus, Sulfurifustis variabilis*. Also identified were 27 MAGS related to sulfide reducing genes (Shmakova, 2020). Within *Lophophysema eversa*, genomic features of sulfite-oxidizing genes were found (Tian et al., 2016)

I believe it is essential to understand if these are true symbionts of the sponge. To determine that, we need to determine if the sponge can continue to live without these symbionts. The inflation-contraction response and uptake of $H_2S$ caused by microbes may not be connected. If the sponge can live without the symbionts, there would be evidence to suggest the adaptation was occurring by microbes, not sponges, suggesting a more commensal relationship.

## XI. REFERENCES:


Altamia M. A., Shipway J. R., Concepcion G. P., Haygood M. G., and Distel D. L. 2019. Thiosocius Teredinicola Gen. Nov., Sp. Nov., a Sulfur-Oxidizing Chemolithoautotrophic Endosymbiont Cultivated from the Gills of the Giant Shipworm, Kuphus Polythalamius. *International journal of systematic and evolutionary microbiology* 69:638-644

Aragno. 1992. The Aerobic, Chemolithoautotrophic, Thermophilic Bacteria. In: Kristjansson J, and Stetter K, eds. *Thermophilic Bacteria*. Boca Raton, FL, USA: CRC Press, 77-104.

Balter M. 2015. Oldest Known Sponge Pushes Back Date for Key Split in Animal Evolution. *Science* 14: 10.1126/science.aab0292

Barton L. L., Fardeau M.-L., and Fauque G. D. 2014. Hydrogen Sulfide: A Toxic Gas Produced by Dissimilatory Sulfate and Sulfur Reduction and Consumed by Microbial Oxidation. *The Metal-Driven Biogeochemistry of Gaseous Compounds in the Environment*. Springer, 237-277.

Bayer K., Schmitt S., and Hentschel U. 2008. Physiology, Phylogeny and in Situ Evidence for Bacterial and Archaeal Nitrifiers in the Marine Sponge Aplysina Aerophoba. *Environmental microbiology* 10:2942-2955

Bergquist P. 2004. The Porifera. In: Ruppert EE, ed. *Invertebrate Zoology : A Functional Evolutionary Approach*. Belmont: Thomson Brooks/Cole,

Björk J. R., Díez-Vives C., Coma R., Ribes M., and Montoya J. M. 2013. Specificity and Temporal Dynamics of Complex Bacteria–Sponge Symbiotic Interactions. *Ecology* 94:2781-2791

Bright M., and Giere O. 2005. Review Article Microbial Symbiosis in Annelida. *Symbiosis*

Brune D. C. 1989. Sulfur Oxidation by Phototrophic Bacteria. *Biochimica et Biophysica Acta (BBA)-Bioenergetics* 975:189-221

Campbell A. M., Fleisher J., Sinigalliano C., White J. R., and Lopez J. V. 2015. Dynamics of Marine Bacterial Community Diversity of the Coastal Waters of the Reefs, Inlets, and Wastewater Outfalls of Southeast Florida. *MicrobiologyOpen* 4:390-408

Caporaso J. G., Kuczynski J., Stombaugh J., Bittinger K., Bushman F. D., Costello E. K., Fierer N., Pena A. G., Goodrich J. K., and Gordon J. I. 2010. Qiime Allows Analysis of High-Throughput Community Sequencing Data. *Nature methods* 7:335

Cardenas P., Menegola C., Rapp H. T., and Díaz M. C. 2009. Morphological Description and DNA Barcodes of Shallow-Water Tetractinellida (Porifera: Demospongiae) from Bocas Del Toro, Panama, with Description of a New Species. *Zootaxa* 2276:1-39

Cohen Y., Padan E., and Shilo M. 1975. Facultative Anoxygenic Photosynthesis in the Cyanobacterium Oscillatoria Limnetica. *Journal of Bacteriology* 123:855-861

Conway C. A., Esiobu N., and Lopez J. V. 2012. Co-Cultures of Pseudomonas Aeruginosa and Roseobacter Denitrificans Reveal Shifts in Gene Expression Levels Compared to Solo Cultures. *The Scientific World Journal* 2012:

Cuvelier M. L., Blake E., Mulheron R., Mccarthy P. J., Blackwelder P., Thurber R. L. V., and Lopez J. V. 2014. Two Distinct Microbial Communities Revealed in the Sponge Cinachyrella. *Frontiers in microbiology* 5:581

De Goeij J. M., Van Oevelen D., Vermeij M. J., Osinga R., Middelburg J. J., De Goeij A. F., and Admiraal W. 2013. Surviving in a Marine Desert: The Sponge Loop Retains Resources within Coral Reefs. *Science* 342:108-110

Desplat Y. 2020. Linking Crude Oil and Dispersant Effects to Gene Expression of Marine Sponge Cinachyrella D.Lopez. Masters of Sciences. Thesis, NOVA Southeastern University

Du Z.-J., Wang Y., Dunlap C., Rooney A. P., and Chen G.-J. 2014. Draconibacterium Orientale Gen. Nov., Sp. Nov., Isolated from Two Distinct Marine Environments, and Proposal of Draconibacteriaceae Fam. Nov. *International journal of systematic and evolutionary microbiology* 64:1690-1696

Dubilier N., Bergin C., and Lott C. 2008. Symbiotic Diversity in Marine Animals: The Art of Harnessing Chemosynthesis. *Nature Reviews Microbiology* 6:725-740

Dubilier N., Mülders C., Ferdelman T., De Beer D., Wendeberg A., Klein M., Wagner M., Erséus C., Thiermann F., Krieger J., Giere O., and Amann R. 2001. Endosymbiotic Sulphate-Reducing and Sulphide-Oxidizing Bacteria in an Oligochaete Worm. *Nature* 411:298-302 10.1038/35077067

Dufour S. C. 2005. Gill Anatomy and the Evolution of Symbiosis in the Bivalve Family Thyasiridae. *The Biological Bulletin* 208:200-212

Easson C. G., and Lopez J. V. 2019. Depth-Dependent Environmental Drivers of Microbial Plankton Community Structure in the Northern Gulf of Mexico. *Frontiers in microbiology* 9:3175

Eimhjellen K. 1967. Photosynthetic Bacteria and Carotenoids from a Sea Sponge Halichondrium Panicea. *Acta Chemica Scandinavica* 21:2280

Erwin P. M., Pita L., López-Legentil S., and Turon X. 2012. Stability of Sponge-Associated Bacteria over Large Seasonal Shifts in Temperature and Irradiance. *Applied and Environmental Microbiology* 78:7358-7368

Felbeck H. 1981. Chemoautotrophic Potential of the Hydrothermal Vent Tube Worm, Riftia Pachyptila Jones (Vestimentifera). *Science* 213:336-338

Fike D. A., Bradley A. S., and Leavitt W. D. 2016. Geomicrobiology of Sulfur. *Ehrlich's Geomicrobiology* Boca Raton, FL: Taylor & Francis, 479-515.

Fike D. A., Bradley A. S., and Rose C. V. 2015. Rethinking the Ancient Sulfur Cycle. *Annual Review of Earth and Planetary Sciences* 43:593-622

Fiore C. L., Labrie M., Jarett J. K., and Lesser M. P. 2015. Transcriptional Activity of the Giant Barrel Sponge, Xestospongia Muta Holobiont: Molecular Evidence for Metabolic Interchange. *Frontiers in microbiology* 6:364

Friedrich C., and Mitrenga G. 1981. Oxidation of Thiosulfate by Paracoccus Denitrificans and Other Hydrogen Bacteria. *FEMS Microbiology Letters* 10:209-212

Friedrich C. G., Bardischewsky F., Rother D., Quentmeier A., and Fischer J. 2005. Prokaryotic Sulfur Oxidation. *Current opinion in microbiology* 8:253-259

Friedrich C. G., Rother D., Bardischewsky F., Quentmeier A., and Fischer J. 2001. Oxidation of Reduced Inorganic Sulfur Compounds by Bacteria: Emergence of a Common Mechanism? *Applied and Environmental Microbiology* 67:2873-2882

Garrity G. M. 2005. *Bergey's Manual of Systematic Bacteriology*. Springer.

Garrity G. M., Brenner D. J., Krieg N. R., and Staley J. T. 2005. *The Proeobacteria: Part C the Alpha-, Beta-, Delta-, and Epsilonproteobacteria*. Springer.

Gauthier M.-E. A., Watson J. R., and Degnan S. M. 2016. Draft Genomes Shed Light on the Dual Bacterial Symbiosis That Dominates the Microbiome of the Coral Reef Sponge Amphimedon Queenslandica. *Frontiers in Marine Science* 3:196

Giovannoni S., and Rappé M. 2000. Evolution, Diversity, and Molecular Ecology of Marine Prokaryotes. In: Kirchman, ed. *Microbial Ecology of the Oceans*. Wiley,

Hadas E., Ilan M., and Shpigel M. 2008. Oxygen Consumption by a Coral Reef Sponge. *Journal of Experimental Biology* 211:2185-2190

Han S., Narasingarao P., Obraztsova A., Gieskes J., Hartmann A. C., Tebo B. M., Allen E. E., and Deheyn D. D. 2010. Mercury Speciation in Marine Sediments under Sulfate-Limited Conditions. *Environmental Science & Technology* 44:3752-3757

Hardoim C. C. P., Esteves A. I. S., Pires F. R., Gonçalves J. M. S., Cox C. J., Xavier J. R., and Costa R. 2012. Phylogenetically and Spatially Close Marine Sponges Harbour Divergent Bacterial Communities. *PloS one* 7:e53029 10.1371/journal.pone.0053029

He L., Liu F., Karuppiah V., Ren Y., and Li Z. 2014. Comparisons of the Fungal and Protistan Communities among Different Marine Sponge Holobionts by Pyrosequencing. *Microbial Ecology* 67:951-961 10.1007/s00248-014-0393-6

Heidelberg J. F., Seshadri R., Haveman S. A., Hemme C. L., Paulsen I. T., Kolonay J. F., Eisen J. A., Ward N., Methe B., and Brinkac L. M. 2004. The Genome Sequence of the Anaerobic, Sulfate-Reducing Bacterium Desulfovibrio Vulgaris Hildenborough. *Nature biotechnology* 22:554-559

Hentschel U., Fieseler L., Wehrl M., Gernert C., Steinert M., Hacker J., and Horn M. 2003. Microbial Diversity of Marine Sponges. *Sponges (Porifera)*. Springer, 59-88.

Hentschel U., Hopke J., Horn M., Friedrich A. B., Wagner M., Hacker J., and Moore B. S. 2002. Molecular Evidence for a Uniform Microbial Community in Sponges from Different Oceans. *Applied and Environmental Microbiology* 68:4431-4440

Hentschel U., Piel J., Degnan S. M., and Taylor M. W. 2012. Genomic Insights into the Marine Sponge Microbiome. *Nature Reviews Microbiology* 10:641-654

Hoffmann F., Larsen O., Thiel V., Rapp H. T., Pape T., Michaelis W., and Reitner J. 2005. An Anaerobic World in Sponges. *Geomicrobiology Journal* 22:1-10

Hoffmann F., Radax R., Woebken D., Holtappels M., Lavik G., Rapp H. T., Schläppy M. L., Schleper C., and Kuypers M. M. 2009. Complex Nitrogen Cycling in the Sponge Geodia Barretti. *Environmental microbiology* 11:2228-2243

Hoffmann F., Rapp H. T., and Reitner J. 2006. Monitoring Microbial Community Composition by Fluorescence in Situ Hybridization During Cultivation of the Marine Cold-Water Sponge Geodia Barretti. *Marine Biotechnology* 8:373-379

Hoffmann M., Fischer M., Ottesen A., Mccarthy P. J., Lopez J. V., Brown E. W., and Monday S. R. 2010. Population Dynamics of Vibrio Spp. Associated with Marine Sponge Microcosms. *The ISME Journal* 4:1608-1612

Hoffmann M., Monday S. R., Allard M. W., Strain E. A., Whittaker P., Naum M., Mccarthy P. J., Lopez J. V., Fischer M., and Brown E. W. 2012. Vibrio Caribbeanicus Sp. Nov., Isolated from the Marine Sponge Scleritoderma Cyanea. *International journal of systematic and evolutionary microbiology* 62:1736-1743

Holmes B., and Blanch H. 2007. Genus-Specific Associations of Marine Sponges with Group I Crenarchaeotes. *Marine Biology* 150:759-772 10.1007/s00227-006-0361-x

Huber G., Drobner E., Huber H., and Stetter K. O. 1992. Growth by Aerobic Oxidation of Molecular Hydrogen in Archaea—a Metabolic Property So Far Unknown for This Domain. *Systematic and Applied Microbiology* 15:502-504

Huber R., and Eder W. 2006. Aquificales. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K, and Stackebrandt E, eds. *The Prokaryotes*. New York: Springer,

Imhoff J. F., Süling J., and Petri R. 1998. Phylogenetic Relationships among the Chromatiaceae, Their Taxonomic Reclassification and Description of the New Genera Allochromatium, Halochromatium, Isochromatium, Marichromatium, Thiococcus, Thiohalocapsa and Thermochromatium. *International journal of systematic and evolutionary microbiology* 48:1129-1143

Janda J. M., and Abbott S. L. 2007. 16s Rrna Gene Sequencing for Bacterial Identification in the Diagnostic Laboratory: Pluses, Perils, and Pitfalls. *Journal of clinical microbiology* 45:2761-2764

Jensen S., Fortunato S. A., Hoffmann F., Hoem S., Rapp H. T., Øvreås L., and Torsvik V. L. 2017. The Relative Abundance and Transcriptional Activity of Marine Sponge-Associated Microorganisms Emphasizing Groups Involved in Sulfur Cycle. *Microbial Ecology* 73:668-676

Kelly D. P., Mcdonald I. R., and Wood A. P. 2000. Proposal for the Reclassification of Thiobacillus Novellus as Starkeya Novella Gen. Nov., Comb. Nov., in the Alpha-Subclass of the Proteobacteria. *International journal of systematic and evolutionary microbiology* 50:1797-1802

Kelly D. P., and Smith N. A. 1990. Organic Sulfur Compounds in the Environment Biogeochemistry, Microbiology, and Ecological Aspects. *Advances in Microbial Ecology*. Springer, 345-385.

Kelly D. P., and Wood A. P. 2000. Reclassification of Some Species of Thiobacillus to the Newly Designated Genera Acidithiobacillus Gen. Nov., Halothiobacillus Gen. Nov. And Thermithiobacillus Gen. Nov. *International journal of systematic and evolutionary microbiology* 50:511-516

Kennedy J., Flemer B., Jackson S. A., Morrissey J. P., O'gara F., and Dobson A. D. W. 2014. Evidence of a Putative Deep Sea Specific Microbiome in Marine Sponges. *PloS one* 9:e91092 10.1371/journal.pone.0091092

Kim M.-K., and Zoh K.-D. 2012. Fate and Transport of Mercury in Environmental Media and Human Exposure. *Journal of Preventive Medicine and Public Health* 45:335

King G. M. 2018. Microbiomes of the Enteropneust, Saccoglossus Bromophenolosus, and Associated Marine Intertidal Sediments of Cod Cove, Maine. *Frontiers in microbiology* 9:3066

Klenk H.-P., Clayton R. A., Tomb J.-F., White O., Nelson K. E., Ketchum K. A., Dodson R. J., Gwinn M., Hickey E. K., and Peterson J. D. 1998. Erratum: The Complete Genome Sequence of the Hyperthermophilic, Sulphate-Reducing Archaeon Archaeoglobus Fulgidus. *Nature* 394:101-101

Kletzin A. 2007. Metabolism of Inorganic Sulfur Compounds in Archaea. *Archaea: evolution, physiology, and molecular biology*261-274

Kletzin A. 2008. Oxidation of Sulfur and Inorganic Sulfur Compounds in Acidianus Ambivalens. *Microbial Sulfur Metabolism*. Springer, 184-201.

Knight R., Jansson J., Field D., Fierer N., Desai N., Fuhrman J. A., Hugenholtz P., Van Der Lelie D., Meyer F., and Stevens R. 2012. Unlocking the Potential of Metagenomics through Replicated Experimental Design. *Nature biotechnology* 30:513

Lavy A., Keren R., Yu K., Thomas B. C., Alvarez-Cohen L., Banfield J. F., and Ilan M. 2018. A Novel Chromatiales Bacterium Is a Potential Sulfide Oxidizer in Multiple Orders of Marine Sponges. *Environmental microbiology* 20:800-814

Lee K.-B., Liu C.-T., Anzai Y., Kim H., Aono T., and Oyaizu H. 2005. The Hierarchical System of the 'Alphaproteobacteria': Description of Hyphomonadaceae Fam. Nov., Xanthobacteraceae Fam. Nov. And Erythrobacteraceae Fam. Nov. *International journal of systematic and evolutionary microbiology* 55:1907-1919

Lee O. O., Wang Y., Yang J., Lafi F. F., Al-Suwailem A., and Qian P.-Y. 2011. Pyrosequencing Reveals Highly Diverse and Species-Specific Microbial Communities in Sponges from the Red Sea. *The ISME Journal* 5:650-664 10.1038/ismej.2010.165

Lenk S., Moraru C., Hahnke S., Arnds J., Richter M., Kube M., Reinhardt R., Brinkhoff T., Harder J., and Amann R. 2012. Roseobacter Clade Bacteria Are Abundant in Coastal Sediments and Encode a Novel Combination of Sulfur Oxidation Genes. *The ISME Journal* 6:2178-2187

Leys S., Kahn A., Fang J., Kutti T., and Bannister R. 2018. Phagocytosis of Microbial Symbionts Balances the Carbon and Nitrogen Budget for the Deep-Water Boreal Sponge Geodia Barretti. *Limnology and Oceanography* 63:187-202

Li Z., Wang Y., Li J., Liu F., He L., He Y., and Wang S. 2016. Metagenomic Analysis of Genes Encoding Nutrient Cycling Pathways in the Microbiota of Deep-Sea and Shallow-Water Sponges. *Marine Biotechnology* 18:659-671 10.1007/s10126-016-9725-5

Lopez J. V. 2019. After the Taxonomic Identification Phase: Addressing the Functions of Symbiotic Communities within Marine Invertebrates. *Symbiotic Microbiomes of Coral Reefs Sponges and Corals*. Springer, 105-144.

Lopez J. V., Peterson C. L., Willoughby R., Wright A. E., Enright E., Zoladz S., Reed J. K., and Pomponi S. A. 2002. Characterization of Genetic Markers for in Vitro Cell Line Identification of the Marine Sponge Axinella Corrugata. *Journal of Heredity* 93:27-36

Lopez J. V., Ranzer L., Ledger A., Schoch B., Duckworth A., Mccarthy P., and Kerr R. 2008. Comparison of Bacterial Diversity within the Coral Reef Sponge, Axinella Corrugata, and the Encrusting Coral Erythropodium Caribaeorum. Proceedings of the 11th International Coral Reef Symposium, Ft Lauderdale, Florida. p 1362.

Ludeman D. A., Farrar N., Riesgo A., Paps J., and Leys S. P. 2014. Evolutionary Origins of Sensation in Metazoans: Functional Evidence for a New Sensory Organ in Sponges. *BMC Evolutionary Biology* 14:3 10.1186/1471-2148-14-3

Ludeman D. A., Reidenbach M. A., and Leys S. P. 2017. The Energetic Cost of Filtration by Demosponges and Their Behavioural Response to Ambient Currents. *The Journal of Experimental Biology* 220:995 10.1242/jeb.146076

Lurgi M., Thomas T., Wemheuer B., Webster N. S., and Montoya J. M. 2019. Modularity and Predicted Functions of the Global Sponge-Microbiome Network. *Nature communications* 10:1-12

Mardis E. R. 2008. The Impact of Next-Generation Sequencing Technology on Genetics. *Trends in genetics* 24:133-141

Mardis E. R. 2011. A Decade's Perspective on DNA Sequencing Technology. *Nature* 470:198-203

Massaro A. J., Weisz J. B., Hill M. S., and Webster N. S. 2012. Behavioral and Morphological Changes Caused by Thermal Stress in the Great Barrier Reef Sponge Rhopaloeides Odorabile. *Journal of Experimental Marine Biology and Ecology* 416-417:55-60 https://doi.org/10.1016/j.jembe.2012.02.008

Mcfall-Ngai M. 2014. Divining the Essence of Symbiosis: Insights from the Squid-Vibrio Model. *PloS one* 12:e1001783 10.1371/journal.pbio.1001783

Meyer B., and Kuever J. 2008. Phylogenetic Diversity and Spatial Distribution of the Microbial Community Associated with the Caribbean Deep-Water Sponge Polymastia Cf. Corticata by 16s Rrna, Apra, and Amoa Gene Analysis. *Microbial Ecology* 56:306-321

Mohamed N. M., Rao V., Hamann M. T., Kelly M., and Hill R. T. 2008. Monitoring Bacterial Diversity of the Marine Sponge Ircinia Strobilina Upon Transfer into Aquaculture. *Applied and Environmental Microbiology* 74:4133-4143

Mohamed N. M., Saito K., Tal Y., and Hill R. T. 2010. Diversity of Aerobic and Anaerobic Ammonia-Oxidizing Bacteria in Marine Sponges. *The ISME Journal* 4:38-48

Moore R., Weiner R., and Gebers R. 1984. Genus Hyphomonas Pongratz 1957 Nom. Rev. Emend., Hyphomonas Polymorpha Pongratz 1957 Nom. Rev. Emend., and Hyphomonas Neptunium (Leifson 1964) Comb. Nov. Emend.(Hyphomicrobium Neptunium). *International journal of systematic and evolutionary microbiology* 34:71-73

Morrow C., and Cárdenas P. 2015. Proposal for a Revised Classification of the Demospongiae (Porifera). *Frontiers in Zoology* 12:7 10.1186/s12983-015-0099-8

Müller W. E. 2003. The Origin of Metazoan Complexity: Porifera as Integrated Animals. *Integrative and Comparative Biology* 43:3-10

Muyzer G., Sorokin D. Y., Mavromatis K., Lapidus A., Clum A., Ivanova N., Pati A., D'haeseleer P., Woyke T., and Kyrpides N. C. 2011. Complete Genome Sequence of "Thioalkalivibrio Sulfidophilus" Hl-Ebgr7. *Standards in genomic sciences* 4:23-35

Muyzer G., and Stams A. J. 2008. The Ecology and Biotechnology of Sulphate-Reducing Bacteria. *Nature Reviews Microbiology* 6:441-454

Nguyen M. T., Liu M., and Thomas T. 2014. Ankyrin-Repeat Proteins from Sponge Symbionts Modulate Amoebal Phagocytosis. *Molecular ecology* 23:1635-1645

Orem W. H. 2007. Sulfur Contamination in the Florida Everglades: Initial Examination of Mitigation Strategies. Open-File Report. p 1374.

Orphan V., Hinrichs K.-U., Ussler W., Paull C. K., Taylor L., Sylva S. P., Hayes J. M., and Delong E. 2001. Comparative Analysis of Methane-Oxidizing Archaea and Sulfate-Reducing Bacteria in Anoxic Marine Sediments. *Applied and Environmental Microbiology* 67:1922-1934

Pape T., Hoffmann F., Quéric N.-V., Von Juterzenka K., Reitner J., and Michaelis W. 2006. Dense Populations of Archaea Associated with the Demosponge Tentorium Semisuberites Schmidt, 1870 from Arctic Deep-Waters. *Polar Biology* 29:662-667 10.1007/s00300-005-0103-4

Pawlik J. R., Loh T.-L., Mcmurray S. E., and Finelli C. M. 2013. Sponge Communities on Caribbean Coral Reefs Are Structured by Factors That Are Top-Down, Not Bottom-Up. *PloS one* 8:

Petersen J., Carlson J., Yockey B., Pillai S., Kuske C., Garbalena G., Pottumarthy S., and Chalcraft L. 2009. Direct Isolation of Francisella Spp. From Environmental Samples. *Letters in applied microbiology* 48:663-667

Peterson B. J., Chester C. M., Jochem F. J., and Fourqurean J. W. 2006. Potential Role of Sponge Communities in Controlling Phytoplankton Blooms in Florida Bay. *Marine Ecology Progress Series* 328:93-103

Pile A., Patterson M., and Witman J. 1997. In Situ Grazing on Plankton< 10 Mm by the Boreal Sponge Mycale Lingua. *Oceanographic Literature Review* 4:340

Pisani D., Pett W., Dohrmann M., Feuda R., Rota-Stabelli O., Philippe H., Lartillot N., and Wörheide G. 2015. Genomic Data Do Not Support Comb Jellies as the Sister Group to All Other Animals. *Proceedings of the National Academy of Sciences* 112:15402-15407

Plugge C. M., Zhang W., Scholten J., and Stams A. J. 2011. Metabolic Flexibility of Sulfate-Reducing Bacteria. *Frontiers in microbiology* 2:81

Pohlner M., Dlugosch L., Wemheuer B., Mills H., Engelen B., and Reese B. K. 2019. The Majority of Active Rhodobacteraceae in Marine Sediments Belong to Uncultured Genera: A Molecular Approach to Link Their Distribution to Environmental Conditions. *Frontiers in microbiology* 10:659

Pujalte M., Lucena T., Ruvira M., Arahal D., and Macián M. 2014a. Alphaproteobacteria and Betaproteobacteria. In: Rosenberg E, Delong EF, Lory S, Stackebrandt, and E. T, F. , eds. *The Prokaryotes*. Springer-Verlag Berlin Heidelberg, 1013.

Pujalte M., Lucena T., Ruvira M., Arahal D., and Macián M. 2014b. The Family Rhodobacteraceae. . In: Rosenberg E, Delong EF, Lory S, Stackebrandt E, and Thompson FL, eds. *The Prokaryotes: Alphaproteobacteria and Betaproteobacteriarosenberg E, Editor*. Berlin, Heidelberg: Springer, 439-512.

Reiswig H. 1971. In Situ Pumping Activities of Tropical Demospongiae. *Marine Biology* 9:38-50

Rützler K., and Smith K. P. 1992. Guide to Western Atlantic Species of Cinachyrella (Porifera: Tetillidae). *Proceedings of the Biological Society of Washington*

Santavy D. L., and Colwell R. R. 1990. Comparison of Bacterial Communities Associated with the Caribbean Sclerosponge Ceratoporella Nicholsoni and Ambient Seawater. *Marine Ecology Progress Series* 67:73-82

Schippers K. J. 2013. Sponge Cell Culture. *Molecular Approaches to the Study of the Ocean*. Dordrecht: Springer,

Schmitt S., Tsai P., Bell J., Fromont J., Ilan M., Lindquist N., Perez T., Rodrigo A., Schupp P. J., and Vacelet J. 2012. Assessing the Complex Sponge Microbiota: Core, Variable and Species-Specific Bacterial Communities in Marine Sponges. *The ISME Journal* 6:564-576

Schönheit P., and Schäfer T. 1995. Metabolism of Hyperthermophiles. *World Journal of Microbiology and Biotechnology* 11:26-57

Seah B. K., Antony C. P., Huettel B., Zarzycki J., Von Borzyskowski L. S., Erb T. J., Kouris A., Kleiner M., Liebeke M., and Dubilier N. 2019. Sulfur-Oxidizing Symbionts without Canonical Genes for Autotrophic Co2 Fixation. *mBio* 10:e01112-01119

Seckbach J. 2006. *Symbiosis: Mechanisms and Model Systems*. Springer Science & Business Media.

Sfanos K., Harmody D., Dang P., Ledger A., Pomponi S., Mccarthy P., and Lopez J. 2005. A Molecular Systematic Survey of Cultured Microbial Associates of Deep-Water Marine Invertebrates. *Systematic and Applied Microbiology* 28:242-264

Sharma V. K., Johnson N., Cizmas L., Mcdonald T. J., and Kim H. 2016. A Review of the Influence of Treatment Strategies on Antibiotic Resistant Bacteria and Antibiotic Resistance Genes. *Chemosphere* 150:702-714

Shmakova E. 2020. Metagenomes of the Domainant Microbial Symbionts of Sponge Genus Cinachyrella Display Common Sulfur Metabolic and Quorum Sensing Functions. D.Lopez. Masters of Science. Thesis, Nova Southeastern University

Sievert S. M., Kiene R. P., and Schulz-Vogt H. N. 2007. The Sulfur Cycle. *Oceanography* 20:117-123

Sipkema D., De Caralt S., Morillo J. A., Al-Soud W. A., Sørensen S. J., Smidt H., and Uriz M. J. 2015. Similar Sponge-Associated Bacteria Can Be Acquired Via Both Vertical and Horizontal Transmission. *Environmental microbiology* 17:3807-3821

Slack M. P. E. 2010. Chapter 172 - Gram-Negative Coccobacilli. In: Cohen J, Opal SM, and Powderly WG, eds. *Infectious Diseases* London: Content Repository Only!, 1738-1756.

Smith E. 2013. De Novo Transcriptome Analysis of the Marine Sponge Cinachyrella Spp: A Potential Model Organism for Oil and Dispersant Ecotoxicology. D.Lopez. Marine Biology. Thesis, Nova Southeastern University.

Sogin M. L., Morrison H. G., Huber J. A., Welch D. M., Huse S. M., Neal P. R., Arrieta J. M., and Herndl G. J. 2006. Microbial Diversity in the Deep Sea and the Underexplored "Rare Biosphere". *Proceedings of the National Academy of Sciences* 103:12115-12120

Steindler L., Schuster S., Ilan M., Avni A., Cerrano C., and Beer S. 2007. Differential Gene Expression in a Marine Sponge in Relation to Its Symbiotic State. *Marine Biotechnology* 9:543-549

Stewart F. J., and Cavanaugh C. M. 2005. Symbiosis of Thioautotrophic Bacteria with Riftia Pachyptila. *Molecular Basis of Symbiosis*. Springer, 197-225.

Suzuki Y., Sasaki T., Suzuki M., Nogi Y., Miwa T., Takai K., Nealson K. H., and Horikoshi K. 2005. Novel Chemoautotrophic Endosymbiosis between a Member of the Epsilonproteobacteria and the Hydrothermal-Vent Gastropod Alviniconcha Aff. Hessleri (Gastropoda: Provannidae) from the Indian Ocean. *Applied and Environmental Microbiology* 71:5440-5450

Taylor M. W., Radax R., Steger D., and Wagner M. 2007. Sponge-Associated Microorganisms: Evolution, Ecology, and Biotechnological Potential. *Microbiology and molecular biology reviews* 71:295-347

Taylor M. W., Schupp P. J., Dahllöf I., Kjelleberg S., and Steinberg P. D. 2004. Host Specificity in Marine Sponge-Associated Bacteria, and Potential Implications for Marine Microbial Diversity. *Environmental microbiology* 6:121-130

Taylor M. W., Schupp P. J., De Nys R., Kjelleberg S., and Steinberg P. D. 2005. Biogeography of Bacteria Associated with the Marine Sponge Cymbastela Concentrica. *Environmental microbiology* 7:419-433

Taylor M. W., Tsai P., Simister R. L., Deines P., Botte E., Ericson G., Schmitt S., and Webster N. S. J. T. I. J. 2013. 'Sponge-Specific'bacteria Are Widespread (but Rare) in Diverse Marine Environments. *The ISME Journal* 7:438

Thomas, Moitinho-Silva, Lurgi, Björk, Easson, Astudillo-García, Olson, Erwin, López-Legentil, Luter, Chaves-Fonnegra, Costa, Schupp, Steinberg, Erpenbeck, Gilbert, Knight, Ackermann, Lopez, Taylor, Thacker, Montoya, Hentschel, and Webster. 2016. Diversity,

Structure and Convergent Evolution of the Global Sponge Microbiome. *Nature communications* 7: https://doi.org/10.1038/ncomms11870

Thomas T., Rusch D., Demaere M. Z., Yung P. Y., Lewis M., Halpern A., Heidelberg K. B., Egan S., Steinberg P. D., and Kjelleberg S. 2010. Functional Genomic Signatures of Sponge Bacteria Reveal Unique and Shared Features of Symbiosis. *The ISME Journal* 4:1557-1567

Thompson L. R., Sanders J. G., Mcdonald D., Amir A., Ladau J., Locey K. J., Prill R. J., Tripathi A., Gibbons S. M., and Ackermann G. 2017. A Communal Catalogue Reveals Earth's Multiscale Microbial Diversity. *Nature* 551:457-463

Tian R.-M., Zhang W., Cai L., Wong Y.-H., Ding W., and Qian P.-Y. 2017. Genome Reduction and Microbe-Host Interactions Drive Adaptation of a Sulfur-Oxidizing Bacterium Associated with a Cold Seep Sponge. *MSystems* 2:e00184-00116

Tian R. M., Sun J., Cai L., Zhang W. P., Zhou G. W., Qiu J. W., and Qian P. Y. 2016. The Deep-Sea Glass Sponge L Ophophysema Eversa Harbours Potential Symbionts Responsible for the Nutrient Conversions of Carbon, Nitrogen and Sulfur. *Environmental microbiology* 18:2481-2494

Tian R. M., Wang Y., Bougouffa S., Gao Z. M., Cai L., Bajic V., and Qian P. Y. 2014. Genomic Analysis Reveals Versatile Heterotrophic Capacity of a Potentially Symbiotic Sulfur-Oxidizing Bacterium in Sponge. *Environmental microbiology* 16:3548-3561

Tremblay J., Singh K., Fern A., Kirton E. S., He S., Woyke T., Lee J., Chen F., Dangl J. L., and Tringe S. G. 2015. Primer and Platform Effects on 16s Rrna Tag Sequencing. *Frontiers in microbiology* 6:771

Trojan D., Schreiber L., Bjerg J. T., Bøggild A., Yang T., Kjeldsen K. U., and Schramm A. 2016. A Taxonomic Framework for Cable Bacteria and Proposal of the Candidate Genera Electrothrix and Electronema. *Systematic and Applied Microbiology* 39:297-306 https://doi.org/10.1016/j.syapm.2016.05.006

Turnbaugh P. J., Ley R. E., Hamady M., Fraser-Liggett C. M., Knight R., and Gordon J. I. 2007. The Human Microbiome Project. *Nature* 449:804-810

Urakawa H., and Feeney M. 2018. Preliminary Sulfur Consumption Test. Unpublished: Florida Gulf Coast University.

Vavourakis C. D., Mehrshad M., Balkema C., Van Hall R., Andrei A.-Ş., Ghai R., Sorokin D. Y., and Muyzer G. 2019. Metagenomes and Metatranscriptomes Shed New Light on the Microbial-Mediated Sulfur Cycle in a Siberian Soda Lake. *BMC biology* 17:69

Vijayan N. 2015. Bacterial Community Dynamics in Marine Sponge Cinachyrella Kuekenthali under Irradiance and Antibiotics. D.Lopez. Marine Biology. Thesis, Nova Southeastern University.

Vogel S. 1977. Current-Induced Flow through Living Sponges in Nature. *Proceedings of the National Academy of Sciences* 74:2069-2071

Wang Y., and Qian P.-Y. 2009. Conservative Fragments in Bacterial 16s Rrna Genes and Primer Design for 16s Ribosomal DNA Amplicons in Metagenomic Studies. *PloS one* 4:

Webster N. S., and Blackall L. L. 2009. What Do We Really Know About Sponge-Microbial Symbioses? *The ISME Journal* 3:1-3

Webster N. S., Cobb R. E., Soo R., Anthony S. L., Battershill C. N., Whalan S., and Evans-Illidge E. 2011. Bacterial Community Dynamics in the Marine Sponge Rhopaloeides Odorabile under in Situ and Ex Situ Cultivation. *Marine Biotechnology* 13:296-304

Webster N. S., Negri A. P., Munro M. M., and Battershill C. N. 2004. Diverse Microbial Communities Inhabit Antarctic Sponges. *Environmental microbiology* 6:288-300

Webster N. S., and Taylor M. W. 2012. Marine Sponges and Their Microbial Symbionts: Love and Other Relationships. *Environmental microbiology* 14:335-346

Webster N. S., Webb R. I., Ridd M. J., Hill R. T., and Negri A. P. 2001. The Effects of Copper on the Microbial Community of a Coral Reef Sponge. *Environmental microbiology* 3:19-31

Weisz J. B., Lindquist N., and Martens C. S. 2008. Do Associated Microbial Abundances Impact Marine Demosponge Pumping Rates and Tissue Densities? *Oecologia* 155:367-376

White J. R., Patel J., Ottesen A., Arce G., Blackwelder P., and Lopez J. V. 2012. Pyrosequencing of Bacterial Symbionts within Axinella Corrugata Sponges: Diversity and Seasonal Variability. *PloS one* 7:

Whittaker R. H. 1972. Evolution and Measurement of Species Diversity. *Taxon* 21:213-251

Wilkinson C. 1979. Nutrient Translocation from Symbiotic Cyanobacteria to Coral Reef Sponges. *Hydrobiologia*

Wilson M. J., Weightman A., and Wade W. 1997. Applications of Molecular Ecology in the Characterization of Uncultured Microorganisms Associated with Human Disease. *Reviews in Medical Microbiology* 8:91-102

Woese C., Magrum L., Gupta R., Siegel R., Stahl D., Kop J., Crawford N., Brosius R., Gutell R., and Hogan J. 1980. Secondary Structure Model for Bacterial 16s Ribosomal Rna: Phylogenetic, Enzymatic and Chemical Evidence. *Nucleic acids research* 8:2275-2294

Woese C., Stackebrandt E., Weisburg W., Paster B., Madigan M., Fowler V., Hahn C., Blanz P., Gupta R., and Nealson K. 1984. The Phylogeny of Purple Bacteria: The Alpha Subdivision. *Systematic and Applied Microbiology* 5:315-326

Woese C. R. 1982. Archaebacteria and Cellular Origins: An Overview. *Zentralblatt für Bakteriologie Mikrobiologie und Hygiene: I Abt Originale C: Allgemeine, angewandte und ökologische Mikrobiologie* 3:1-17

Woese C. R. 1987. Bacterial Evolution. *Microbiological reviews* 51:221

Woese C. R., and Fox G. E. 1977. Phylogenetic Structure of the Prokaryotic Domain: The Primary Kingdoms. *Proceedings of the National Academy of Sciences* 74:5088-5090

Yoch D. C. 2002. Dimethylsulfoniopropionate: Its Sources, Role in the Marine Food Web, and Biological Degradation to Dimethylsulfide. *Applied and Environmental Microbiology* 68:5804-5815

## XII. APPENDICES:

Appendix A - Tables

**Table 12: Similar taxa within algae using simper.** The below table shows the results of a Simper test to determine similar major ASVs in all Algae. Overall, similarly of 8.99% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Simper results for Dominant Algae ASVs***

| *ASVs* | Taxonomy | Average Abundance | Average Similarity | Percent Contribution |
|---|---|---|---|---|
| *1* | Genus *Hyphomonas* | 0.15 | 0.77 | 8.59 |
| *2* | Genus *Parvularcula* | 0.15 | 0.74 | 8.27 |
| *3* | Genus *Diplosphaera* | 0.04 | 0.26 | 2.92 |
| *4* | Genus *Pseudoalteromonas* | 0.09 | 0.25 | 2.79 |
| *5* | Genus *Pseudohaliea* | 0.05 | 0.23 | 2.53 |
| *6* | Genus *Phormidium* | 0.07 | 0.21 | 2.35 |
| *7* | Genus *Pyruvatibacter* | 0.09 | 0.21 | 2.34 |
| *8* | Family Nodosilineaceae | 0.17 | 0.2 | 2.2 |
| *9* | Family Phycisphaeraceae - SM1A02 | 0.07 | 0.19 | 2.09 |
| *10* | Class Ignavibacteria- Uncultured | 0.07 | 0.18 | 1.96 |
| *11* | Family Cryomorphaceae- Uncultured | 0.07 | 0.16 | 1.81 |
| *12* | Genus Ruegeria | 0.05 | 0.15 | 1.7 |
| *13* | Genus Gambierdiscus | 0.05 | 0.15 | 1.68 |
| *14* | Family Rhodobacteraceae - Unknown | 0.16 | 0.12 | 1.3 |
| *15* | Order Kordiimonadales - Uncultured | 0.04 | 0.12 | 1.29 |
| *16* | Family Rhodobacteraceae- Unknown | 0.08 | 0.12 | 1.28 |
| *17* | Class Gammaproteobacteria-Unknown | 0.04 | 0.11 | 1.25 |
| *18* | Genus Oleiphilus | 0.05 | 0.11 | 1.17 |
| *19* | Genus Aestuariibacter | 0.05 | 0.1 | 1.12 |
| *20* | Family Rhodobacteraceae- Unknown | 0.04 | 0.1 | 1.08 |

**Table 13: Similar taxa within microbial mat using simper**. Below were the results of a Simper test to determine similar major ASVs in all Microbial mat samples. Overall, similarly of 16.74% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Simper results for Dominant Similar Microbial Mat ASVs***

| *ASVs* | Taxonomy | Average Abundance | Average Similarity | Percent Contribution |
|---|---|---|---|---|
| *21* | Genus *Arcobacter* | 0.09 | 0.53 | 3.16 |
| *22* | Oder Clostridiales - Unknown | 0.09 | 0.5 | 2.97 |
| *23* | Genus *Arcobacter* | 0.17 | 0.44 | 2.62 |
| *24* | Genus *Desulfuromusa* | 0.12 | 0.43 | 2.55 |
| *25* | Family Rhodobacteraceae - Unknown | 0.06 | 0.41 | 2.47 |
| *26* | Genus *Marinifilum* | 0.06 | 0.41 | 2.45 |
| *27* | Family Rhodobacteraceae - Unknown | 0.1 | 0.41 | 2.42 |
| *20* | Family Rhodobacteraceae - Unknown | 0.07 | 0.39 | 2.34 |
| *28* | Genus *Halodesulfovibrio* - Uncultured | 0.06 | 0.39 | 2.33 |
| *29* | Genus Cohaesibacter - Uncultured | 0.05 | 0.36 | 2.17 |
| *30* | Genus *Marinifilum* - Unknown | 0.08 | 0.35 | 2.1 |
| *31* | Family Rhodobacteraceae - Unknown | 0.04 | 0.32 | 1.92 |
| *32* | Class Ignavibacteria - Unknown | 0.07 | 0.31 | 1.84 |
| *33* | Family Rhodobacteraceae - Unknown | 0.04 | 0.31 | 1.83 |
| *34* | Order Chitinophagales - Unknown | 0.08 | 0.29 | 1.71 |
| *35* | Family Rhodobacteraceae - Unknown | 0.05 | 0.27 | 1.59 |
| *36* | Genus *Arcobacter* | 0.07 | 0.26 | 1.56 |
| *11* | Family Cryomorphaceae - Unknown | 0.05 | 0.25 | 1.47 |
| *38* | Genus *Draconibacterium* | 0.07 | 0.23 | 1.39 |
| *39* | Genus *Neptuniibacter* | 0.05 | 0.23 | 1.38 |
| *40* | Genus *Phaeodactylibacter* | 0.05 | 0.23 | 1.38 |
| *41* | Order Clostridiales - Unknown | 0.04 | 0.22 | 1.3 |
| *42* | Order Clostridiales - Family XII | 0.05 | 0.22 | 1.3 |
| *43* | Genus *Desulfovibrio* | 0.04 | 0.21 | 1.28 |

**Table 14: Similar taxa within sponge samples using simper.** The above was the results of a Simper test to determine similar major ASVs in all Sponge samples. Overall, similarly of 10.83% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Simper results for Dominant Similar Sponge ASVs***

| *ASVs* | Taxonomy | Average Abundance | Average Similarity | Percent Contribution |
|---|---|---|---|---|
| *52* | Family Rhodobacteraceae - Unknown | 0.27 | 1.49 | 13.81 |
| *53* | Family Nitrosopumilaceae - Unknown | 0.22 | 0.85 | 7.87 |
| *54* | Class Gammaproteobacteria - Unknown | 0.12 | 0.72 | 6.69 |
| *55* | Class Deltaproteobacteria - NB1-J | 0.12 | 0.61 | 5.66 |
| *12* | Genus *Ruegeria* | 0.09 | 0.52 | 4.85 |
| *56* | Order Betaproteobacteriales - Unknown | 0.1 | 0.31 | 2.86 |
| *57* | Class Alphaproteobacteria - uncultured | 0.09 | 0.27 | 2.52 |
| *28* | Genus *Halodesulfovibrio* | 0.09 | 0.24 | 2.23 |
| *58* | Genus *Draconibacterium* | 0.15 | 0.24 | 2.19 |
| *20* | Family Rhodobacteraceae - Unknown | 0.03 | 0.22 | 2.01 |
| *59* | Class Alphaproteobacteria - Unknown | 0.04 | 0.19 | 1.79 |
| | Family Lachnospiraceae - | 0.07 | 0.19 | 1.77 |

**Table 15: Similar taxa within water samples using simper.** The above was the results of a Simper test to determine similar major ASVs in all Water samples. Overall, similarly of 8.33% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Simper results for Dominant Similar Water ASVs***

| *ASVs* | Taxonomy | Average Abundance | Average Similarity | Percent Contribution |
|---|---|---|---|---|
| *1* | Genus *Hyphomonas* | 0.09 | 0.63 | 7.55 |
| *65* | Family Flavobacteriaceae - Uncultured | 0.24 | 0.49 | 5.92 |
| *31* | Family Rhodobacteraceae - Unknown | 0.13 | 0.35 | 4.14 |
| *66* | Class Oxyphotobacteria- Unknown | 0.13 | 0.28 | 3.41 |
| *40* | Genus *Phaeodactylibacter* | 0.08 | 0.27 | 3.19 |
| *67* | Genus *Thalassobius* - Uncultured | 0.06 | 0.26 | 3.11 |
| *11* | Family Cryomorphaceae - Uncultured | 0.05 | 0.24 | 2.93 |
| *68* | Genus *Cylindrotheca* | 0.12 | 0.21 | 2.55 |
| *69* | Family Mitochondria | 0.07 | 0.21 | 2.55 |
| *70* | Family Alteromonadaceae - Uncultured | 0.06 | 0.18 | 2.16 |
| *71* | Genus *Francisella* | 0.04 | 0.16 | 1.88 |
| *10* | Class Ignavibacteria - OPB56 | 0.04 | 0.14 | 1.7 |
| *72* | Genus *Mesoflavibacter* | 0.05 | 0.13 | 1.55 |
| *73* | Family Thiotrichaceae - Uncultured | 0.05 | 0.13 | 1.51 |
| *74* | Family Rhodobacteraceae - Unknown | 0.09 | 0.12 | 1.44 |
| *49* | Order Bacteroidales - Unknown | 0.04 | 0.09 | 1.13 |
| *75* | Genus *Francisella* | 0.03 | 0.09 | 1.13 |
| *16* | Family Rhodobacteraceae - Unknown | 0.04 | 0.09 | 1.12 |
| *76* | Family Cyclobacteriaceae - Unknown | 0.05 | 0.09 | 1.03 |
| *20* | Family Rhodobacteraceae - Unknown | 0.03 | 0.08 | 1.02 |
| *48* | Family Rhodobacteraceae - Unknown | 0.07 | 0.08 | 1 |

**Table 16: Similar taxa within before samples using simper.** The above was the results of a Simper test to determine similar major ASVs in all samples taken before exposure. Overall, similarly of 8.42% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Simper results for Dominant Similar Before Exposure ASVs***

| *ASVs* | Taxonomy | Average Abundance | Average Similarity | Percent Contribution |
|---|---|---|---|---|
| *52* | Family Rhodobacteraceae - Uncultured | 0.15 | 0.36 | 4.28 |
| *21* | Genus Arcobacter | 0.06 | 0.32 | 3.86 |
| *14* | Family Rhodobacteraceae - Unknown | 0.11 | 0.28 | 3.31 |
| *83* | Genus Vibrio | 0.05 | 0.26 | 3.08 |
| *40* | Genus Phaeodactylibacter | 0.07 | 0.22 | 2.64 |
| *20* | Family Rhodobacteraceae - Unknown | 0.03 | 0.2 | 2.35 |
| *12* | Genus Ruegeria | 0.05 | 0.18 | 2.19 |
| *27* | Genus Shimia | 0.04 | 0.18 | 2.19 |
| *77* | Genus Pseudofulvibacter | 0.06 | 0.18 | 2.09 |
| *54* | Class Gammaproteobacteria - Unknown | 0.07 | 0.17 | 2.07 |
| *1* | Family Cryomorphaceae | 0.04 | 0.17 | 2.05 |
| *55* | Class Deltaproteobacteria - NB1-j | 0.08 | 0.17 | 2.03 |
| *53* | Family Nitrosopumilaceae | 0.13 | 0.16 | 1.88 |
| *83* | Family Rhodobacteraceae - Unknown | 0.04 | 0.15 | 1.83 |
| *84* | Genus Tropicibacter | 0.04 | 0.15 | 1.77 |
| *72* | Genus Mesoflavibacter | 0.04 | 0.15 | 1.74 |
| *29* | Genus Cohaesibacter | 0.04 | 0.14 | 1.72 |
| *76* | Family Flavobacteriaceae | 0.04 | 0.13 | 1.54 |
| *51* | Family Rhodobacteraceae - Unknown | 0.03 | 0.13 | 1.53 |
| *23* | Genus Arcobacter | 0.08 | 0.13 | 1.52 |
| *48* | Family Rhodobacteraceae - Unknown | 0.06 | 0.13 | 1.49 |
| *85* | Genus Phaeocystidibacter | 0.06 | 0.12 | 1.37 |
| *60* | Genus Halodesulfovibrio | 0.03 | 0.11 | 1.36 |
| | Family Rhodobacteraceae - | 0.04 | 0.11 | 1.34 |

**Table 17: Similar taxa within after samples using simper.** The above was the results of a Simper test to determine similar major ASVs in all samples taken after exposure. Overall, similarly of 11.11% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Simper results for Dominant Similar After Exposure ASVs***

| *ASVs* | Taxonomy | Average Abundnce | Average Similarity | Percent Contribution |
|---|---|---|---|---|
| *58* | Genus *Draconibacterium* | 0.15 | 0.41 | 3.68 |
| *1* | Genus *Hyphomonas* | 0.08 | 0.38 | 3.45 |
| *34* | Order Chitinophagales - Uncultured | 0.07 | 0.26 | 2.34 |
| *12* | Genus *Ruegeria* | 0.06 | 0.23 | 2.11 |
| *2* | Genus *Parvularcula* | 0.06 | 0.19 | 1.74 |
| *10* | Class Ignavibacteria - OPB56 | 0.05 | 0.17 | 1.57 |
| *20* | Family Rhodobacteraceae - Unknown | 0.04 | 0.17 | 1.54 |
| *28* | Genus *Halodesulfovibrio* | 0.07 | 0.16 | 1.48 |
| *27* | Genus *Shimia* | 0.07 | 0.16 | 1.41 |
| *43* | Genus *Desulfovibrio* | 0.05 | 0.15 | 1.37 |
| *82* | Order Clostridiales - Family XIII | 0.05 | 0.14 | 1.25 |
| | Order Rhycisphaerales | 0.06 | 0.14 | 1.22 |

**Table 18: Dissimilar taxa within the microbial mat and water samples using simper. The** above was the results of a Simper test to determine major dissimilar ASVs of water samples compared to Microbial Mat samples. Overall, a dissimilarly of 88.24% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Simper results for Dominant Dissimilar ASVs for Microbial Mat compared to Water***

| *ASVs* | Taxonomy | Algae Average Abundance | Water Average Abundance | Average Dissimilarity | Percent Contribution |
|---|---|---|---|---|---|
| *65* | Genus Flavobacteriaceae | 0 | 0.24 | 1.19 | 1.33 |

**Table 19: Dissimilar taxa within sponges and algae samples using simper.** The above was the results of a Simper test to determine major dissimilar ASVs of sponge samples compared to algae samples. Overall, a dissimilarly of 94.41% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Simper results for Dominant Dissimilar ASVs for Algae compared to Sponge***

| *ASVs* | Taxonomy | Algae Average Abundance | Sponge Average Abundance | Average Dissimilarity | Percent Contribution |
|---|---|---|---|---|---|
| *52* | Family Rhodobacteraceae | 0.01 | 0.27 | 1.28 | 1.36 |
| *53* | Family Nitrosopumilaceae | 0 | 0.22 | 1.28 | 1.35 |

**Table 20: Dissimilar taxa within algae and water samples using simper**. The above was the results of a Simper test to determine major dissimilar ASVs of Water samples compared to Algae samples. Overall, a dissimilarly of 88.24% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Simper results for Dominant Dissimilar ASVs for Algae compared to Water***

| *ASVs* | Taxonomy | Algae Average Abundance | Water Average Abundance | Average Dissimilarity | Percent Contribution |
|---|---|---|---|---|---|
| *65* | Genus *Flavobacteriaceae* | 0.03 | 0.24 | 1.14 | 1.26 |

**Table 21: Dissimilar taxa within the sponge and microbial mat samples using simper.** The above was the results of a Simper test to determine major dissimilar ASVs of sponge samples compared to Microbial Mat samples. Overall, a dissimilarly of 88.24% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Simper results for Dominant Dissimilar ASVs for Microbial Mat compared to Sponge***

| *ASVs* | Taxonomy | Microbial Average Abundance | Sponge Average Abundance | Average Dissimilarity | Percent Contribution |
|---|---|---|---|---|---|
| *53* | Family Nitrosopumilaceae | 0 | 0.22 | 1.41 | 1.6 |
| *52* | Family Rhodobacteraceae | 0.02 | 0.27 | 1.36 | 1.55 |
| *58* | Genus *Draconibacterium* | 0.13 | 0.15 | 1.01 | 1.14 |
| *23* | Genus *Arcobacter* | 0.17 | 0.02 | 0.88 | 1 |

Note: All ASVs that were dissimilar for the microbial mat and algae did not contribute to more than 1%, thus the table is not shown.

Appendix B – DRAFT MANUSCRIPT

**ABSTRACT:**

Recent unpublished research suggests the symbionts within marine sponges are actively participating in the sulfur cycle. This study measured the abundance of microbes within the genus *Cinachyrella* before and after exposure to hydrogen sulfide. A four-part study was conducted: a) five-hour drop experiments, b) vertical distribution experiments, c) five-hour uptake experiments, and d) long-term exposure experiments. The five-hour drop experiment utilized a microsensor to measure sulfide levels, which was lowered 1.0 mm every thirty minutes for a total of 5 hours. Three trials were performed, each with one sponge and a control with no sponge. The vertical distribution experiments measured hydrogen sulfide levels throughout 9.0 mm. A five-hour uptake experiment measured hydrogen sulfide over five hours without the use of microsensors. The bacterial composition was detailed during long-term exposure experiments, where three sponges were exposed to 60 μmol/L for several weeks. Tissue samples collected from the long-term exposure experiment underwent microbial DNA extractions and high-throughput sequencing. Hydrogen sulfide concentrations from the five-hour drop, vertical-distribution, and five-hour experiments underwent various generalized additive models and generalized linear models. A significant relationship between time (depth for the vertical-distribution) and hydrogen sulfide concentration ($p$-value$<0.05$) resulted. A significant difference based on the type (sponge and control group) of sample ($p$-value$<0.05$) was also seen. Long-term exposure indicated that hydrogen sulfide affected the relative abundance of genus *Draconibacterium*, family Rhodobacteraceae, and genus *Halodesulfovibrio* within Sponges. This data suggests that *Cinachyrella* spp. can filter and process hydrogen sulfide from the water column with help from its microbiome.

KEYWORDS: *Cinachyrella* sp., sulfur cycle, symbionts, 16S rRNA, Sulfur-reducing/oxidizing bacteria

## INTRODUCTION:

Sponge abundance and filter-feeding lifestyle allow sponges to fill a significant ecological niche by removing suspended matter (e.g., dissolved organic matter (DOM), picoplankton, and bacterioplankton) from the water column (Reiswig, 1971; Pile, Patterson & Witman, 1997; Peterson et al., 2006). A kilogram sponge can filter up to 24,000 L of seawater per day (Vogel, 1977), making poriferans highly efficient at removing particulate organic matter. For example, other organisms consume discarded choanocytes, which allows dissolved organic carbon to be accessible to various marine organisms (De Goeij et al., 2013). Thus, linking pelagic and benthic systems (Webster et al., 2011).

A high abundance of symbionts have been found within the tissues extracellularly and intracellularly, totaling up to 50% of sponge biomass (Santavy & Colwell, 1990; Cuvelier et al., 2014). These symbionts are phylogenetically diverse, comprising of 48 bacterial phyla, 3 archaeal phyla, 3 fungal phyla, and phylogenetically diverse algae (Webster et al., 2004; Pape et al., 2006; Holmes & Blanch, 2007; Lee et al., 2011; He et al., 2014; Li et al., 2016; Thomas et al., 2016).

Once microbes capable of sulfur metabolism were isolated from marine sponges, research began to focus on the sulfur cycle to understand the contribution from this host-symbiont interaction. The most intensive research for sulfur metabolism within sponges has been done on *G. barretti*. Sulfate reduction rates from *G. barretti* are among the highest recorded in natural systems, up to 1,200 nmol $SO_4^{2-}$ $cm^{-3}$ per sponge per day (Hoffmann et al., 2005).

*Cinachyrella* spp., a genus that is extremely hard to identify down to species, has even been documented to have many possible microbes performing sulfur-reduction and oxidation, such as Acidobacteria, Cellvibrionaceae, Colwelliaceae, Rhodobacteraceae, and Gammaproteobacteria (Vijayan, 2015). Within *Cinachyrella* spp., Shmakova recently described sulfur metabolism in five metagenomically assembled genomes (MAGs): *Opitutaceae bacterium, Thioalkalivibrio paradoxus, Desulfobacterium autotrophicum, Thioalkalivibrio sulfidiphilus, Sulfurifustis variabilis*. This study also identified 27 other MAGS with sulfide reducing genes (**Fig. 4**) (Shmakova, 2020). Sulfatase hydrolase/transferase, along with other genes, has been found in the *Cinachyrella* spp. holobiont (Desplat, 2020). Preliminary data on this species has suggested that there is an uptake in hydrogen sulfide over time (Urakawa & Feeney, 2018).

These studies are limited to identifying various taxa that have demonstrated the ability to metabolize sulfur (Meyer & Kuever, 2008; Tian et al., 2014; Tian et al., 2016; Jensen et al., 2017; Tian et al., 2017). Understanding the relationship between host and symbiont can reveal how the symbiosis occurs and persists. Symbiosis is not merely an interaction between organisms but an innovative mechanism of survival (Seckbach, 2006; Mcfall-Ngai, 2014). Thus, this study's objective was to investigate the role of bacteria in the sulfur cycle within *Cinachyrella* spp. of the Florida reefs. *Cinachyrella* has been chosen as a model sponge sue to many positive features (extended survival in aquaculture, natural along nearby reefs, the possibility of reproduction, etc.) (Barton, Fardeau & Fauque, 2014; Vijayan, 2015).

Four different experimental methods were used to determine: if a significant relationship between time and uptake of $H_2S$ (by a natural and sponge environment) would be seen (refer to five-hour uptake), if significant relationship between depth and uptake of $H_2S$ (by a natural and sponge environment) would be seen (refer to vertical-distribution), If a significant relationship between time (with the interaction of depth) and uptake of $H_2S$ (by a natural and sponge environment) would be seen (refer to five-hour uptake experiments), if a there was a statistical differences in the control (used to represent the diffusion rate of $H_2S$) and sponge (refer to five-hour uptake, vertical-distribution, and five-hour uptake experiments), if *Cinachyrella* spp. host SRB and SOB (refer to long-term experiments), if there would be a change of relative bacterial abundance after the $H_2S$ exposure (refer to long-term experiments)

## MATERIALS AND METHODOLOGY:

**Collection.** A total of 9 sponge specimens were collected off Halmos College of Nova Southeastern University on the Florida Reef Tract. Samples were collected while diving; careful measures were taken such that specimens did not have air exposure. The Molecular Microbiology and Genomics Laboratory of Halmos College of Nova Southeastern University obtained Florida permits for sponge collection. The specimens were then taken back to Halmos College of Nova Southeastern University and placed within an aquarium system. Marine sponges can be affected by transfer into aquaculture (Webster & Blackall, 2009); thus, sponges were used within 24 hours of collection.

**Five-Hour Drop Experiments.** To determine the relationship between the interaction of time and depth and uptake of $H_2S$ (by a natural and sponge environment), fresh sponges (n= 3,

Sponges 3, 4, and 5) were collected, maintained under normal aquarium conditions. They were then exposed to hydrogen sulfide. Two experimental beakers (500 mL) were used with normal aquarium seawater and 60 μmol/L of $H_2S$. The concentration of hydrogen sulfide was chosen due to previous research done at Florida Gulf Coast University. $H_2S$ was concentrated in DI water utilizing a hydrogen sulfide salt. This solution had all oxygen removed from the solution such that the solution could be stored. If the solution were stored with oxygen, the $H_2S$ would react with the oxygen lowering the overall concentration of $H_2S$. Note only one of the two 500L-beakers contained a *Cinachyrella* spp, which was in a smaller beaker to prevent movement. Sponges were allowed to acclimate to the experimental beaker condition for 30 minutes before $H_2S$ exposure. There were three tests per environment, i.e., a total of 6 trials, with only 3 sponges. Natural microbial populations are known to shift within sponges after separation from natural environments and culture in aquaria (Cardenas et al., 2009; Webster & Blackall, 2009). Due to this, sponge samples were used within 24 hours. Sulfur was routinely monitored for 5 hours in intervals of 30 seconds by microsensors. Unisense microsensors recorded a gradual change but moving the sensor by 1000 μm every 30 minutes. Oxygen levels of each trial were measured before and after experimentation. The microelectrode measurements were taken using a glass 100 μm diameter sensor, and microelectrodes were manipulated using a motorized micromanipulator. There was only one arm to hold the sensor; thus, the control did not move every 30 minutes, and a microsensor was placed at the bottom of the beaker.

**Vertical distribution Experiments.** During the five-hour drop experiments, two sponges (Sponge 4 and 5) had the vertical distribution profiles of $H_2S$. A Unisense microsensor measured the concentration of $H_2S$ continuously over a 9 mm depth. A Sponge 4 was measured before the five-hour drop experiment, while the other was measured after the five-hour drop experiment.

**Five-Hour Uptake Experiments.** Fresh sponges were collected (n=3, Sponges 2, 6, and 9), maintained under normal conditions, then placed under experimental conditions to determine the relationship between time and uptake of $H_2S$. Two experimental beakers (2 L) were set up with normal aquarium water and 60 μmol/L of $H_2S$. This concentrated solution using hydrogen sulfide salt was made with DI water immediately before experimentation. The solution was slowly and gently, to reduce oxygenation, drained into the beaker immediately before the first measurement. Only one of the two beakers contained a *Cinachyrella* spp. Sponges were allowed to acclimate to the experimental beaker condition for 30 minutes before $H_2S$ exposure. There

were three tests per environment, i.e. a total of 6 trials, with only 3 sponges. A GENESYS 20 without printer spectrometer was utilized to measure absorbance. Absorbance (at 690 nm) was measured using the Sulfide Reagent Set, Methylene Blue (Hach product number 181732; methodology DOC316.53.01136) every 30 minutes for five hours. This kit allows the absorbance to be converted to $H_2S$ µmol/L utilizing a standard curve. The curve was made before experimentation to determine the relationship between $H_2S$ and absorbance. Although very similar to five-hour drop experiments, the five-hour uptake experiments do not utilize microsensors and only had concentrations measured from the beakers' top.

**Statistical Analysis and Modeling Techniques of Five-Hour Drop, Vertical-Distribution, and Five-Hour Uptake Experiments.** All data collected was recorded in Excel, under a comma-separated value file format (CSV). Sponge 4 was tested with a five-hour drop experiment, then vertical distribution was performed. Sponge 5 had a vertical distribution preformed, then underwent a five-hour drop experiment. Both data sets were treated as independent events.

Statistical analysis was used to determine if the natural-uptake was significantly different from sponge uptake. A GAM was performed on the average values of the five-hour drop experiments to determine if the type of sample (control or sponge) and hour influenced the $H_2S$ concentration. A Generalized Linear Model (Poisson Distribution) (GLM) was performed on the accumulation of data from five-hour uptake experiments to determine if the type of sample (control or sponge) and hour influenced the $H_2S$ concentration. Long term exposure experiments were excluded because no chemical measurements were acquired during that test. All statistical tests were performed at a 95% confidence interval.

**Long-Term Exposure to Hydrogen Sulfide.** An aquaculture tank system was maintained to house three *Cinachyrella* spp. (Sponges 1, 7, and 8). Different sponges were utilized than those used in the five-hour drop experiments and five-hour uptake experiments to reduce the sponge's stress. Sponges were given 60 µmol/L of $H_2S$ twice weekly until the sponge appeared to decay. This concentrated solution using hydrogen sulfide salt was made with DI water immediately before experimentation. The solution was slowly and gently, to reduce oxygenation, drained into the beaker immediately before the first measurement. Water was routinely monitored visually; temperature and salinity remained constant to environmental conditions. The five-hour drop experiments sponge samples were taken before and after

experimentation. Any changes in the sponge's appearance were observed and recorded. A microbial mat formed was collected and stored at -80$^{o}$C without any solution. Water was collected and filtered using a 0.2 µm filter before and after experimentation. Triangle tissue samples from the bottom, the previous collection cut, were taken before and after exposure to $H_2S$. These samples were then stored at -80$^{o}$C.

**DNA Extraction and Sequencing Methods for Long-Term Exposure.** Tissue samples from long term exposed sponges then underwent DNA extraction using the Qiagen Powersoil PowerLyzer protocol. A 1% agarose gel was used to confirm a successful extraction. After confirmation, samples underwent polymerase chain reaction (PCR) using universal primers (MIDf-515F and 806rc) and Platinum 2X polymerase (Illumina) (Lopez et al., 2008). The PCR thermocycler followed an initial denaturation at 94°C for 3 minutes (one cycle). Then, denaturation at 94$^{o}$C for 45 seconds followed by annealing at 50°C for 1 minute, and finally, extension at 72°C for 1 minute and 30 seconds. This step was repeated for 29 cycles. There was a final extension at 72°C for 10 minutes, with the reactions held at 4°C indefinitely. Confirmation on 1% agarose gel was performed to ensure the presence of DNA.

The 16S rRNA gene's amplicon was sequenced per the EMP sequencing protocol for the Illumina MiSeq platform. This sequencing was completed using Illumina barcoded primers for the 16S rRNA region (MIDf-515F and 806rc) with Platinum 2X polymerase (Illumina) (Promega). PCR was performed using the same procedure within the previous paragraph. Unique barcodes provide samples with an Id, which allows samples to be traced through data analysis. PCR was then checked on a 1% agarose gel for proper amplification with clean bands.

Samples were purified using AMPure bead as outlined in the 16S metagenomic library prep guide (Illumina, 2013). Final DNA concentrations were determined using a Qubit 2.0 fluorometer for normalization (Life Technologies), then underwent library pooling. Sample's quality was checked by Agilent Bioanalyzer tape station 2200 as outlined in the Agilent High Sensitivity D1000 ScreenTape System Quick Guide (Agilent Technologies, 2013). A high-throughput Illumina MiSeq sequencing approach targeting the 16S rRNA gene V4 regions was applied to verify specific microbial groups' presence and abundances. Upon sequencing completion, two FASTQ files, a forward and a reverse read, were used for downstream analysis.

**Data Analysis of 16S rRNA data for Long-Term Exposure.** Sponge 1, 7 and 8 16S rRNA FASTQ DNA sequence files were run through Quantitative Insights into Microbial

Ecology (QIIME2) for demultiplexing, quality filtering, ASV picking, taxonomic assignment, phylogenetic reconstruction, diversity analysis, and all visuals. Mapping files were compared for errors using “validate_mapping_file.py”, before demultiplexing and quality filtering with “split_libraries_fastq.py”. Sequences were filtered to remove chimeras and any score under 25 (1 error in 10,000 base pairs based on the PHRED system). The sequences were then sorted into ASVs with a 99% or more significant similarity for the Silva database using the “pick_open_reference_otus.py”. All reads (forward and reverse) were combined into one "qza" file using the "demuc" command, then imported into QIIME2 with the "emp-import" command. Then filtered and trimmed using the "dada2 denoise" command creating a feature-table, which was used to generate phylogenetic reconstruction using the "phylogeny fastttree" command.

Alpha and beta diversity community structures were determined in R Studio. Alpha diversity describes the number of taxa and abundance within communities or habitats (species richness and species evenness), while beta diversity is variation in community composition (Knight et al., 2012). The phyloseq package with R was used to assess alpha diversity. Beta diversity was measured with VEGAN. Bray-Curtis values, quantifying dissimilarities between the type of experiment (Sponge: Before, Sponge: After, Water: Before, Water: After, Microbial Mat, and Algae) were used. In both packages, Shannon's index and Inverse Simpson's index calculated alpha and beta diversity. Two t-tests were performed at a 95% interval to determine if sponge samples, before vs. after exposure, had a significantly different beta and alpha diversity.

Within primer, a non-metric multidimensional scaling (NMDS) plot was constructed using relative abundance. Analysis of similarities (ANOSIM) was performed in the following groups: sponge and water; before and after; before: sponge and before: water; after: sponge and after: water; after: algae, after: microbial mat, after: sponge, and after: water; before: sponge and after: sponge; before: water and after: water. A shaded plot was constructed in PRIMER to show the differences in classes, orders, and families within all samples. The topmost 30 abundant taxa were displayed. A Simper analysis was performed in PRIMER to determine the top similar and dissimilar ASVs. Any abundant taxa with a percent contribution under 1% were discarded. A Simper analysis was also performed in R Studio to identify the significant contributions of taxa differing between water and sponges before and after exposure, independently.

## RESULTS:

**Five-Hour Drop Experiments.** A GAM also demonstrated that the type of sample (control or sponge) and time had a significant impact on the rate of uptake (p-value<$2e^{-16}$and $R^2$>92) (**Figs. 1**).

**Vertical distribution Experiments.** Vertical distribution profiles of $H_2S$, using microsensors, were taken of Sponge 4 and Sponge 5. The profile of Sponge 4 was taken before the five-hour drop experiments, while the vertical distribution of Sponge 5 was taken after the five-hour drop experiments. This measurement was taken with the microsensor, moving a total of 9000 µm. The depth significantly affected the $H_2S$ measurement for sponge 4 (F=86.91, p-value<$2e^{-16}$), explaining 93.2% variation ($R^2$ = 0.932) (**Fig. 1)**. Sponge 5 data indicated that depth significantly affected the $H_2S$ measurement (F=38.61, p-value<$2e^{-16}$). Depth explains 87.2% $H_2S$ measurement for sponge 5 ($R^2$ = 0.872) (**Fig. 1**).

**Five-Hour Uptake Experiments.** A GLM determined that time significantly influenced the consumption of $H_2S$ (p-value <$2e^{-16}$). Additionally, the type of sample does significantly influence the consumption of $H_2S$ (p-value=$5.019e^{-12}$ There is a significant interaction between the type of sample and hour (p-value =$6.793e^{-11}$). The GLM explains 80.61% of deviations within the data (**Fig. 1**).

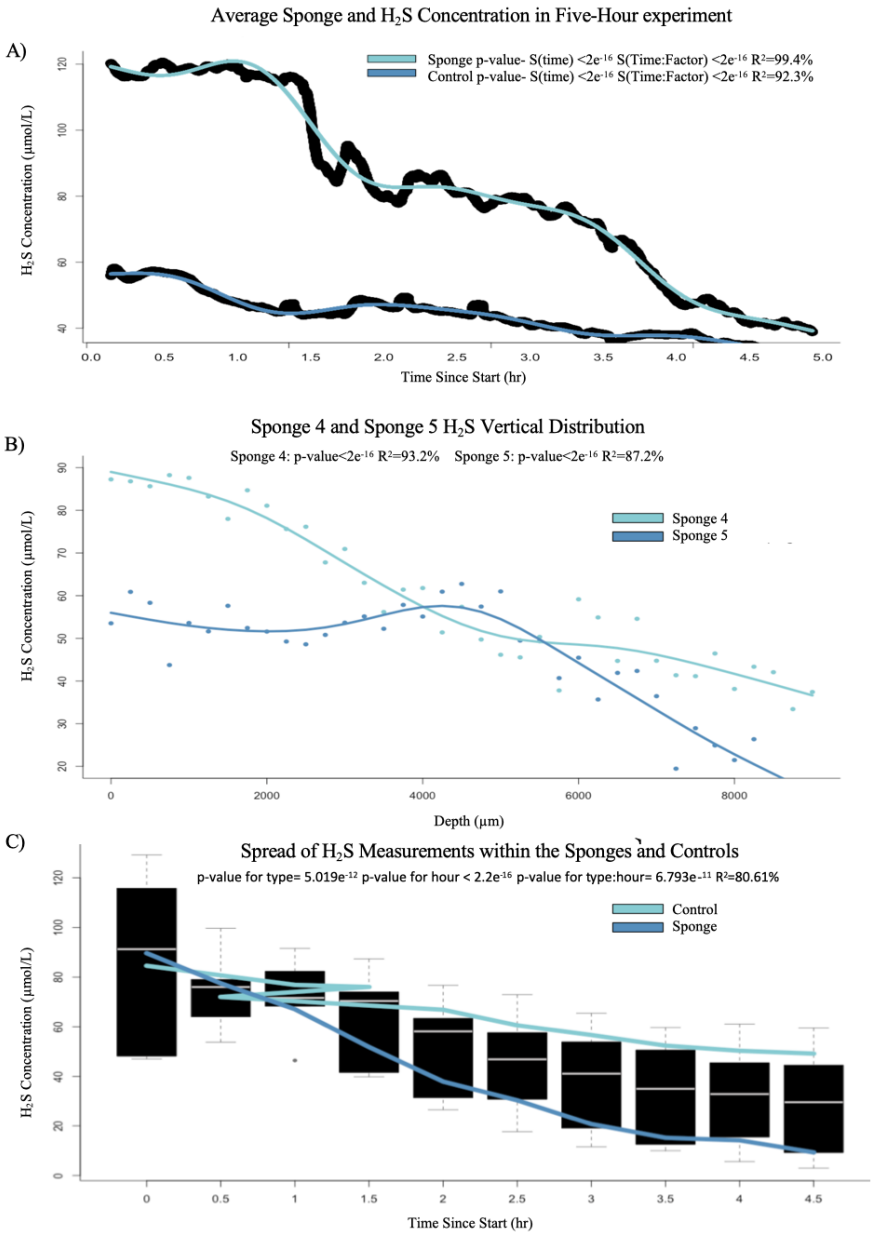


**Figure 1: Hydrogen Sulfide Concentration Measurements.** The graphs above detail the various measurements taken in the five-hour drop (A), vertical distribution experiments (B), and five-hour uptake (C). All models demonstrated a significant relationship with hydrogen sulfide concentration (p-value<0.05). A significant relationship between type of sample (sponge and control) was also seen (p-value<0.05). $R^2$ values are displayed on each graph. Part A demonstrates the model constructed for the average five-hour drop experiments. Every 30 microsensor dropped 1000 µm. Sponge samples are indicated in light blue, and control samples are indicated in dark blue. Part B demonstrates the vertical distribution of Sponge 4 and Sponge 5 is seen above. The light blue represents Sponge 4, and the dark blue represents Sponge 5. Note the vertical distribution of Sponge 4 and Sponge 5 was performed before and after the five-hour drop experiments, respectively. Part C shows all possible $H_2S$ measurements for sponge and control samples are displayed in the boxplot.

**Microbiome Analyses of Long-Term Exposure to Hydrogen Sulfide.** Sponges 1, 7, and 8 were kept over several weeks and were exposed to 60 μmol/L of $H_2S$ twice weekly. Samples of each sponge were taken before exposure (S#B) and after (S#A). Water samples were also taken before exposure (W#B) and after (W#A). Note water from the tank of Sponge 7 was mistakenly not taken after. Algae formed on all long-term sponges, and samples were taken (A#). A microbial mat formed on Sponge 7 before the first exposure of $H_2S$. Thus, a sample was taken when initially forming (MM7B) and when the experimentation was complete (MM7A). Sponge 8 was also seen to have a microbial mat form (MM8).

Seventeen samples were sequenced using a MiSeq sequencer (**Table 1**). A total of 1,100,167 raw 16S rRNA amplicon sequences were obtained. After filtration with dada2, 824,409 reads were generated. The average number of reads in each sample was 48,495, with a 41,926 standard deviation. The cut off for quality scores was 25, as default in QIME2. The average length of the samples was about 251 base pairs.

The alpha and beta diversity metrics were determined for Long-term Exposure experiments. Alpha diversity describes the number of taxa and abundance within communities or habitats (species richness and species evenness), while beta diversity is variation in community composition (Knight et al. 2012). The phyloseq package with R was used to assess alpha diversity. Beta diversity was measured with the vegan package. Bray-Curtis values, a method for quantifying dissimilarities between different types, were used. The types used here were Sponge: Before, Sponge: After, Water: Before, Water: After, Microbial Mat, and Algae. In both packages, Shannon's index and Inverse Simpson's index were used. Alpha diversity appears to be in two groups. One group appears to contain sponge samples after exposure, microbial mat, and algae. The second group contains sponge samples before exposure, water samples before exposure, and water samples after exposure. This separation is seen in Shannon's Index and Inverse Simpson's Index. The same trend is seen with beta diversity**.** A t-test was performed at a 95% interval to determine if sponge samples (after and before exposure) had a significantly different beta diversity and alpha diversity (beta: t = 2.5749, df = 3.9593, p-value = 0.06228 alpha: t = 2.5789, df = 3.9604, p-value = 0.062).

An NMDS was plotted in PRIMER utilizing relative abundance (**Fig. 2**). Using the ANOSIM (Analysis of similarities) function, no significance was seen between the relative abundance and the type of experiment (p-value=0.073). The same trend was seen when comparing the

following: sponge and water (p-value=0.054), After samples (p-value=0.567), Before and After (p-value=0.14), sponge samples after exposure and water samples after exposure (p-value>0.05), sponge samples before exposure and water samples before exposure (p-value= 0.20), sponge samples before exposure and sponge samples after exposure (p-value>0.05). An ANOSIM was also performed to determine if individual sponge (Sponge 1, 7, and 8) influenced relative abundance. A significant relationship between the individual sponge and relative abundance was seen (p-value=0.01, R=55.8%).

A shaded plot with clustering was constructed to dominate 30 classes within the Long-Term samples (**Fig. 3**). Clostridia was seen to be high in Sponge 7 before exposure, which decreased in abundance after exposure. Bacteroides was seen to increase in relative abundance within all sponge samples after exposure compared to before exposure. Gammaproteobacteria was seen to decrease in all samples after exposure compared to before exposure. Deltaproteobacteria increased in Sponge 7 and 8 after exposure compared to before. However, Deltaproteobacteria decreased in Sponge 1 after exposure compared to before exposure. Alphaproteobacteria increased after exposure compared to before exposure in Sponge 1 and 7. Alphaproteobacteria decreased after exposure compared to before exposure in Sponge 8. Water from the tank of sponge 7 showed a high abundance of Alphaproteobacteria, but the sample was not taken after exposure. Water from the tank of sponge 1 decreased in Alphaproteobacteria, decreased in Bacteroidia, increased in Oxyprotobacteria after exposure compared to before exposure. Water from the tank of sponge 8 increased in Deltaproteobacteria after exposure compared to before exposure. Water from the tank of Sponge 8 decreased in Gammaproteobacteria, Alphaproteobacteria, and Verrucomicrobiae after exposure compared to before exposure. The microbial mat consisted of Alphaproteobacteria, Clostridia, Deltaproteobacteria, and Bacteroides. Algae had a high amount of Alphaproteobacteria, Gammaproteobacteria, Bacteroidia, and Oxyphotobacteria.

A shaded plot with clustering was constructed to dominate 30 orders and families within the long-Term samples (**Fig. 4**). Within sponge 1 and 7, there were abundant Rhodobacterales before exposure, but the relative abundance still increased after exposure. This trend was not seen for Sponge 8, who decreased in relative abundance after exposure. The majority of this abundance can be attributed to the family Rhodobacteraceae (Genera *Rhodobacter, Paracoccus, Desulfovibrio, Loktanella, and Oceanicella*). Sponge 1 samples of Flavobacteriales and

Cytophagales increased after exposure, while decreased after exposure in Sponges 7 and 8. Sponges 8 and 7 both increased Desulfovibrionales (Family Desulfovibrionaceae) and Desulfuromonadales (Family Desulfuromonadaceae). Sponge 8 and 7 also increased in the order Bacteroidetes. Sponge 7 had an abundant Clostridia (Family XII), which was seen to decrease after exposure.

Within water samples, Rhodobacterales (family Rhodobacteraceae) were seen to be abundant. This family increased in the water from Sponge 8 and decreased from Sponge 1. In water from the tank of Sponge 1, a high abundance of Flavobacteriales (Family Flavobacteriuaceae) slightly increased after exposure. In Sponge 8, these taxa decreased. Recall the water from the tank of Sponge 7 did not get collected after exposure. Sponge 8 decreased in Oceanospirillales (From Saccharospirillaceae and Nitrinoclaceae) and Verrucomirobiales (From the Family Rubritaleaceae). An increase of Bacteriodales and Clostridiales (families of XII, family XIII, and Lachnospiraceae) within Sponge 8 after exposure.

Microbial mat samples contained an abundance of the order Rhodobacterales (family Rhodobacteraceae), Oceanspirillales (Nitrincolaceae, and Oceanospirillales), Clostridiales (Lechnospiraceae, families of XII, and family XIII), Desuldovibrionales (Family Desulfovibrionacaea), and Desulfuromonadales (Family Desulfuromonadaceae), Bacteriodales, Campylobacterales, Flavobacteriales. Algae showed an abundant of Rhodobacterales (Family Hyphomonadaceae and Rhodobacteraceae), Oceanospirillales (Family Nitrincolaceae), Flavobacteriales (Family Crymorphoraceae), Rickettsiales, Alteromonadales (Family Alteromonadaceae, Colwelliaceae), Caulobacterales (Family Parvularculaceae), Chitinophagales (Family Saprospiraceae), Cytophagales (Family Cyclobacteriaceae), Nostocales, and Phormidesmiales (Family Nodosilineaceae).

Simper analysis was implemented on all samples. The major contributing taxa for the dissimilarity of sponge samples compared before and after exposure had a dissimilarity of 89.66% (**Table 2**). Simper showed a dissimilar rate of 89.41% between water samples before and after exposure (**Table 3**). Water and sponge samples showed a dissimilar rate of 92.57%.

**Table 1: MiSeq sequencing read statistics.** The sequencing reads per sample are detailed below. Filtered reads are the reads that were kept after the filtration with dada2. The date at which the experiment was started and ended can be seen on the right-hand side. Average and standard deviation reads can be seen in the last two rows.

| ***Sequencing Reads per Samples*** | | | | | | |
|---|---|---|---|---|---|---|
| ***Sample ID*** | **Raw Reads** | **Filtered Reads** | **Percentage of filtered reads in raw reads** | **Date Experiment Started** | **Date Experiment Ended** | **Total Experimental Time** |
| ***W1B*** | 166987 | 145070 | 86.88 | Sept 24, 2019 | Oct 22, 2019 | 28 days |
| ***S1B*** | 60933 | 21022 | 34.5 | | | |
| ***A1*** | 49921 | 23301 | 46.68 | | | |
| ***S1A*** | 23940 | 19968 | 83.41 | | | |
| ***W1A*** | 66931 | 36766 | 54.93 | | | |
| ***W7B*** | 50099 | 44451 | 88.73 | Feb 4, 2019 | Feb 22, 2019 | 18 days |
| ***MM7B*** | 28948 | 25103 | 86.72 | | | |
| ***S7B*** | 34602 | 22990 | 66.44 | | | |
| ***A7*** | 150992 | 131440 | 87.05 | | | |
| ***S7A*** | 38523 | 34562 | 89.72 | | | |
| ***MM7A*** | 68949 | 57706 | 83.69 | | | |
| ***W8B*** | 48819 | 22443 | 45.97 | Feb 4, 2019 | Feb 29, 2019 | 25 days |
| ***S8B*** | 168651 | 123540 | 73.25 | | | |
| ***A8*** | 36469 | 31509 | 86.4 | | | |
| ***MM8*** | 27108 | 20297 | 74.87 | | | |
| ***S8A*** | 29681 | 23363 | 78.71 | | | |
| ***W8A*** | 48614 | 40878 | 84.09 | | | |
| ***Average*** | **64715.7059** | **48494.6471** | - | - | - | |
| ***SD*** | **48516.574** | **41925.6992** | - | - | - | |


**Figure 2: NMDS plot based on various samples and exposures.** The above illustration shows the non-metric multidimensional scaling ran on multiple groups. The analysis is shown for all samples based on the type of sample (part A), for all sponge and water samples (part B), all samples based on time (part C), and based on sponge number (part D).

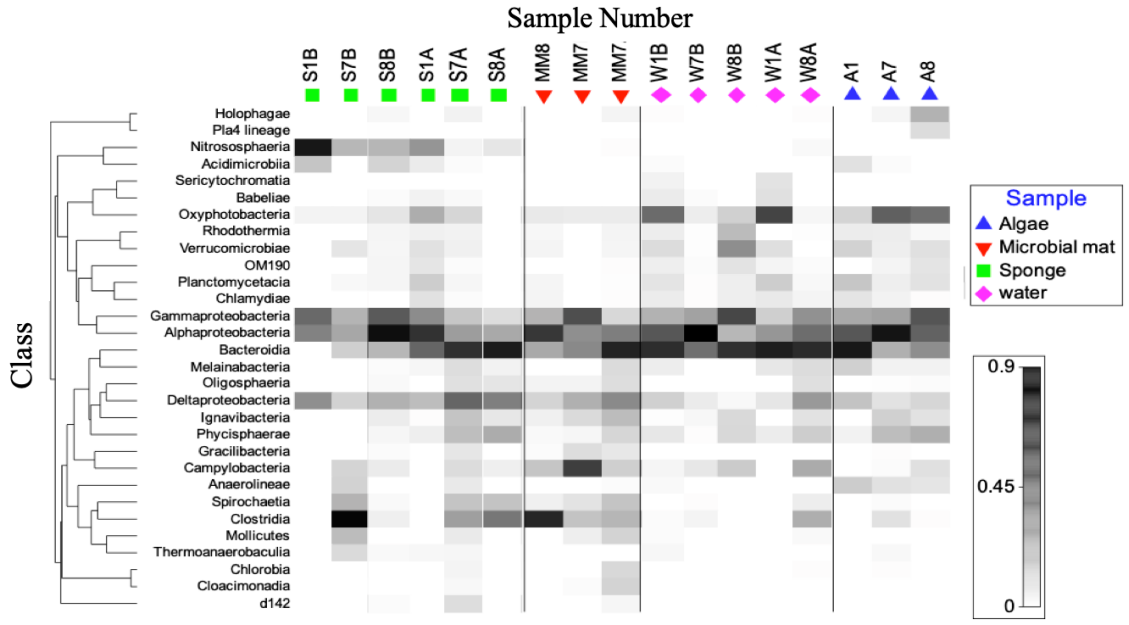


**Figure 3: Shaded plot based on the relative abundance of classes.** The shaded plot and clustering based on the relative abundance of classes in all samples tested. The sample legend is seen on the right. Recall only the top 30 abundant classes were graphed.

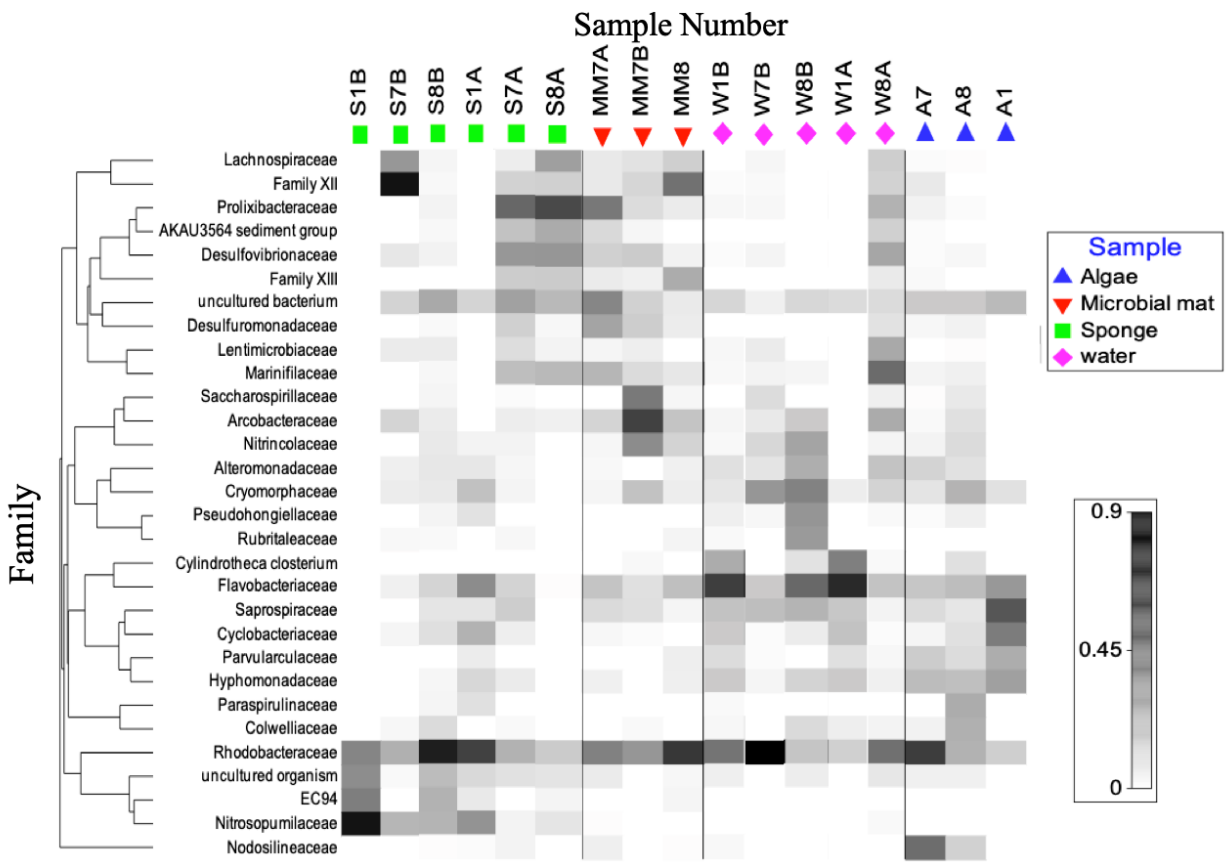


**Figure 4: Shaded plot based on the relative abundance of families.** The shaded plot and clustering based on the relative abundance of the family in all samples tested. The sample legend is seen on the right. Recall only the top 30 abundant families were graphed.

**Table 2: Dissimilar taxa within the sponge sample before and after exposure using Simper percent contribution.** The above was the results of a Simper test to determine major dissimilar ASVs within sponges before and after the exposure to $H_2S$. Overall, a dissimilarly of 89.66% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Dissimilar Taxa within before Sponges Sample before and After Exposure using Simper***

| *ASVs* | Taxonomy | After Average Abundance | Before Average Abundance | Average Dissimilarity | Percent Contribution | Sulfur Metabolism | P-value |
|---|---|---|---|---|---|---|---|
| *53* | Family Nitrosopumilaceae | 0.13 | 0.31 | 2.04 | 2.28 | - | 0.03 |
| *58* | Genus *Draconibacterium* | 0.29 | 0.01 | 2.02 | 2.25 | Possible Sulfate Reduction | 0.45 |
| *52* | Family Rhodobacteraceae - Unculutred | 0.18 | 0.35 | 1.57 | 1.76 | Possible Thiosulfate oxidation | 0.03 |
| *50* | Order Clostridiales - Family XII | 0.03 | 0.25 | 1.48 | 1.65 | - | 0.08 |
| *56* | Order Betaproteobacteriales - EC94 | 0.03 | 0.17 | 1.15 | 1.29 | Possible sulfur reduction | 0.02 |
| *55* | Class Deltaproteobacteria - NB1-j | 0.06 | 0.18 | 1.04 | 1.16 | Possible sulfur Reduction | 0.02 |
| *28* | Genus *Halodesulfovibrio* | 0.16 | 0.02 | 1.01 | 1.13 | Known sulfur Reduction | 0.22 |
| *63* | Order Phycisphaerales - AKAU3564 | 0.13 | 0.01 | 0.89 | 1 | - | 0.1 |

**Table 3: Dissimilar taxa within before sponges water before and after exposure using Simper.** The above was the results of a Simper test to determine major dissimilar ASVs within water samples before and after the exposure to $H_2S$. Overall, a dissimilarly of 89.41% was seen. Sulfur metabolism, if any is known, is marked on the left.

***Dissimilar Taxa within before Sponges Water before and After Exposure using Simper***

| *ASVs* | Taxonomy | After Average Abundance | Before Average Abundance | Average Dissimilarity | Percent Contribution | Sulfur Metabolism | P-value |
|---|---|---|---|---|---|---|---|
| *65* | Family Flavobacteriaceae | 0.19 | 0.32 | 2.02 | 2.25 | Possible Sulfate Reduction | 0.13 |
| *80* | Genus *Cylindrotheca* | 0.08 | 0.18 | 1.15 | 1.29 | | 0.06 |
| *68* | Class Oxyphotobacteria | 0.11 | 0.17 | 1.1 | 1.23 | | 0.12 |
| *81* | Genus *Marinifilum* | 0.01 | 0.21 | 1.08 | 1.21 | | 0.46 |
| *14* | Family Rhodobacteraceae | 0.16 | 0 | 1.02 | 1.14 | Possible sulfur oxidation | 0.72 |
| *31* | Family Rhodobacteraceae | 0.13 | 0.14 | 0.98 | 1.09 | Possible sulfur oxidation | - |
| *40* | Genus *Phaeodactylibacter* | 0.14 | 0 | 0.91 | 1.01 | | 0.16 |

## DISCUSSION:

**Five-Hour Drop Experiments:** The consumption rate of hydrogen sulfide ($H_2S$) within both sponge and the control (non-sponge) samples had a significant relationship with time (p-value<0.05). It should be noted that a dramatic drop in concentration (about 1.5 hours) can be seen. This extreme drop could result from a change in sponge pumping, directly correlated to the amount of water flow through the sponge (Massaro et al., 2012; Ludeman et al., 2014; Ludeman, Reidenbach & Leys, 2017). Water and other molecules move at a faster speed when near the sponge. Molecules farther from the oscula will move slowly, potentially taking hours to reach the sponge. This change in current could create a dramatic decrease in $H_2S$. It was noted but not measured that the osculum would become larger throughout the experiment. Current research has suggested sponges have a sensory ability termed 'inflation-contraction response'. This response suggests that the sponge increase pumping to eliminate unwanted waste (Ludeman et al., 2014). No water flow was within the experiment, which could influence the rate of sulfur exposure and sulfur processing rate.

GAM models indicated a significance based on the type of sample (control or sponge) **Fig. 1**). The control sample indicates the natural diffusion of $H_2S$ into the atmosphere. The significance supports that the sponge does have an impact on the uptake of $H_2S$. An impact on the uptake of $H_2S$ suggests that sponges have an active role in the sulfur cycle.

There are several oscillations that the GAM does not explain. They may be due to the improper handling of the probe. If the lab bench was bumped or disturbed, the probe could have varying measurements. The probe is extremely sensitive. Thus, these varying measurements could be the movement of water and $H_2S$ molecules.

Additionally, the solutions were not mixed. The solution was not mixed because it would cause increased oxidation. Hence, the solution may not have been homogeneous. The probes are extremely precise, down to the µmol. If there is a change, the probe will detect it. All experiments had noticeable increases of $H_2S$ within the sponge compared to the Control. This could be due to the hydrogen sulfide previously present in the tissues of the sponges. If the sponge is already producing $H_2S$, it could be transferred into the experiment. Overall, the rate is what was being compared, not the starting concentration.

**Vertical distribution Experiments:** Both Sponge 4 and Sponge 5 uptake of $H_2S$ concentration have a significant relationship with respect to depth (p-value>0.5). Sponge 4 showed more of a

linear decrease, while Sponge 5 showed a plateau from depth 0 to about 400 µm followed by a linearly decrease. Sponge 4 had its vertical distribution measure before the five-hour drop measurements were taken, while Sponge 5 had its vertical distribution take after the five-hour drop measurements. The solution was not mixed because it would cause increased oxidation. Therefore, the plateau seen in Sponge 5 was most-likely caused by the threshold of pumping action seen in the five-hour drop experiments. Recall the solutions were not mixed. Thus, the increased starting concentration of sponge 4 could be due to a non-homologous mixture.

**Five-Hour Uptake Experiments:** All samples showed a significant relationship between $H_2S$ consumption and time ($p<0.05$). All functions were of the Gaussian Family and Identify link function with a formula of $H_2S$_measurment ~ s(Hour). The GLM shows a significant difference for the average sponge samples and average control samples ($p<0.05$), meaning they do not have the same uptake rate. This difference did have significant interaction between type (sponge or control) and hour. The boxplot demonstrates that the control and sponge values begin around the sample value; the control then consistently stays above the sponge values, indicating that the sponge has an increased uptake rate compared to the control.

**Microbiome analyses to characterize of Long-Term Exposure to Hydrogen Sulfide:** The alpha and beta diversity structures were determined for long-term exposure experiments. Alpha diversity appears, by studying the boxplot, to be separated into two groups (one containing Sponge: After, Microbial Mat, Algae and Sponge: Before, another containing Water: Before, Water: After). This trend was also seen for beta diversity. A t-test did show light insignificance in alpha and beta diversity of sponge samples before compared to after at a 95% interval (p-value≈0.06). This difference was expected as a change in nutrients should, over time, change the bacterial composition, suggesting that the species composition and abundance changes in sponges before and after the exposure. The significance in alpha and beta diversity suggests that bacterial composition did change after exposure. However, relative abundance did not show a significant difference in any groups (p-value>0.05). It should be noted that the significance of the relative abundance of families was slightly insignificant (p-value≈0.06). The slight insignificance suggests that there are not large community differences, but there are differences seen on the microscale.

The NMDS plots did not show any specific trends when looking at all samples in sample type (**Fig. 2, part A**). No trend was seen when comparing all samples based on time (**Fig. 2, part**

**C**). The NMDS show the water samples clustered more closely together than throughout sponge samples (**Fig. 2, part B**). This clustering was not significant but noticeable. *Cinachyrella* sponges and surrounding water have been found to have a significantly different microbiome (Cuvelier et al., 2014). Data compiled here may not have enough replicates to see a significant difference.

The NMDS did demonstrate samples taken from the same sponge are more similar than samples from the same type (sponge, microbial matt, algae, and water) or the same exposure (before and after) (**Fig. 2, part D)**. With this seen, an ANOSIM was performed to determine if the samples taken from the same sponge influenced relative abundance. A significant relationship between sponge number and relative abundance was seen (p-value>0.05). This relationship could be due to a difference in species. Cuvelier et al. (2014) demonstrated that different species of *Cinachyrella* have distinct microbial communities. However, species were unable to be determined in this study.

A shaded plot was constructed and allowed the determination of enrichment of specific samples (**Figs. 3 and 4**). Within microbial mat samples, only one group is knowns to undergo sulfur metabolism, Rhodobacteraceae (Pujalte et al., 2014a). This group is highly abundant in the microbial mat formed on Sponge 8. Rhodobacteraceae is considered one of the most diverse bacterial lineages in the marine habitat (Giovannoni & Rappé, 2000; Garrity et al., 2005; Pohlner et al., 2019). Rhodobacteraceae is found readily in the waters of Ft. Lauderdale (Campbell et al., 2015) and *Cinachyrella* (Cuvelier et al., 2014). This lineage undergoes sulfur metabolism, aerobic anoxygenic photosynthesis, carbon monoxide oxidation, and the use of organic or inorganic compounds (Pujalte et al., 2014a). The ASVs found in this study did not indicate a particular species or genus. All were listed as uncultured. Thus, it is highly debatable that these isolates engage in sulfur metabolism.

Samples from Sponge 1 showed elevated counts of ASVs in the order Rhodobacterales and the class Deltaproteobacteria. Both of these groups contain sulfur metabolism (Garrity, 2005; Muyzer & Stams, 2008), with Deltaproteobacteria engages in sulfur reduction while Rhodobacterales engages in sulfur oxidation. These taxa's presence indicates that SRB and SOB in the sponge tissue perform a functional role in the sulfur cycle. However, after being enriched with $H_2S$, these bacterial counts were depleted. Sponge 7 and Sponge 8 samples also showed ASVs in the order Rhodobacterales. After enrichment, this bacterial order was depleted, but

Desulfvibrionacaea and Prolixibacteraceae (genus *Draconibacterium*), a well-known family of sulfur metabolites, was enriched. Order Rhodobacterales was seen in the water samples taken from the tank of Sponge 7 before exposure. The water from Sponge 8 after exposure showed enrichment of Rhodobacteraceae and Desulfvibrionacaea. This particular sponge appeared to disintegrate towards the end of the exposure. This desecration could have easily mixed spongy tissue into the water column, meaning transfer from sponge symbionts to the water column. It is also possible the disintegration of sponge tissue trapped water, meaning the transfer of microbes from the water to the sponge. It can be concluded that enrichment did occur. Because Sponge 1 also contains both taxa within sponge tissue, it is more likely the enrichment was initiated by the sponge, then transferred to the water column.

Simper files were constructed using PRIMER. A variety of sulfur metabolizing microbes, including genus *Desulfuromusa* (ASV 24), family Rhodobacteraceae (ASV 25, 27, 20,31, 33, 35, 16, 48, and 51), genus *Halodesulfovibrio* (ASV 28), and genus *Desulfovibrio* (ASV 43), was seen to contribute up to 22% of the microbial mat samples (**Appendix A, Table 13**). This high abundance of sulfur cycle engaging microbes suggests that the microbial mat was formed by SRB and SOB bacteria due to the addition of $H_2S$.

Before exposure, sponge samples had the highest abundance of microbes from Rhodobacteraceae (ASV 52), Gammaproteobacteria (ASV 54), Deltaproteobacteria (ASV 55), Nitrosopumilaceae (ASV 53), totaling more than 48% combined (**Table 5**). The major contributing taxa within sponge samples after exposure included *Desulfovibrio* (ASV 43), Halodesulfovibrio (ASV 28), and *Desulfobacter* (ASV 91). These taxa are known to be sulfate reducers. These taxa showed a percent contribution of 6.49% together. Overall the number of sulfur metabolites was 18 out of the 24 top contributors. Taxa that contributed to the sponge's most different composition before exposure compared to after was Nitrosopumilaceae (ASV 53) at 2.28%. This particular family was seen to have a higher abundance before exposure. On the other hand, Genus *Draconibacterium* (ASV 58) was not in sponge samples before exposure but increased to 0.29 count after exposure (**Table 2**). *Draconibacterium* is a relatively new bacterial taxa, only proposed in 2014 (Du et al., 2014). NCBI taxonomy browser recognizes three species: *Draconibacterium filum*, *Draconibacterium orientale*, and *Draconibacterium sediminis*. Kegg currently only recognizes *D. orienta* as a sulfate reducer.

The highest abundance of taxa in tank water before exposure stemmed from *Phaeodactylibacter* (ASV 40), *Hyphomonas* (ASV 1), *Cryomorphaceae* (ASV 11), *Mesoflavibacter* (ASV 72), and *Francisella* (ASV 75), totaling 42%. After exposure, tank water increased in *Francisella* (ASV 71) and *Hyphomonas* (ASV 1) to 80% contribution. *Francisella* is of order Thiotrichales. Individuals of this genus strictly aerobic and contain the species *Francisella tularensis*, which causes tularemia in animals and humans (Slack, 2010). It is not unusual for this group to be isolated from the marine habitat (Petersen et al., 2009). No sulfur metabolism was found in the literature for *Francisella* (ASV 71). *Hyphomonas* is a genus within the order Rhodobacterles. This group is mainly found in the seawater (Lee et al., 2005) and is known to undergo sulfur oxidation (Moore, Weiner & Gebers, 1984). Flavobacteriaceae (ASV 65) and Cylindrotheca (ASV 80) drove key differences between tank water before and after exposure. Both taxa have a higher abundance before exposure compared to after exposure. It should be noted that Rhodobacteraceae (ASV 14) and Phaeodactylibacter (ASV 40) increased from a zero abundance before exposure to 0.15 relative abundance after exposure (**Table 3**). It is not abnormal to see an increase in Rhodobacteraceae because it is known to have members undergo sulfur oxidation (Pujalte et al., 2014b). No sulfur metabolism was identified for Phaeodactylibacter. The influential taxa contributing to the differences in all sponge and water samples were Nitrosopumilaceae (ASV 53) and Rhodobacteraceae (ASV 52). Neither group was present in water, but rather in sponge samples.

It should be noted that sulfur metabolism was inferred through a literature search. I would have applied functional analysis, such as using PICRUSt2 analyses of KEGG pathways, but I ran out of time. As in Vijayan (2015) Acidobacteria, Cellvibrionaceae, Colwelliaceae, Rhodobacteraceae, and Gammaproteobacteria were documented in *Cinachyrella* spp. A small abundance of Chromatiales, purple sulfur bacteria, and family Chlorobiaceae, green sulfur bacteria, was seen. Dominant microbial phyla associated with marine sponges are Proteobacteria (especially the classes Alpha-, Gamma- and Deltaproteobacteria), Chloroflexi, Actinobacteria, Acidobacteria, Nitrospirae, and the candidate phylum Poribacteria (Hentschel et al., 2012). Various Proteobacteria, Chloroflexi, actinobacteria, Acidobacteria, and Nitrospirae, were observed in the data. However, Poribacteria was curiously not seen even though Cuvelier et al. (2014) determined that this was an abundant phylum in *Cinachyrella* spp.

## CONCLUSION:

Five-hour drop and five-hour uptake experiments showed a significant relationship between time and $H_2S$ consumption, while vertical distribution showed a significant relationship between depth and $H_2S$ consumption. A GAM was the best model for all experiments. These experiments show over time and depth that $H_2S$ is consumed readily in a sponge environment. In each of these instances, the sponge always increased consumption compared to the control, representing the natural diffusion rate. When a GLM and GAM compared the natural diffusion rate to the uptake rate caused by a sponge, there was a significant difference; meaning the $H_2S$ consumption rate was significantly affected when a marine sponge was introduced.

Long-Term exposures did not show a significant difference in relative abundance on a community scale, not supporting hypothesis 6. There was a significant difference in beta and alpha diversity. Sponge samples were seen to host SRB and SOB before exposure supporting hypothesis 5 and was seen to be enriched when introducing $H_2S$ supporting hypothesis 8. Using 16S rRNA data, the microbial mat appeared to host SRB and SOB bacterial taxa, specifically genus *Desulfuromusa*, family Rhodobacteraceae, genus *Halodesulfovibrio,* and genus *Desulfovibrio,* supporting hypothesis 7. This abounding data indicates that SRB and SOB within *Cinachyrella* spp. play a functional role in the sulfur cycle.

Sponges evolved in prevalent sulfur oceans (Balter, 2015; Fike, Bradley & Rose, 2015). A High amount of sulfide is extremely toxic to many animals. By partnering with an organism that can remove toxins from an environment, individuals can continue to live. This relationship may have begun this way, a way for both parties to survive, the microbe getting housing and protection, while the sponge was getting toxins removed from its tissues. The inflation-contraction response seen was the sponge's attempt to remove the toxin faster. Over time the ocean became less sulfur concentrated, possibly influencing the sulfur metabolites by shrink in number but not disappearing. The sponge still needed to remove harmful sulfur toxins but did not necessarily need a high abundance, leading to a lower abundance of sulfur metabolites than other metabolites. Studies on inverts, such as oligochaete worms (Dubilier et al., 2001), have similar SOB and SRB relationships.. The host receives carbohydrates, while the microbes receive protection, housing, and nutrients. Thus, it is thought that sponges also receive a carbohydrate

benefit (Tian et al., 2016). This benefit could be one reason this relation continues to survive in a less sulfur-concentrated ocean.

Sulfate-reducing bacteria use sulfate as the electron acceptor producing sulfide. Sulfide-oxidizing bacteria utilize sulfide to produce biological sulfur and sulfate. This study saw taxa such as *Desulfobacter* producing $H_2S$ and *Ruegeria* removing $H_2S$. Thus, SRB may produce sulfide for SOB, which produces sulfate for SOB and continue in a cycle, utilizing the same sulfur molecules. Thus, isotopic tracing of sulfur should be conducted to determine the converted molecules produced, helping determine what carbohydrates are being produced. A more functional-based study should be done to determine what genes and pathways produce the carbohydrates or other molecules produced. Targeted sequencing of sulfur metabolite microbes should be completed to get a complete look at the sulfur cycle of sponges. To date, only one study has identified sulfur metabolite genes in *Cinachyrella* spp. Shmakova (2020) identified characterize five sulfur related metagenomically assembled genomes (MAGs) (Shmakova 2020): *Opitutaceae bacterium, Thioalkalivibrio paradoxus, Desulfobacterium autotrophicum, Thioalkalivibrio sulfidiphilus, Sulfurifustis variabilis*. Also identified were 27 MAGS related to sulfide reducing genes (Shmakova, 2020). Within *Lophophysema eversa*, genomic features of sulfite-oxidizing genes were found (Tian et al., 2016)

I believe it is essential to understand if these are true symbionts of the sponge. To determine that, we need to determine if the sponge can continue to live without these symbionts. The inflation-contraction response and uptake of $H_2S$ caused by microbes may not be connected. If the sponge can live without the symbionts, there would be evidence to suggest the adaptation was occurring by microbes, not sponges, suggesting a more commensal relationship.