

CHARACTERIZATION OF THE MICROBIOME OF FRESHWATER SPONGES UNDERGOING
ASEXUAL REPRODUCTION

A Thesis
by
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Abstract

CHARACTERIZATION OF THE MICROBIOME OF FRESHWATER SPONGES UNDERGOING ASEXUAL REPRODUCTION

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Sponges are sessile filter-feeding organisms that are found in both marine and freshwater habitats and are known to harbor symbiotic microbial communities. There is strong interest in understanding the taxonomic and functional composition of these microbial communities due to their integral roles in sponge ecology, nutrient cycling, and production of natural products. Particularly with freshwater sponges, there is limited work on the functional roles of the sponge microbiome. The goals of the current work were to first understand how the composition of these microbial communities change prior to, and post production of asexual reproductive bodies known as gemmules in freshwater sponges. I also aimed to determine what role, if any, the microbiome may have in the facilitating developmental change of the sponge host. Preliminary taxonomic profiling by 16S rRNA gene sequencing of sponges and river water indicated the presence of putatively sponge-enriched bacterial taxa with approximately 60% of taxa unique to the sponges. Metatranscriptome analysis of five sponge microbiomes collected prior to gemmule formation (“pre” samples) and three separate sponge microbiomes post gemmule formation (“post” samples) highlighted four key results: 1) taxa within the phyla *Bacteroidetes* and *Proteobacteria* (class

Alphaproteobacteria) were significantly different between pre and post samples in the gene expression analysis and these taxa were identified as enriched in the sponges based on 16S taxonomic profiling, 2) the presence of eukaryotic-like domains and a type IX secretion system that may be important in symbiosis, 3) multiple functional transcripts were enriched in the post samples (e.g., porin protein, pilus assembly protein) potentially indicative of long-term symbionts that may be present in all life stages of the sponge, 4) multiple taxa within the class *Flavobacteriales* that decreased in the post samples, potentially indicative of facultative symbionts. This study has implications for better understanding the functional roles of the microbiome in freshwater sponges and draws implications that may be relevant to marine species and other microbial symbiont systems.

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1. Introduction

1.1 Introduction to Freshwater Sponges

Sponges are in Phylum Porifera, the most basal clade of metazoans and appear in the fossil record approximately 600 million years ago (*Love et al., 2009*). The Porifera have proliferated to many diverse forms and lineages in the ocean, while only one major lineage is present in freshwater worldwide: Order Spongillida in Class Demospongiae (*Manconi & Pronzato, 2002*). The suborder Spongillida, contains 6 families, 45 genera, and 219 species currently described (*Manconi & Pronzato, 2008*). In contrast, marine sponges have over 10,000 species belonging to 107 genera within 21 orders (*Zhang et al., 2003*). Most marine species are within the Class Demospongiae, although sponges in other classes are also represented (i.e., glass sponges, calcareous sponges, and encrusting homoscleromorph sponges).

Freshwater sponges are sessile, filter feeding animals that inhabit a variety of freshwater systems including streams, lakes, rivers and ponds and can be found in both lentic (still water) and lotic (flowing water) habitats (*Manconi & Pronzato, 2008*). Sponges attach themselves to various substrata submerged in the water, including rocks, bivalve shells, and man-made debris (*Pronzato & Manconi, 2002; Ricciardi & Resiwig, 1992*). These organisms also have the ability to tolerate extreme chemico-physical conditions (e.g., desiccation, cold temperatures) (*Harrison, 1974; Pronzato & Manconi, 2002*). The ability to survive such conditions is in part, due to an asexual life cycle that includes a resting stage called gemmules, which can survive harsh environmental conditions to produce a new sponge when the environment is favorable again. The production of gemmules is characteristic of freshwater sponges, with very few marine sponges carrying this trait.

Although freshwater sponges are not as abundant or speciose as marine sponges, they still play an important role in the ecology of freshwater environments. They are prolific filter feeders and play an active role in recycling suspended organic materials that reside in the water column (*Manconi & Pronzato, 2008*). Many freshwater sponges are also important in primary production and nitrogen cycling with some sponges hosting high numbers of unicellular algal symbionts (*Frost, 1978; Frost & Williamson, 1980*). Freshwater sponges also host other microbial symbionts that may have important roles in nutrient dynamics and in host function (e.g., *Gaikwad et al., 2016*). However, the characterization of the microbiome of freshwater sponges lags behind that of marine sponges (*Thomas et al., 2016*). Sponges are also an important food source for other animals that inhabit freshwater systems, including insects, waterfowl, fishes, and turtles (*Manconi & Pronzato, 2007; Ricciardi & Resiwig, 1992*). Thus, there is interest in better understanding their ecological roles and the factors that influence their development and survival. Such factors include environmental parameters as well as associated microbes within the sponge (*Gaikwad et al., 2016; Pronzato & Manconi, 2002*). While the exact nature of the relationship of the associated microbes within freshwater sponges is not known, most are considered as commensals (similar to marine sponges) (*Keller-Costa et al., 2014; Schmitt et al., 2007; Taylor et al., 2007*), where the host may not benefit from harboring the microbes, but it is also not harmed. I use the term microbiome to include the community of associated microbes within the sponge and the term symbiont to refer to sponge-associated microbes regardless of their beneficial, negative, or neutral interaction with the host (*de Bary, 1879*).

1.2 Identification of Freshwater Sponges

Freshwater sponges are among the least studied faunal group for several reasons (*Ricciardi & Resiwig, 1992*); the primary challenge is their highly variable morphologies (e.g.,

body shape, dimension, texture, color) between species and even among individuals (*Manconi & Pronzato, 2008*). The lack of distinguishable morphological features also makes differentiating between species in the field difficult (*Ricciardi & Resiwig, 1992*). Furthermore, there are few informative taxonomic keys for freshwater sponges as the taxonomy is not well resolved. Most of the current taxonomic keys lack basic information on the different species, such as ecological variation, environmental factors that can be correlated with individual species, and a range of morphological variation among the different species (*Ricciardi & Resiwig, 1992*).

The skeletal make up of freshwater sponges, which is used in species identification, is comprised of collagen and spongin fibers embedded with spicules to provide the animal with strength and flexibility (*Aguliar-Camacho et al., 2019*). Spicules are siliceous, needle-like structures that comprise the mineral skeleton of the sponges and form the structural support for the sponge's filtration system (*Bart et al., 2019*). Taxonomy for sponges is based on the morphology (e.g., shape and size) and arrangement of these spicules (*Hooper & Van Soest, 2002; Manconi & Pronzato, 2008; Morrow & Cárdenas, 2015*). Spicules are classified in the following three general classes: megascleres, microscleres, and gemmuloscleres (*Ricciardi & Resiwig, 1992*). Megascleres are large spicules that form the sponge's main skeleton, while microscleres are smaller spicules (although not always present in some species) that are free-floating in the tissue. Freshwater sponges have only one type of megascleres, called oxeas, whereas microscle spicules can vary in shape, but are typically monoaxial (*Manconi & Pronzato, 2008; Morrow & Cárdenas, 2015*). Lastly, gemmuloscleres are spicules that form around the resilient coat of the gemmule (*Ricciardi & Resiwig, 1992*), which are asexually produced, spherical-shaped resting bodies of the sponge (*Penney & Racek, 1968*). These gemmuloscleres are typically required for correct species identification, as the variations in size and morphology are often unique to that species (*Penney & Racek, 1968*). However, gemmules are only formed at certain times of the year, hindering identification if they are not present.

1.3 Reproduction of Freshwater Sponges with a Focus on Asexual Reproduction

Freshwater sponges can undergo two types of reproduction: sexual and asexual. In Figure 1, both reproductive cycles are shown for *Ephydatia fluviatilis*, a common and model species for freshwater sponges. Sponges in general, are hermaphroditic, but the timing in production of sperm and eggs is often separate, where an individual may even be one sex for a year then switch the following year (Gilbert & Simpson, 1976). Sexual reproduction occurs when male sponges release sperm into the water column and fertilize eggs in nearby female individuals, where they will begin development of larvae. Sponge larvae will leave the maternal sponge and settle on substrata before beginning to metamorphize into a fully, functioning adult (Funayama et al., 2005). In the asexual cycle, adult sponges produce gemmules, a process triggered by environmental cues (e.g., temperature, water level) and are typically formed toward the end of the growing season and before onset of harsh environmental conditions such as cold temperatures or desiccation (Bart et al., 2019). The process of gemmule formation is usually inhibited by a factor from the parent sponge tissue. The parent sponge postpones this process until these environmental cues occur (Funayama et al., 2005). As the parent tissue begins to degrade, likely cued by abiotic environmental factors, these inhibitors, which postpone the germination (hatching) process, are eliminated (Funayama et al., 2005). Cues for germination, are similar to those that initiate gemmule formation, and include a significant change in temperature, while other factors may be included but not as heavily studied such as: light exposure, pH, water level, and water turbidity. Following these environmental cues, as well as, increases in certain metabolites (such as retinoic acid) have also been documented and may be part of a larger physiological change, but this has been investigated in only a few species (Funayama et al., 2005; Harsha et al., 1983; Ilan et al., 1996; Loomis et al., 2008; Simpson, 1984; Wilkinson, 1980).

Gemmules are spherical, about 100-1200 μm in diameter, and contain totipotent cells that can rapidly proliferate and are able to form a new adult sponge when conditions are suitable again (*Manconi & Pronzato, 2008*). This allows sponges to re-establish the population in a habitat suitable to host the mature form. The totipotent cells are able to overcome extreme environmental conditions, which would typically kill the maternal, adult sponge (*Manconi & Pronzato, 2008*). Depending on the climate, in some regions, most freshwater sponges are typically found in their resting stage of gemmules with limited time represented by their adult sessile form (*Manconi & Pronzato, 2007*). Cells within gemmules are in a state of endogenously controlled diapause development arrest until germination is initiated (*Loomis et al., 2008*).

A unique feature of freshwater sponges is that they are relatively amenable to maintaining them in the laboratory. While sponges typically do not survive in the laboratory beyond two to three weeks, they can be grown from a gemmule to a fully formed, albeit small, individual sponge on a glass slide. Because of this trait, freshwater sponges have been the target of many physiological and evolutionary-development studies (e.g., *Errekes-Medrano et al., 2014*). These traits also make freshwater sponges well-suited to study the relationship between the host life cycle (section 1.4 below) and the microbiome (section 1.5 below), particularly in the formation of gemmules and subsequent germination into a new sponge, the primary goal of the present study.

1.4 Sponge Cell Types, Structures, and Development from a Gemmule

There are multiple types of cells present in sponges, most of which are defined by their morphology. However, the literature has not described in detail the majority of these and mainly focuses on only a few. The major cell types of sponges are listed in Table 1 (*Funayama et al., 2005; Simpson, 1984*). These cell types each exhibit distinct and well-defined morphology that is fairly similar across different species, thus making them easy to identify and study

widely. In addition to their stable morphology, their function is consistent in the role they play in sponge development and their location within the sponge body plan. Below, I highlight major structural components of sponges and gemmules, and the relevant cell types involved. These components are likely important in both the formation of gemmules and the germination process; however, the latter process is better characterized and is the focus in this section.

Sponge gemmules are characterized by two distinct features: an acellular envelope or coat, and a mass of internal cells containing totipotent cells and other cell types, including archeocytes, that have an abundance of cytoplasmic reserve substances (*Simpson & Fell, 1974*). The theca, or gemmule coat, is comprised of a spongin protein covering that protects the cells within it. There can be one, two, or three layers that create the theca and siliceous spicules may be wholly or partially embedded in it, as well. The theca layers are clearly separated and defined in freshwater gemmules, with internal, pneumatic, and external layers. The morphological differences in the layers can also help identify freshwater sponges. Some species exhibit simple structured gemmules with only one or two layers, while others exhibit a gemmule with a tri-layered pneumatic theca or one that displays spiny gemmuloscleres radially arranged around the coat (*Manconi & Pronzato, 2007*).

Gemmules only start to 'hatch' when delivered environmental cues that the adult sponge tissue could survive. The process of a gemmule hatching is called germination. There are roughly six stages of active germination in *E. fluviatilis* (*Funayama et al., 2005*) as seen in Figure 2. Stage 0 consists of the gemmule in a resting state and can be in this stage for many months, even years. Upon active germination, stage 1 typically occurs around day 2 when cells are actively migrating from the gemmule and differentiating into epithelial cells to form an envelope. Around days 3 and 4, the gemmule enters stage 2. Stage 2 consists of cells moving into the epithelial envelope, from stage 1, to proliferate and start differentiating into several different types of cells. Stage 3 of germination takes place when the sponge is about 5 days old.

During this time the aquiferous system within the sponge is formed, as well as, choanocyte chambers (chambers with flagellated collar cells for filter feeding). During this stage, a key process in the development occurs, which is the formation of the skeleton during the vegetative growth phase (*Bart et al., 2019; Funayama et al., 2005*) (Figure 2). Once the sponge is about a week old, stage 4 begins, where the sponge becomes fully functional with the formation of the oscula (an opening where waste water is expelled). The final stage of germination is stage 5. In stage 5 the sponge maintains its current body form and gradually increases in size from this point on (note the scale bar in Figure 2 for size of the germinated sponge).

Some of the cellular processes occurring during each stage of germination has been documented; however, there are still few studies overall on this topic with many physiological components not yet described. When germination is initiated, totipotent stem cells contained within the gemmule start to differentiate into different cells types (*Höhr, 1977*). These totipotent stem cells are referred to as thesocytes. Thesocytes are a double nuclei cell rich in vitelline platelets, or storage organelles, whose purpose is to provide energy for the archeocytes (*Imsiecke et al., 1995*). Thesocytes start to undergo mitosis within the gemmule coat and become archeocytes (stem cells, mononucleated), which further differentiate into histoblasts (nuclei lacking nucleolous, containing few or no vitelline platelets) (*Imsiecke et al., 1995; Simpson, 1984*). These cells (histoblasts and archeocytes) start to migrate outwards from the gemmule coat onto the substrate in which the sponge is developing on. Archeocytes also differentiate into various cell types- including choanocytes and gametes (*Harrison et al., 1977; Höhr, 1977; Rozenfeld, 1970*). Archeocytes are known to contain stored cytoplasmic reserve substances such as RNA, lipids, and polysaccharides that help nurture the sponge as it develops (*Ruthmann, 1965*). While in the early stages of germination, the histoblasts are thought to become the basopinacocytes (basal epithelial cells) that attach the fully functional sponge to the substrate. Figure 3 represents a vertical sectioned view of a gemmule hatching sponge.

Once the sponge has become attached, the migrated cells continue to proliferate into a fully functional sponge (*Funayama et al., 2005; Höhr, 1977*). A key process in the development of the sponge is the construction of its skeleton, which forms the structural support for the sponge's three-dimensional filtration system. The spicule skeleton is comprised of individual, silica spicules embedded in a fibrous organic matrix made from chitin and spongin. Spicules are produced by cells called sclerocytes. The process of skeleton formation in sponges is the only known biological mechanism in which a sequence of cooperative behaviors of individual, specialized cells lead to a self-organized biological structure using non-cellular materials (*Nakayama et al., 2010*).

Sclerocytes are located at various locations within the hatching gemmule, and transported by transport cells to their final position, where they are positioned and anchored by a third type of specialized cell, called basopinacocytes (*Nakayama et al., 2010*). Sclerocytes have been studied in-depth and are characterized by having a well-developed rough endoplasmic reticulum, abundant mitochondria, and several small refracting inclusions (*Simpson & Vaccaro, 1974*). As the cell starts the formation of a spicule, an appearance of an organic axial filament can be seen; as the process continues, the cells are flat with a large contact area to the substrate. As the spicules increase in size, they can be seen protruding from the body of the sclerocytes. Once fully formed, the spicules are embedded in a matrix of collagen-fiber bundles, often referred to as spongin (*Imsiecke et al., 1995*). This spongin is what helps support the surface of the sponge, which is formed by a single layer of thin, pentagonal cells called pinacocytes (pavement cells) that enclose a collagenous mesohyl (middle layer).

Once the spicules are set in an upright position within this spongin, the filter system of the sponge begins to develop, allowing the sponge to start the process of obtaining nutrients via active filter-feeding (*Funayama et al., 2005*). Food particles such as bacteria and single-celled algae are removed from the water and translocated into the mesohyl interior. Some of these

microorganisms can take resident in the extracellular region of the mesohyl, such as mobile amoeboid cells, where they become part of the commensal microbiota of the sponge host (Gernet *et al.*, 2005; review by Hentschel *et al.*, 2012).

Gemmules can be hatched in the laboratory using an iterative washing protocol to isolate from the parent sponge tissue. Then stored and maintained at 4°C with no or limited light exposure for several months to a year prior to initiating active germination (see Appendix I). In contrast to germination, there is little documentation on how the gemmules are formed within the parental sponge. Limited previous work suggests that gemmules are a pocket of resting archeocytes that have migrated within the parental sponge and formed these large masses (Funayama *et al.*, 2005). However, how they become enwrapped by a gemmule coat is unclear. Additionally, whether symbiotic microbes are present in the gemmules, either by a selective process or as an artifact of capturing archaeocytes within the gemmules, has not been documented. Furthermore, any potential role of symbionts in the development of the sponge from gemmules has not been investigated. Rather, most research has been focused on the biology of the hatching process.

1.5 Microbiome of Phylum Porifera

Sponges, as the most basal metazoan, also offer insight into an ancient animal-microbiome relationship. Members of the phylum Porifera are hosts to diverse microbial communities with a range of symbiotic relationships including commensalism, parasitism, and mutualism (Manconi & Pronzato, 2008). Recent work has shown that sponges are selective in hosting specific lineages of bacteria, suggesting there are some selective pressures from the host in establishing and maintaining these symbiotic communities (Costa *et al.*, 2013; Keller-Costa *et al.*, 2014). Furthermore, some symbionts are vertically transferred to the sponge larvae, potentially indicating evolutionary investment in maintaining certain microbial symbionts

across generations (*Hoffmann et al., 2009; Sharp et al., 2007; Steger et al., 2008*). While there are now many studies on sponge microbe phylogeny (e.g., *Hentschel et al., 2002; Taylor et al., 2007; Thomas et al. 2016*), knowledge about the functions and host-interactions remains limited, particularly in freshwater sponges (*Hoffmann et al., 2009*).

Symbiotic microbial communities have major roles in the development, metabolism, and health of their host organisms (*Lloyd-Price et al., 2016; Petersen & Osvatic, 2018*). For example, in the bobtail squid, the host uses the light produced by bioluminescent *Vibrio fischeri*, a horizontally transmitted symbiont, to avoid predation. This squid-Vibrio system has been well characterized, where a series of developmental events is induced by the *V. fischeri* upon colonization in the host light organ (Reviewed by *Nyholm & McFall-Ngai, 2004*). The *V. fischeri* is able to alter the morphology of the light organ to a fully mature and functioning organ (*McFall-Ngai & Ruby, 1998*). In another classic system, the pea aphid *Acyrtosiphon pisum* and its bacterial symbiont *Buchnera*, the symbionts provide essential amino acids to the host, while the host drives the production of these specific amino acids through interactions with the symbiont (*Douglas, 2011*). The symbionts are intracellular, maintained in specialized cells called bacteriocytes, and are critical for the survival of the host (*Gunduz & Douglas, 2009*).

In sponges, some functions of the associated bacteria and archaea have been linked to defense mechanisms, vitamin production for the host, and nutrient utilization and transport, among other roles (*Kiran et al., 2018*). However, few of these putative roles have been empirically tested. Symbiotic *Cyanobacteria*, in particular, has been shown to provide photosynthetically derived carbon to the host sponge (*Freeman & Easson, 2016; Wilkinson & Fay, 1979*), or in some cases, bioavailable nitrogen produced by fixation of atmospheric nitrogen (*Mohamed et al. 2010; Wilkinson & Fay 1979*). Some marine sponges host *Cyanobacteria* within bacteriocytes similar to some insect host-microbe systems (*Wilkinson & Fay 1979*). Freeman and Thacker (2011) documented nutritional (photosynthate) transfer from photosymbionts,

namely *Cyanobacteria*, to the host, and found it to be highly dependent upon sponge species. Some species obtain significant amounts of carbon and/or nitrogen from these symbionts, whereas others receive little carbon or nitrogen from symbionts (*Freeman & Thacker, 2011*).

Aside from photosynthetically derived carbon or nitrogen, there are other possible contributions from diverse microbes. Nitrifying microbes, which convert ammonia to nitrate as a means to produce energy, are present in many diverse sponges and may be important for removal of ammonia produced as a waste product of metabolism by the host sponge (*Hoffmann et al., 2009*). Nitrifying microbes and other microbes with an autotrophic metabolism, where carbon is fixed from dissolved carbon dioxide (CO₂), likely support very little transfer of carbon to the host (*Hoffmann et al., 2009*). Additionally, marine sponges are a major source of bioactive compounds which are of interest as antitumor and anticancer compounds and these may be produced as a means of chemical defense (*Blunt et al., 2004; Kiran et al., 2018; Loh & Pawlik, 2014; Mayer et al., 2010*). Some studies have shown that most of these compounds are biosynthesized by sponge specific microbial symbionts (*Sacristan-Soriano et al., 2011; Wilson et al., 2014*). A study by *Gaikwad et al. (2016)* showed that bioactive compounds are also present in freshwater sponges.

Some morphological differences in cells and body structures are relevant to the ability to host microbial symbionts. For example, of the three types of gemmules produced by the freshwater species *Spongilla lacustris*, the gemmule with the triple-layered coat possessed very few symbionts as compared to the two single-layered coat gemmules (*Simpson, 1984*). Suggesting that the additional layers provided an obstacle or deterrent for symbionts. In marine demosponges, most microorganisms were located extracellularly of the adult sponge and mostly within the sponge mesohyl. There are only a few examples of symbionts located intracellularly within vacuoles of sponge archeocytes and even within the nuclei of specific sponge cells have been observed (*Hentschel, et al., 2003; Vacelet & Donadey, 1977; Wilkinson,*

1978). The sponge structure with the largest effect on the microbial community is the mesohyl, which comprises all of the sponge tissue inside of the outer pinacoderm layer. The mesohyl is where most of the symbionts in marine sponges tend to reside. However, with disruption of the mesohyl, symbionts can relocate deeper in the sponge tissue (*Williamson, 1979*). Although, it is not known if such relocation of symbionts has any effect on the host sponge, it is interesting to note that the symbionts only seem to exhibit this shift in localization upon changes or disturbances to the mesohyl and not at random.

While there are limited studies thus far on the microbiome of freshwater sponges, these communities are likely structured differently from marine communities by (1) the location of the microbes within the sponge as freshwater sponges tend to be thin encrusting forms, (2) likely higher diversity of symbionts are present in marine sponges compared to freshwater sponges, and (3) marine microbes are highly sponge-specific and absent from most environmental libraries that have been constructed already, whereas in freshwater samples the 16S rRNA gene pool have larger similarities with its environment (*Gaikwad et al., 2016; Gernert et al., 2005; Hentschel et al., 2002*). The microbial community found in freshwater sponges, however, may be more specific in its localization within the sponge and so far, are only found intracellularly. For example, zoochlorellae, an alga and commonly found symbiont in freshwater sponges, occur in locations such as: (1) within cytoplasmic vacuoles in archeocytes, (2) within choanocytes of vegetative tissue, and (3) in the thesocytes of gemmules. In a more recent study of *S. lacustris*, a freshwater species, the majority of its putative bacterial symbionts were found in the vacuoles of other cells, while the mesohyl appeared to be lacking any microorganisms (*Gernert et al., 2005; Williamson, 1979*). It is unclear whether the bacteria present in the sponge represent food particles or are symbionts and further work in localization of bacteria in freshwater sponges is necessary (*Gernert et al., 2005*). An interesting observation indicated that archeocytes containing a superabundance of microbes may preclude further

ingestion of food particles as they do not ingest as many particles as those hosting fewer symbionts. This led to the suggestion that the number of occupied vacuoles in the cytoplasm is somehow involved in the uptake rate regulation of additional particles (*Van Weel, 1949*). Two recent studies that leveraged high throughput sequencing, however, have identified putatively sponge-enriched and divergent lineages of bacteria in freshwater sponges (*Gaikwad et al., 2016; Gladkikh et al., 2014*), lending some support to the notion of a truly symbiotic bacterial community in freshwater sponges rather than simply reflecting the same microbes as in the surrounding water.

In the present study, I conducted microbiome profiling analysis on previously collected and sequenced sponge and water samples from the New river, near Boone, N.C. (collected in 2017 and 2018). This profiling analysis leveraged well-characterized protocols for amplification, sequencing, and taxonomic analysis of the 16S rRNA gene, specific to prokaryotes (*Caporaso et al., 2018; Ul-Hasan et al., 2019*;). The microbiome profiling analysis provided a preliminary overview of the composition of sponge microbiome. I then wanted to investigate a potential role of the microbiome in the development of a sponge from gemmule to adult by examining gene expression during germination with and without antibiotics. However, I found that the sponge gemmules that I collected would not hatch in the laboratory, so I shifted my project to: 1) develop a working protocol for hatching sponges in the laboratory, and 2) leverage sponges collected in the field prior to gemmule formation and post gemmule formation to examine gene expression in the microbiome during these times. To examine gene expression, I used a metatranscriptomic approach, where total RNA from the sponge was extracted and sequenced, allowing for functional characterization based on mRNA produced by the sponge and microbes (*Anwar et al., 2019; Fiore et al., 2015; Leimena, et al., 2013; Westreich et al., 2018*). Due to high natural variability in the presence of sponges and the sponge life cycle, sponges collected in 2018 and 2019, that together span the time before and after the onset of asexual

reproduction, were used in the metatranscriptomics analysis. This work presents the first metatranscriptomics analysis of freshwater sponges and lays the groundwork for future experiments aimed at understanding the potential role of the microbiome in the sponge life cycle.

2. Methods

2.1 Field Methods

Freshwater sponge samples were collected for three consecutive years from New river state park in Ashe County, North Carolina (36.4615° N, 81.3387° W). Sponges collected in 2017 were collected for 16S rRNA microbiome profiles and preserved in DNA buffer, whereas sponges collected during 2018-2019 were preserved in RNAlater (Invitrogen, USA) for metatranscriptome analysis. The experimental design was to collect sponges prior to gemmule formation and post gemmule formation for metatranscriptome analysis. In 2018, two individuals were collected in July prior to gemmule formation and two in August post gemmule formation. In 2019, another three individuals were collected in July and August prior to gemmule formation and one individual in September post gemmule formation (Table 2). The individuals collected prior to gemmule formation and after gemmule formation are not the same individuals.

Each sample for microbiome profiling and for metatranscriptome analysis was collected using a sterile razor blade to scrape a portion of the sponge off the rock, or substrate, into a sterile microcentrifuge tube filled with 2mL of DNAbuffer or RNAlater. Those samples collected in 2017 for microbiome profiles of 16S rRNA were stored in 2mL of DNAbuffer (*Seutin et al., 1991*). Each tube was individually labeled with the date and location of each sample and placed on ice until they were returned to Appalachian State University. Samples were stored at -20°C, until further analysis. From these freshwater sponge samples one set (n=5 individuals) from pre-gemmule formation (July 2018 and 2019, August and September 2019) and one set (n=3 individuals) from post-gemmule formation (August 2018, September 2019) were used to generate the metatranscriptome dataset (Table. 2).

Environmental data including three independent water samples (500 mL each) for nutrient analysis and microbiome analysis were collected at each time point. The water samples

were vacuum filtered using a sterile 0.2 μm polyethersulfone filter; the filters were stored in RNAlater and frozen, while approx. 30 mL of the filtrate was stored in a 50 mL conical tube and frozen for inorganic nutrient analysis. Inorganic nutrient analysis of anions (nitrate, phosphate, sulfate, chloride), was performed using an ion chromatograph with quantification based on an external standard curve. The analysis was performed previously by Allison Griggs with Dr. Carol Babyak in Chemistry and Fermentation Sciences at ASU. During 2019, a subset of water samples (1mL each sample) were fixed in filtered paraformaldehyde (0.5% final concentration) and frozen for flow cytometry analysis at Bigelow Facility for Aquatic Cytometry (ME, USA). Additionally, in 2019, temperature, pH, conductivity data were collected using a handheld multiparameter meter (Oakton, IL, USA).

2.2 Laboratory Methods

2.2.1 Preliminary Microbiome Profiling of 16S rRNA Genes

DNA was extracted from sponge and filters collected in 2017 using hexadecyltrimethylammomium bromide (CTAB) extraction. The 16S rRNA genes were amplified using the earth microbiome project (EMP) standard primer set (515F and 806R) and EMP protocol (*Caporaso et al., 2018; Ul-Hasan et al., 2019*). Paired-end library construction and high throughput sequencing using an Illumina MiSeq was performed by Dr. Cole Easson at Nova Southeastern University in January 2018. Sponge and water samples from the New river were analyzed for microbiome taxonomy using the Dada2 pipeline in R (*Callahan et al., 2016*), which produces amplicon sequence variants (ASVs) that differ from each other by a minimum of one nucleotide. The ASVs as well as read abundance within each ASVs were used in alpha and beta diversity metrics using Phyloseq (*McMurdie & Holmes, 2013*) in the R environment. Analysis of similarity (ANOSIM) was used to compare microbiome composition across samples. Further analysis and tests, such as PERMANOVA, will be performed prior to manuscript publication.

2.2.2 RNA and DNA Extractions and Sequencing for Metatranscriptome

Extraction of total RNA and DNA from each of the freshwater sponge samples was performed using a TRIzol Reagent (Sigma Aldrich, USA) per the manufacturer's protocol for simultaneous extraction of RNA and DNA, where RNA- and DNA-containing layers are separated by centrifugation and extracted separately. The extracted RNA was then incubated with DNase I Treatment (Promega, WI, USA) to remove genomic DNA and Monarch RNA Cleanup Kit to remove protein and other contaminants (New England Biolabs, MA, USA). DNA extraction was performed using the TRIzol Reagent standard protocol. The concentration and quality of both the RNA and DNA were analyzed via NanoDrop 2000. Due to low concentration and quality, the extracted RNA was further purified with a phenol-chloroform extraction. The acceptance of these RNA products was selected upon quality ratios of 260/280 with values upwards of 2.0 or greater, except samples 16 and 17 which had values of 1.60 and 1.35 respectively. The extracted RNA was processed by GeneWiz (NJ, USA) to analyze quality and concentration by a bioanalyzer, remove eukaryotic ribosomal RNA (rRNA), which generally comprises the majority of RNA in a total RNA pool (Ribo-Zero rRNA removal kit (Illumina, CA, USA)), construct the paired-end cDNA library, and sequence on one lane of an Illumina HiSeq 2000 (150 nt read length).

All extracted DNA products were low in concentration (average 69 ng/ μ L) and were therefore selected based on the absorbance ratio of 260/280 value of typically greater than 1.40. The extracted DNA samples were processed for 16S rRNA gene amplification with the Earth Microbiome primers (515F and 806R (*Caporaso et al., 2018; Ul-Hasan et al., 2019*)) and sequenced using an Illumina MiSeq.

2.2.3 Sequence Assembly of Symbiont Metatranscriptome

Symbiont sequence analysis was performed using the SAMSA pipeline (Simple Analysis of Metatranscriptomes through Sequence Annotation), version 2.0 (*Westreich et al., 2018*) on a high-performance cluster computer. The pipeline consists of three main processing steps (preprocessing, annotation, and aggregation/downstream processing) and uses a set of python scripts that brings together multiple programs involved in processing, a flow chart of this process can be seen in Figure 4. For SAMSA2 to provide the best results, metatranscriptomes need to be ribodepleted (removal of ribosomal RNA) and sequenced in a paired-end format, both of which were completed with the sponge metatranscriptome dataset (*Westreich et al., 2018*). The sequencing of RNA (RNAseq) produced two FastQ files for each sample (n = 8 total) were produced, containing the forward and reverse reads respectively. Here I focus on the symbiont (i.e., bacterial) metatranscriptome, specifically using the bacterial RefSeq database for annotation within the SAMSA2 pipeline, and future work will include analysis of the host sponge transcriptome.

2.2.3.1 Preprocessing

RNAseq produced forward and reverse reads for each RNA (cDNA) that was sequenced, therefore the first step in preprocessing was to merge paired reads. PEAR 0.9.8 was used to merge paired reads (*Zhang et al., 2014*), which produced a single file of extended fragments from the two (forward and reverse) FASTQ sequencing files per sample. The command for PEAR was:

```
$pear_location/pear -f $forward_reads.fastq -r $reverse_reads.fastq -o $out_name
```

Where, -f points to the forward reads, -r points to the reverse reads, and -o designates the output file of merged reads. Next, low-quality sequences and/or adaptor contamination were removed using the program Trimmomatic 0.36 (*Bolger et al., 2014*). Trimmomatic was specifically designed to be used for Illumina sequences and thus includes the adapter and primer sequences for these machines. Trimmomatic was also used to remove low quality reads (below phred = 33) or trim the reads to produce an average phred score of 33. The command for Trimmomatic was as follows:

```
java -jar $./trimmomatic-0.36.jar SE -phred33 $infile $outfile_name SLIDINGWINDOW:4:15
MINLEN:99
```

Where, SE is the flag for single end mode (only one input file-the merged reads file from PEAR), -phred33- specifies the base quality encoding (FASTQ quality score), SLIDINGWINDOW-<windowSize>:<requiredQuality>, and MINLEN- specifies the minimum length for reads to be kept. For the sliding window, the 'windowSize' specifies the number of bases to produce an average as the program moves across the read, and 'requiredQuality' specifies the average quality threshold. The phred score is the quality score of base quality in DNA sequencing.

A 'cleaned' output file for each sample is produced from Trimmomatic for the final preprocessing step. Although a prior ribodepletion has already been performed, due to the naturally high rRNA counts present in microbiome samples, a digital ribodepletion is included in the SAMSA2 pipeline as the final preprocessing step. SortMeRNA 2.1 (*Kopylova et al., 2012*) removes any ribosomal reads left in the samples that may affect annotation of the metatranscriptome. SortMeRNA incorporates several reference databases for both bacterial and

eukaryotic sequences for rRNA identification: SILVA, GreenGenes, RDP. The command for SortMeRNA was as follows:

```
$sortmerna_location/sortmerna --ref $sortmerna_location/rRNA_databases/silva-bac-16s-id90.fasta,$sortmerna_location/index/silva-bac-16s-db --reads $file --aligned $file.ribosomes --other $file.ribodepleted --fastx --num_alignments 0 --log -v
```

Where, --ref points to the FASTA reference file, --reads points to the FASTA/FASTQ reads files, --aligned points to the aligned reads filepath, --other points to the rejected reads filepath, --fastx indicates to make the output file in a FASTA/FASTQ format, --num_alignments 0 indicates all alignments reaching the E-value threshold are reported; very slow, --log creates an output of overall statistics, and -v stands for verbose. Two files were produced: \$file.ribosomes which contains all the rRNA sequences, and \$file.ribodepleted which contain all the reads not identified as ribosomes. The ribodepleted file was used in the annotation step.

2.2.3.2 Annotation

SAMSA2 uses a BLAST-like aligner called DIAMOND (*Buchfink et al., 2015*) for the annotation process. This is because DIAMOND was designed to handle the large numbers of reads metatranscriptomes may possess at a rate 10,000 times faster than BLASTX (*Westreich et al., 2018*). DIAMOND has the ability to annotate sequences against one or more reference databases. The databases selected were the NCBI bacterial RefSeq (*Tatusova et al., 2014*) and SEED Subsystems hierarchical databases (*Overbeek et al., 2014*) for functional activities. The commands for DIAMOND were as follows: to perform the annotation process and to covert the generated results file into a data table for the aggregation step, respectively.

```
(1) $diamond_location blastx --db $diamond_database -q $file -a $file.RefSeq -t ./ -k 1 -sensitive
```

```
(2) $diamond_location view --daa $file.RefSeq.daa -o $shortname -f tab
```

Where, in command 1 , -db specifies the reference database to use, -q indicates the query file name, -a indicates the name of results file in DIAMOND format, -t sets the temporary directory locations needed as the program runs, -k 1 indicates the number of hits above cutoff (e-value cutoff = 0.001) to return, and settings changed from “-fast” to “-sensitive” to increase accuracy. In command 2, --daa points to the output file in .daa format, -o determines the output file name, and -f indicates separator of values in final output.

2.2.3.3 Aggregation and Downstream Processing

DIAMOND produced annoated files are returned in line-by-line results, each sequence from the metatranscriptome that had a corresponding match with a reference base sequence occupied one line in the output file. The next step was to use Python 2.7 scripts to aggregate these line-by-line files into condensed, summary tables (*Westreich et al., 2018*). Python is able to create two files from the same DIAMOND output reads: one for organism annotations and the other for functional annotations into 3 column tables. The 3 columns are broken down into 1) percentage of total reads, 2) read count, 3) annotated organism or function. This is generated by the following two commands:

```
(1) python $python_programs/standardized_DIAMOND_analysis_counter.py -I $file -D $RefSeq_db -O
```

```
(2) python $python_programs/standardized_DIAMOND_analysis_counter.py -I $file -D
$RefSeq_db -F
```

Where, -I points to the infile, -D indicates the specific reference database to search against, -O (in command 1) indicates to aggregate all reads by organism, and -F (in command 2) indicates to aggregate all reads by function. In addition, Python can also produce hierarchical annotations using SEED Subsystems to include abundance counts with all hierarchy level information, using the command:

```
python $python_programs/DIAMOND_subsystems_analysis_counter.py -I $file -D $Subsys_db -O
$file.hierarchy -P $file.receipt
```

These count files are then saved as the final product of the automated workflow for the SAMSA2 pipeline before moving into R for the statistical analysis portion (*Westreich et al., 2018*).

2.2.3.4 Additional Applications

SAMSA2 also has the ability to annotate functions by specific organisms, using a Python program named “DIAMOND_specific_organism_retriever.py”. This is because each processed read already receives both an organism and functional annotation. The command for the specific organism retriever was:

```
python $python_programs/DIAMOND_specific_organism_retriever.py -I $file -SO (genus or
species) -D $RefSeq_db
```

Where, -I points to the input file, which is the DIAMOND results file from annotation step above, -SO specifies which specific organism, either genus or species, to be isolated, and -D specifies the reference database to use. This creates an output file containing all the individual transcripts that originated from the specified organism. Following this, the produced file will have to be aggregated as well using the command:

```
python $python_programs/DIAMOND_analysis_counter.py -I $file -D $RefSeq_db -F
```

Where -I points to the infile, -D indicates the specific reference database to search against, and -F indicates to aggregate all reads by function. These files were saved as the final product and ready for further statistical analysis using R (*Westreich et al., 2018*).

2.2.3.5 Statistical Analysis and Visualization of RNAseq

All statistical analyses were performed in RStudio version 3.6.3 using R scripts from SAMSA2 pipeline. These scripts were used to calculate Shannon and Simpson diversity indices, taxonomic and functional bar plots, and principle coordinates analysis (PCA) analysis. The R scripts also included a specific set of scripts that utilize the program DESeq2 to calculate differential features (i.e., taxa or functions) between pre and post gemmule samples. The pre gemmule samples are: samples 16, 17, 50, 55, and 58; whereas, the post gemmule formation samples are 24, 25, and 57. DESeq2 is a differential expression analysis which takes read count data from these samples and performs an internal normalization, calculating a geometric mean for each gene across all samples, and uses a Wald test to calculate statistical significance and Benjamini and Hochberg test correction for multiple testing (*Love et al., 2014*).

The resulting profile of organisms in each sponge sample, as well as, the functional profiles of gene expression in the sponge host and microbiome were first characterized as an

overview of “who is there” (i.e., taxonomic profile based on RefSeq database taxonomy) and “what are they doing” (i.e., functional profile of transcripts) prior to, and during the time in which gemmules developed in the sponges (“Pre” and “Post” gemmule formation).

Differentially abundant features were calculated for taxa and functional annotation using RefSeq and functional annotation using the SEED Subsystems (*Overbeek et al., 2014*). A subsystem in the SEED database is a collection of functionally related proteins and the SEED subsystem organization provides hierarchical functional categories, facilitating comparisons in function between groups. Specific taxa that were identified as differentially abundant between the two time points were used in organism-specific analysis described above.

2.2.4 Environmental Data

In 2017 and 2018 various environmental data was collected for New river and Jacob Fork river including: phytoplankton and bacteria cell densities, pH, temperature (°C), conductivity, and chloride, nitrate and sulfate concentrations. These datasets were small with n=3 samples each. Therefore, a Wilcoxon test was performed in R for all environmental comparisons (e.g., temperature or pH between sites, phytoplankton abundance vs. bacterial abundance at a given site).

3. Results

3.1 Sponge Identification and Preliminary Microbiome Profiling of 16S rRNA Genes

Morphological and spicule preparations of the collected sponges indicate a putative identification of the sponges as *Ephydatia fluviatilis* with the megascleres being slightly curved, and mostly smooth oxea. Gemmuloscleres were unable to be visualized; therefore, genetic identification is in the process of being performed.

Total input reads ranged from 38,340 to 95,249 with an average of 64,856 across all 27 samples sent for 16S microbiome profiling (Table 3). Between input reads and nonchimeric reads, the percentage loss was an average of <27% for all samples. With the lowest number of nonchimeric reads being 7,813 but the average for all being 17,573.

Microbial community profiling using 16S rRNA genes indicated overlap in taxonomy at the phylum level between water and sponges with the major taxonomic groups including Proteobacteria, Bacteroidetes, and Actinobacteria (Figure 5). However, at low taxonomic resolution, analysis of similarity (ANOSIM) applied to the sponge and water ASVs communities indicated a significant difference in composition the level of operational taxonomic units (ASVs, as with operational taxonomic units (OTUs) these are a taxonomic unit loosely used as a bacterial 'species') between the two habitats (ANOSIM, $R=0.23$, $p=0.01$). Time of sampling (July, August, September) was also shown to have an effect on community structure in all samples (ANOSIM, $R=0.54$, $p=0.001$), which is clearly visualized by network analysis showing the separation of water and sponge communities with those being more similar indicated with shorter and thicker lines. As well as, samples in July, both water and sponge, are completely isolated in comparison to August and September (Figure 6).

Taxonomic comparisons at the ASV-level, however, highlighted certain taxa that were detected only in the sponge or water (Figure 7). There was a total of 594 ASVs produced, with

312 ASVs in the sponges and 415 ASVs detected in the water. Of the ASVs observed in the sponges, 189 (60%) were present only in the sponges and not in the surrounding water. The ASV table was not rarefied (McMurdie & Homes, 2014). Specifically, within the most abundant 300 operational taxonomic units (ASVs) at the phylum level, several ASVs were observed to be abundant in the sponge samples while being absent from the water samples (Figure 7), thereby identifying these taxa as putatively enriched or specific to sponges. Some of the ASVs specific to sponges included taxa within Classes *Flavobacteriales* and *Sphingobacteriales* within the phylum *Bacteroidetes* (Figure 8), as well as *Betaproteobacteriales*, *Xanthomonadales*, and *Sphingomonadales* within the phylum *Proteobacteria*.

3.2 Metatranscriptome Results

Total reads generated per samples ranged from 54,321,279 to 37,446,129 (Table 4). Between raw reads and final read counts, the percentage lost was <31% for all samples with an average loss of 25% after the merging of paired reads and removal of low-quality reads. With the lowest number of final reads over 27,000,000 and the most being over 40,000,000 (Table 4).

In 2018, a total of four individuals were collected between July and August. The two collected in July showed no evidence of gemmule formation, whereas the other two collected in August gemmules were present. In 2019, another four individuals were collected over the course of three months (July, August, and September) (Table 2). The resulting profile of organisms in each sponge sample, as well as, the functional profiles of gene expression in the sponge host and microbiome were first characterized as an overview of “organism” (i.e., taxonomic profile based on RefSeq database taxonomy) and “function” (i.e., functional profile of transcripts based on RefSeq database gene annotations) prior to, and during the time in which gemmules developed in the sponges (“Pre” and “Post” gemmule formation). Shannon and

Simpson diversity metrics were similar between the pre-gemmule (Pre) and post-gemmule (Post) sponge samples. For organismal results the Shannon diversity for Pre was 6.12 and for Post it was 6.18, and the Simpson diversity was 0.98 for Pre and 0.98 for Post. For functional results, Pre was 4.65 for Shannon and 0.87 for Simpson and Post was 4.67 for Shannon and 0.88 for Simpson (Table 5).

As an overview, PCA plots, which visualize the community composition data (taxonomy or function profile and abundance) in a two-dimensional space, did not show separate groups for Pre and Post sponge samples by organism taxonomy (Figure 9) or by functional annotation from the RefSeq database (Figure 10). The lack of separation of the Pre and Post sponge samples is supported by the results of a permutational multivariate analysis of variance test between the two groups (ADONIS: $R^2=0.069$, $p=0.84$ by organism; $R^2=-0.068$, $p=0.74$ by function). In both analyses, organism and function, although particularly for function, the Pre samples tended to be more dispersed than the Post samples (Figures 9, 10). However, there are also more Post samples than Pre samples.

The most prevalent taxa across all sponge samples based on the metatranscriptome analysis included groups mainly within the *Bacteroidetes* and the Class *Gammaproteobacteria* (Phylum *Proteobacteria*). Differential abundance of taxa from Pre to Post samples was detected with DESeq2 analysis (Table 6). The information from DESeq2 includes the means of both sample types (Pre and Post), log2FoldChange, lfcSE (error associated with the fold change), the statistic, p -value, and adjusted p -value. The log2FoldChange is the indicator as to whether the organism increased or decreased in abundance. A negative log2FoldChange means there was a decrease in abundance from Pre to Post. A positive log2FoldChange means there was an increase. Organisms that showed differential abundance between time points include *Actinobacteria* including taxa within the Family Streptomycetaceae (i.e., *Kitasatospora* and *Streptomyces* genera), *Flavobacterium* within the *Bacteroidetes* phylum, *Betaproteobacteria*

such as *Polynucleobacter* spp., Alphaproteobacteria such as *Sphingomonas* spp., an *Epsilonproteobacteria*, *Sulfurimonas*, and one taxon within Firmicutes (*Bacillus*). There was no clear trend for a broad taxonomic group (e.g., all *Flavobacterium* and all *Polynucleobacter*) to increase and decrease (respectively) in abundance from Pre to Post gemmule time points.

The most abundant functions detected in the sponge samples, based on RefSeq functional annotation were involved in energy and maintenance metabolism such as electron transport chain enzymes and the chaperone protein DnaK (Figure 11). Note that proteins of unknown function were also prevalent (Figure 11). Differential abundance of specific functional annotations was detected for 12 functions assigned from RefSeq between Pre and Post samples, where five functions decreased and seven increased (Table 7). Functions that increased in abundance in Post relative to Pre samples include transcripts for catechol 2,3-dioxygenase, involved in aromatic compound degradation, porin proteins, which are channel proteins specific to certain molecules and common in Gram negative bacteria, disulfide bond formation protein B, which catalyzes disulfide bond formation in periplasmic proteins (thus would be found in Gram negative bacteria), enzymes in nitrogen metabolism (type III glutamate—ammonia lyase), pilus assembly protein, a domain of unknown function (DUF4293), and an elongation factor involved in proteins translation and ribosome recycling (EF-G) (Table 7). In contrast, functions that decreased in abundance in the Post samples included transcripts involved in vitamin B12 bioynthesis (Uroporphyrin-III methyltransferase), amino acid metabolism (bifunctional o-acetylhomoserine/o-acetylserine sulfhydrylase), as well as different porin proteins, the chaperone protein Hsp70, and a hydrogenase protein (Table 7).

Some similar functional profiling results were observed with the SEED subsystem categories although at a greater scale (Figure 12). Looking at Figure 11, the top bar graph shows the relative activity of total sample, which is predominantly 'hypothetical protein' and 'other', as well as a couple subunits of cytochrome c oxidase (I, II, and 3) and NADH-quinone

oxidoreductase subunit H, and these appear relatively consistent across both Pre and Post. Differential abundance of SEED subsystems indicated a marginal but statistically significant increase in respiration, cofactors, virulence, and photosynthesis and iron acquisition and metabolism (Table 8). SEED subsystems that decreased in abundance in Post samples included motility and chemotaxis, cell wall and capsule, transposable elements, sulfur metabolism, and dormancy and sporulation (Table 8).

Lastly, from the list of taxa that showed a significant difference in abundance between Pre and Post gemmule samples (Table 6), four genera were selected for more in-depth functional analysis: *Polynucleobacter*, *Flavobacterium*, *Sulfurimonas*, and *Sphingomonas*, as they were the most prominently listed or were of interest for their potential role in nutrient cycling. They were also of interest due to also being prominent in the 16S data. These four were analyzed for the functional annotation of transcripts within just these organisms using the NCBI RefSeq database. However, within these four organisms, only two genera displayed functions that were significantly different from pre and post samples. Those two genera were *Polynucleobacter* and *Flavobacterium* (Table 9). There were seven functions in *Polynucleobacter* that were different with six of them increased in abundance from Pre to Post gemmule formation, which were all involved in bacteriochlorophyll biosynthesis or one enzyme, transaldolase, involved in the metabolism of carbohydrates in the pentose phosphate pathway (Table 9). In contrast, *Flavobacterium* had 25 functions that all of them significantly decreased in abundance, including proteins likely involved in maintenance metabolism (e.g., acyl-coA ligase in fatty acid metabolism, NADH-quinone oxidoreductase subunit N) as well as several proteins of unknown function and proteins known to be involved in protein-protein interactions in a host (i.e., fibronectin type III domain-containing protein, ankyrin repeat domain-containing protein, Type IX secretion system (T9SS C-terminal target domain-containing protein)) (Table 9).

3.3 Environmental Results

Flow cytometry of water samples yielded an overview of phytoplankton and bacterioplankton abundances in the water, both of which may be food for the sponges. A comparison of two locations where sponges have been collected, New river and the Jacob Fork river which is at a lower elevation near Hickory, NC, are provided here to add context for the environmental data. Phytoplankton density was always lower than bacterial density by about an order of magnitude (Figure 13). Phytoplankton were more variable in abundance at the New river, with a sharp increase in August (Figure 13). There was no difference in phytoplankton abundance between the New river and Jacob Fork river ($W=57$, $p\text{-value}=0.1615$). Bacteria was significantly more abundant in New river than Jacob Fork ($W=0$, $p\text{-value}=4.1145\text{e-}05$).

Temperature, pH, and conductivity were not significantly different between the two locations (Figure 14a,b,c). Inorganic nutrients were generally low across time points and between locations (Figure 15). Nutrient analysis for 2019 has not yet been conducted, but data from 2017 and 2018 indicate low anion nutrients in those two years (Figure 15).

4. Discussion

Microbial symbionts of metazoans can have a significant role in the development of host structure, immunity, diet and ecological niche (*Brestoff & Artis, 2013; Douglas, 2011; Hentschel et al., 2012; Manconi & Pronzato, 2008; Reviewed by Nyholm & McFall-Ngai, 2004*). In sponges most microbial symbionts are considered to be commensals, although experimental work has mainly been focused on photosymbionts in sponges, for which it is fairly straightforward to identify potential host benefits. Here, I set out to investigate how the composition of these microbial communities change as the sponge undergoes developmental changes involved in asexual reproduction, and determine what role, if any, the microbiome may have in the facilitating developmental change of the sponge host. An experimental manipulation of the sponge microbiome was the original goal; however, due to experimental challenges, I shifted my work to a correlative study and acknowledge both the limitations in interpretation of my results and the foundation that this work provides for future experimental studies.

4.1 16S rRNA Gene Microbiome Profiling

Strong evidence was found in the 16S rRNA gene profiling results of this study that the microbial communities in sponge and water are significantly different from each other. Specifically, while some groups overlap between sponge and river water, there were groups of taxa that were present only in the sponges as well as taxa that were abundant in the sponge and rare in the water. This distinction of the sponge microbiome from that of the surrounding water is similar to findings from other studies on marine and, more recently, on freshwater sponges (*Gaikwad et al., 2016; Reveillaud et al., 2014*). Furthermore, of the taxa observed in sponges in the present study, the best matches to these ASVs in the NCBI database were to taxa known to be symbionts in other sponges. Specifically, this includes ASVs within the phylum *Bacteroidetes* (Orders: *Chitinophagales*, *Cytophagales*, *Flavobacteriales*, and

Sphingobacteriales) and *Proteobacteria* (Classes: *Gamma*- and *Alphaproteobacteria*). Other taxa found to be more prevalent in the sponge host compared to the water were similar to those in other freshwater sponge studies that used high throughput sequencing methods:

Actinobacteria, *Bacteroidetes*, *Chloroflexi*, *Cyanobacteria*, *Nitrospira*, and *Proteobacteria* (*Alpha* and *Gammaproteobacteria*) (Gaikwad *et al.*, 2016; Gladkikh *et al.*, 2014). A notable exception is the low abundance of *Cyanobacteria* that was observed in the sponge and water in the present study, similar to several other freshwater sponge studies (Costa *et al.*, 2012; Gladkikh *et al.*, 2014), but in contrast to observations by Gaikwad *et al.* (2016). The compositional results here are also similar to a study performed on the 16S rRNA microbial profile of the cosmopolitan freshwater sponge *Spongilla lacustris* whose gene library comprised four major lineages: *Alphaproteobacteria*, *Actinobacteria*, *Betaproteobacteria*, and *Chloroflexi* (Gernert *et al.*, 2005). These data overall support both high diversity and potentially divergent symbiotic bacterial taxa within freshwater sponges.

4.2 Metatranscriptome Analysis of Sponge Symbiont Communities

In the metatranscriptome analysis, SEED produced a pie chart showing the large scale of the top 10 functional subsystems functions that were prevalent. The biggest portion of that pie chart is indicated by these three functions: protein biosynthesis, electron accepting reactions and electron donating reactions (Figure 12). RefSeq functions bar chart are more specific gene functions rather than broad scale. The majority of these include hypothetical protein (gene of unknown function), as well as, energy functions being a major component (cytochrome c and NADH-quinone oxidoreductase part of electron transport chain) (Figure 11). Molecular chaperone DnaK is also a key function to note, this falls under housekeeping protein folding in addition to being involved in DNA replication and energy & maintenance processing.

Pre and Post microbial communities did not significantly separate into two different groups based on taxonomy (organismal analysis), but we did see specific taxa increased in one or the other group. Some taxa that were identified as significantly different in abundance between the Pre and Post samples, overlapped with taxa identified as enriched in the sponges based on 16S rRNA gene profiling. These include taxa within the Orders *Flavobacteriales* and *Sphingomonadales*, both of which increased in abundance from Pre to Post metatranscriptome samples and were taxa observed only in the sponges based on 16S microbiome gene profiling (Figure 8). Separately, two taxa that were more abundant in Pre samples include the genera *Streptomyces* sp. and *Xenorhabdus* sp. These are of interest as *Streptomyces* is a recognized symbiont in multiple organisms, including marine sponges (reviewed by Seipke *et al.*, 2012). This group is of particular interest because of the bioactive molecules and antimicrobial properties they may produce (Almeida *et al.*, 2019; Reviewed by Seipke *et al.*, 2012). *Xenorhabdus* sp. also produces bioactive compounds with a broad-spectrum of antimicrobial activity and are well known for their role as symbionts of entomopathogenic nematodes (Dreyer *et al.*, 2018). Thus, these taxa in particular, (Flavobacteriales, Sphingomonadales, *Streptomyces*, *Xenorhabdus*) may represent symbiotic (facultative or obligate) bacteria within the sponges.

Another bacterial group that was prevalent in the results of the differential abundance analysis, was that of the genus *Polynucleobacter*, all of which decreased in abundance from Pre to Post samples. *Polynucleobacter* was not highlighted as a taxon of interest in the 16S microbiome samples, which is interesting to note. Although, that doesn't mean they weren't present and further investigation is needed to explore that idea. *Polynucleobacter* was originally described as endosymbionts that resided in ciliates (*P. necessaries*, Hechmann & Schmidt, 1987), but more recently, multiple *Polynucleobacter* strains were identified as abundant free-living

planktonic bacteria in freshwater systems (*Hoetzing et al., 2018*). I cannot determine from these data alone whether the strains observed here are free living or symbiotic.

4.3 A Potential Framework for Symbiont-Host Interaction in Freshwater Sponges

While it is not possible at this point in time to determine the nature of the relationship for the major taxa observed in the metatranscriptome analysis, I have organized the results into a framework to generate hypotheses for future work (e.g., tracking microbiome changes over time, experimental manipulation of the microbiome in the laboratory). There are three main categories of bacterial life histories that could be present in the sponge samples: 1) free-living, 2) obligate or long-term facultative symbiont that is always present in the sponge and likely to be present in the gemmules and gametes, and 3) short-term facultative symbiont that disperses from the sponges when it disappears.

First, if we are detecting free living bacteria that happened to be included in the sample from water present in the sponge or as food particles, we might expect the following: They would likely exhibit maintenance metabolism that may not change over the two time points sampled here and living in the water, they are likely to be photoautotrophic or heterotrophic. In these bacteria, we may not see changes in metabolic activity over two time points other than photosynthesis genes that may change in expression as irradiance and temperature changes between July and September. In comparison to symbiotic bacteria, there would likely be fewer, or lack of, motility and chemotaxis transcripts, as well as transcripts for transposable elements, viral defense, and proteins with eukaryotic-like domains (*Thomas et al., 2016*).

The results presented here within the functional analysis of the *Polynucleobacter* genus indicated the only significant changes in transcript abundance were those associated with photosynthesis, which increased in the Post samples. This could be driven by environmental factors (e.g., irradiance, temperature) and may indicate some or all of these taxa are free-living.

No other symbiotic traits (e.g., eukaryotic-like domains, chemotaxis) were observed in the *Polynucleobacter* analysis. However, this does not preclude some of these strains from being symbiotic; they may be at low abundance or may not change their activity with changes in the sponge, and several of the best matches in NCBI were to the symbiotic *P. necessaries* specifically. Further monitoring of sponge and water microbial populations should shed more light on the role of this group.

Second, in contrast to free living bacteria, if we are detecting facultative or obligate symbiotic bacteria that associate with the sponge during vegetative growth and are intentionally transmitted into the gemmules, we might expect that some of the bacteria that would be transmitted into the gemmules are also still present in the remaining sponge tissue. Such a relationship would be similar to vertical transmission in many marine sponges which can include high percentages (>30%) of symbionts that are passed to the offspring through gametes (Reveillaud *et al.*, 2014; Schmitt *et al.*, 2012; Webster *et al.*, 2010). In this case, we would expect a certain set of traits to be present and potentially change in expression with the developmental and structural changes in the sponge: 1) bacterial metabolisms that include photoautotrophic, and potentially ammonia oxidizing bacteria and sulfur oxidizers (using waste products from the sponge), as well as heterotrophic bacteria (Liu *et al.*, 2011; Moitinho-Silva *et al.*, 2017; Slaby *et al.* 2017). If these microbes have a long-term and intimate association with the sponge, then the metabolic activity may show no change over time or may increase as sponge begins to disappear and undergoes structural changes. An increase in metabolic activity may be the result of more nutrients released as the sponge degrades or as a result of interaction with and movement within the sponge. 2) Similarly, metabolic functions that could provide certain nutrients (e.g., vitamins, amino acids, organosulfur compounds) to the host may show no change or an increase in expression as the host produces gemmules and begins to disappear. 3) Presence of transposable elements and viral defense would be expected to be higher in the

symbiotic community than in free living microbes based on marine sponge microbiome analyses (*Horn et al., 2016; Thomas et al., 2016*), but may show little to no change over the time points. 4) Disappearance of the sponge tissue may also increase expression of motility and/or chemotaxis genes in symbiotic bacteria as these bacteria respond to remodeling and developmental changes in the sponge. 5) Expression of proteins with eukaryotic-like domains has been documented as an enriched trait in sponge and other symbiont communities in comparison to free living microbes (*Horn et al., 2016; Slaby et al., 2017; Thomas et al., 2016*). Expression of such proteins might decrease upon sponge degradation after gemmules are produced if these bacteria leave the sponge (i.e., facultative symbionts) or may increase if this is involved in transmission into gemmules (i.e., obligate, or at least long-term, symbionts). 6) Lastly, expression of nutrient transporters might increase in expression as the sponge begins to disappear due to longer commitment to living in a sponge and potential release of metabolites as the sponge degrades.

Relevant results that may apply under this second framework would be functions highlighted in Table 7, such as: pilus assembly protein, porin family protein, catechol 2,3-dioxygenase, and type III glutamate-ammonia ligase. These functions increased in expression in the Post samples. Pili are structures used to adhere to surfaces, like a host, or in bacteria-bacteria communication and have been documented in marine sponge symbiotic bacteria (*Liu et al., 2011*). Therefore, an increase in pilus assembly protein could be evidence of increased bacteria-bacteria interaction or potentially bacteria-host interaction in the Post samples. The Porin family of proteins is a type of transport protein usually located in the outer membranes of Gram-negative bacteria, which were the dominant type of bacteria in the New river sponges. The main function of porins is to import specific molecules into the cell, such as certain types of sugars as well as secondary metabolites, any of which would likely to be produced within the sponge (*Fan et al., 2012; Kamke et al., 2013*). In particular, as the sponge

degrades, it may release different metabolites and nutrients, and these bacteria may be well-suited to take advantage of such compounds. Similarly, the increase in transcripts for catechol 2,3-dioxygenase in the Post samples may make sense as this enzyme is involved in degradation of aromatic compounds that could be released by the sponge and/or other bacterial symbionts. Type III glutamate-ammonia ligase, also known as glutamine synthetase, is an important enzyme in nitrogen regulation which forms glutamine from ammonia and glutamate. Nitrogen regulation through the protein PsII, which regulates glutamine synthetase has been documented as enriched in marine sponge symbiont communities in comparison to free-living communities (*Moithinho-Silva et al., 2017; Thomas et al., 2016*). While it is still speculation, the observed increase in transcript abundance of these functional traits in the Post samples may reflect characteristics of a group of bacteria that are committed to residing in the sponge and have adapted to their host environment and life cycle. Potentially these bacteria would be candidates to be transferred into gemmules, if there is selective pressure to maintain these lineages with the sponge life cycle.

Third, if we are detecting facultative symbiotic bacteria that associate with the sponge during vegetative growth and then transition to free-living after the sponge disappears, we might expect that these bacteria would either leave the sponge to become free-living again or remain until they are released as the host disappears. In this case we would expect similar traits to the long-term symbionts described above, but with some potential differences in response to gemmule formation and degradation of the sponge: 1) Such symbionts could have similar metabolic potential as the long-term symbionts described above (i.e, photoautotrophs, potentially ammonia oxidizing bacteria and sulfur oxidizers, and heterotrophs). However, there may be more varied responses to sponge degradation. Heterotrophs may increase in metabolic activity as sponge disappears, while ammonia and sulfur oxidizing may decrease if reliant on host metabolism or increase as sponge tissue degrades. 2) The expression of motility and/or

chemotaxis genes might increase in expression upon sponge degradation if these bacteria leave the sponge. 3) Expression of proteins with eukaryotic-like domains, nutrient importers, presence of transposable elements and viral defense, and metabolism that could provide certain nutrients (e.g., vitamins, amino acids, organosulfur compounds) to the host, might decrease in expression as the sponge begins to disappear.

Results that may apply to this third category include the significant functions observed to decrease in expression in the genus *Flavobacterium*. These include: T9SS C-terminal target domain, fibronectin type III domain-containing protein, and ankyrin repeat domain. T9SS C-terminal target domain, is a type IX secretion system, only found in some species of the *Bacteroidetes* phylum, and has one of two roles: a means of gliding movement for commensal or environmental bacteria or involved in virulence secretion by pathogens (*Lasica et al., 2017*). Interestingly, the non-pathogenic bacteria with T9SS are known to degrade cellulose or chitin (*Lasica et al., 2017*), while other secretion systems enriched in the marine sponge symbiont *Entotheonella* included T2SS associated with degradation of collagen (*Liu et al., 2016*). The ability to degrade collagen was hypothesized to be critical for *Entotheonella* to create space in the sponge mesohyl (*Liu et al., 2016*). This is the first record of T9SS in putative sponge symbionts and may be involved in nutrient acquisition and/or interaction with the host. Fibronectin type III domain-containing protein and, mainly, ankyrin repeat domains are enriched in sponge-symbiotic community in marine sponges compared to free-living communities (*Fan et al., 2012; Fiore et al., 2015; Liu et al., 2016; Thomas et al., 2010*). These eukaryotic-like domains presumably facilitate interaction with host cells or proteins, although the mechanisms for this are not well characterized. All of these traits would be expected in symbiotic bacteria and were observed to decrease in abundance in the *Flavobacterium* genus in Post samples. Therefore, some of the *Flavobacterium* observed here may be facultative

symbionts that leave the host as the sponge tissue degrades and/or cued by other environmental factors.

The sponges collected for metatranscriptome analysis were collected over three months in summer and early fall where the temperature, irradiance levels, turbidity, and water levels change in addition to physiological changes in the sponge. Environmental parameters (pH, temperature, and conductivity) did not appear to vary visibly over the three months at the New River collection site, with the exception of an increase in pH in August. Phytoplankton also increased in abundance in August at the New river site. For comparison, another location that consistently contains sponges, the Jacob Fork river, was also tested for nutrient and environmental parameters. At this particular site, we typically observe larger sponges, sponges that are green in color (harboring algal symbionts), and sponges are generally present in the water for longer into the fall and winter than at the New river site. However, the measurements of environmental parameters at Jacob Fork were generally opposite of what we expected with lower abundance of bacteria (although consistently higher abundances of phytoplankton), lower pH, lower conductivity, and similar water temperatures. Overall, irradiance levels and pH and/or changes in water level could be environmental factors affecting the microbial communities in the water and in the sponges.

The results described here support the presence of some symbiotic bacteria in the sponge and potential for obligate or at least long-term symbiosis that could be important throughout the sponge life cycle. Future work is needed to track sponge microbiome communities over time and between species and compare the phylogeny of these taxa with other freshwater sponge taxa. Additionally, analysis of the host sponge transcriptome as a next step, using the marine sponge genome, *Amphimedon queenslandica*, should shed light on physiological processes occurring in the sponge during gemmule formation. Another consideration is the use of short reads in the annotation analysis as conducted in the present

work. The annotation in the SAMSA2 pipeline relied on reads only, and a logical next step is to create a *de novo* assembly of the metatranscriptome to annotating the contigs, rather than the reads. Ideally, we would be able to then compare active functional annotations between the microbes and sponge. This would also provide insight into the proportions of sponge reads versus reads from microorganisms. Lastly, work is in progress to sequence 16S rRNA genes using DNA from gemmules isolated from sponges in the present study. The work presented here has also laid the foundation for future experimental work with gemmules in the laboratory to ultimately move beyond correlation in the sponge-microbe community addressed here.

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Tables and Figures

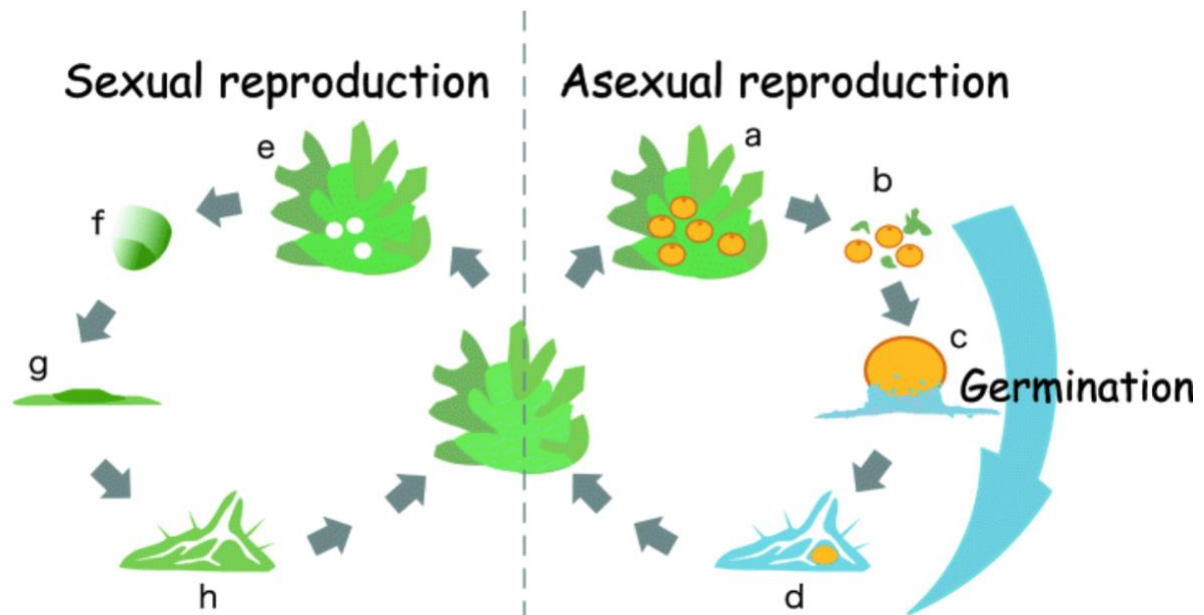


Figure 1: Life cycle of *Ephydatia fluviatilis* in asexual and sexual reproduction. (A) Gemmules formed within the parent sponge tissue. (B) Parent tissue is destroyed, gemmule is exposed and followed by subsequent hatching; germination occurs. (C) cells break out of the gemmule coating. (D) These cells form a small, fully functioning sponge in about 1 week. Sexual reproduction sperm and eggs are formed within male and female sponges. (E) Eggs are fertilized by the sperm in the female sponges and begin to cleavage. (F) Larvae leave parent sponge. (G) Larvae settle and start to metamorphize. (H) A new sponge has formed (Funayama et al., 2005).

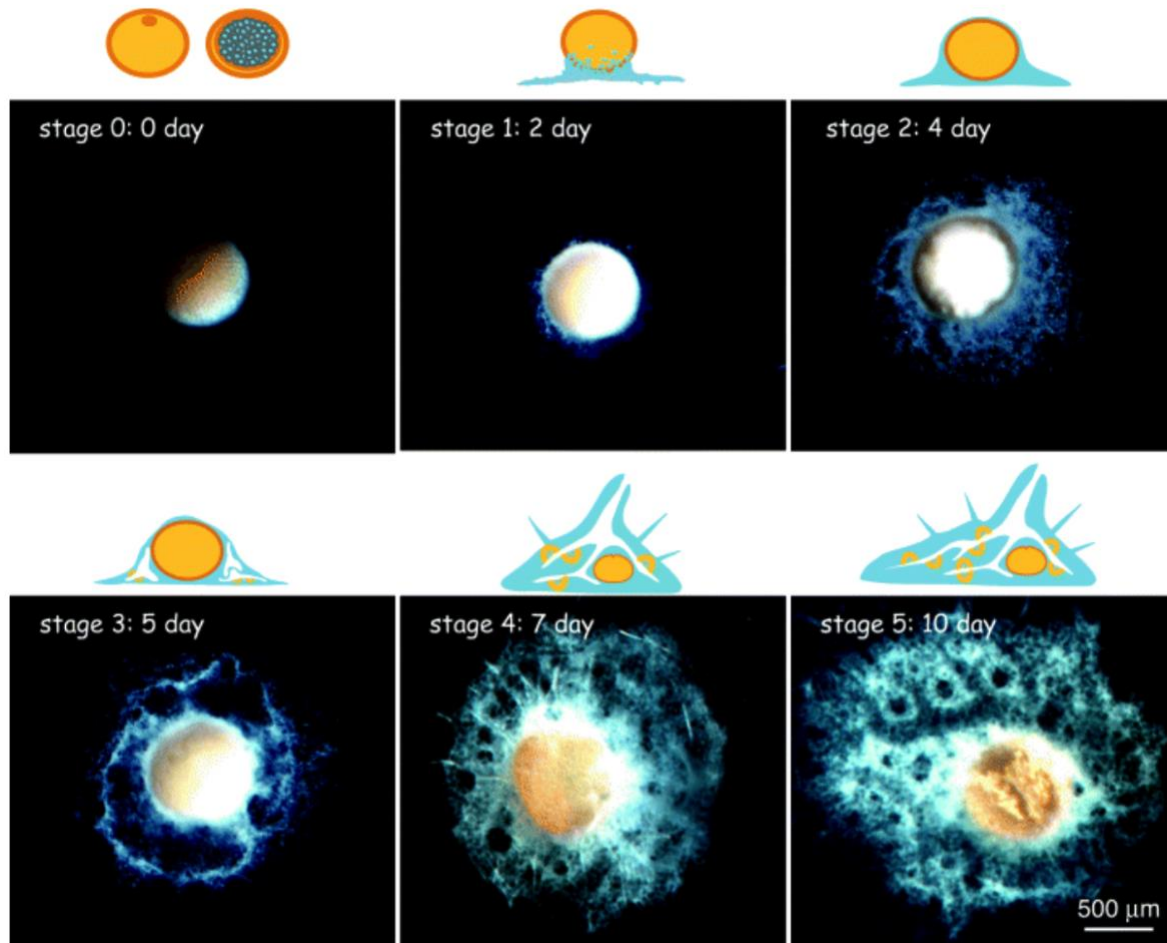


Figure 2: Developmental stages of germination in the freshwater sponge *Ephydatia fluviatilis*. (A) Stage 0: resting gemmule. (B) Stage 1: (2-day-old sponge) cells actively migrate from the gemmule and differentiate into epithelial cells to form an envelope. (C) Stage 2: (3-4-day old sponge) cells move into the epithelial envelope to proliferate and start differentiating into several different types of cells. (D) Stage 3: (5-day-old sponge) the aquiferous system and choanocyte chambers are formed. (E) Stage 4: (6-7-day-old sponge) sponge is fully functional with the formation of the oscula in this miniature sponge. (F) Stage 5: (8-12-day-old sponge) sponge maintains its current body form and gradually increases in size from this point on (Funayama et al., 2005).

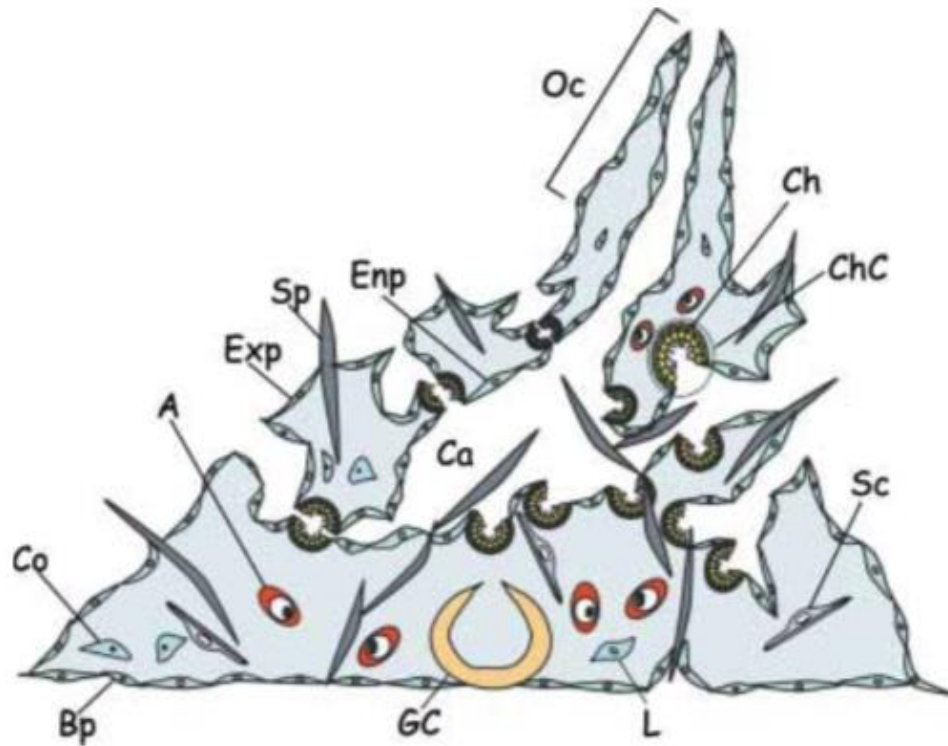


Figure 3: Vertical sectioned view of a hatching sponge from a gemmule. A, archeocyte; Bp, basopinacocyte; Ca, water canal; Ch, choanocyte; ChC choanocyte chamber; Enp, endopinacocyte; Exp, exopinacocyte; GC, gemmule coat; L, lophocyte; Co, collagenocyte; Oc, oscle; Sc, sclerocyte; Sp, spicule (Funayama et al., 2005).

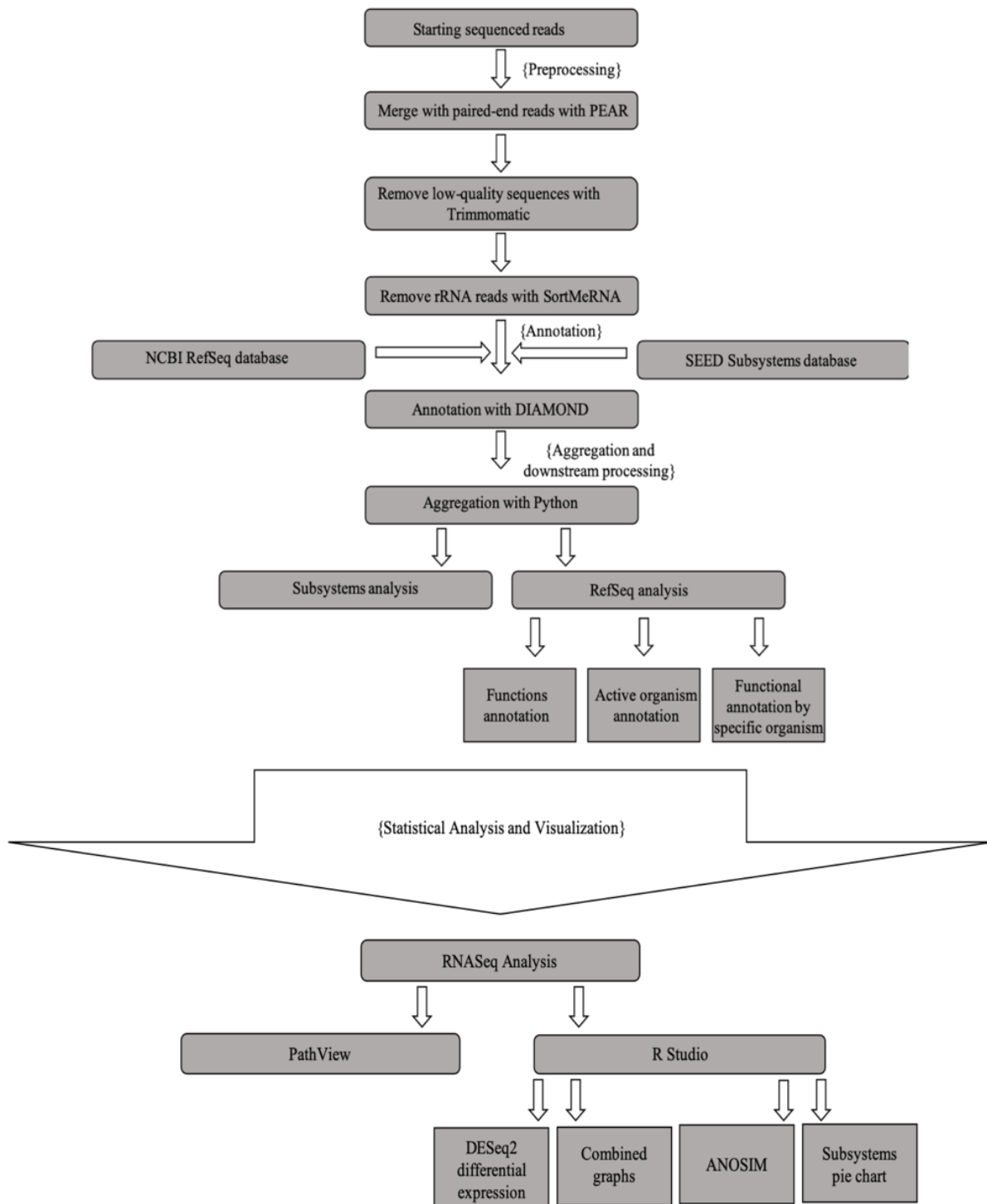


Figure 4: Modified SAMSA2 pipeline flow chart to include all the steps taken during the sequence assembly and data analysis process (Westreich *et al.*, 2018).

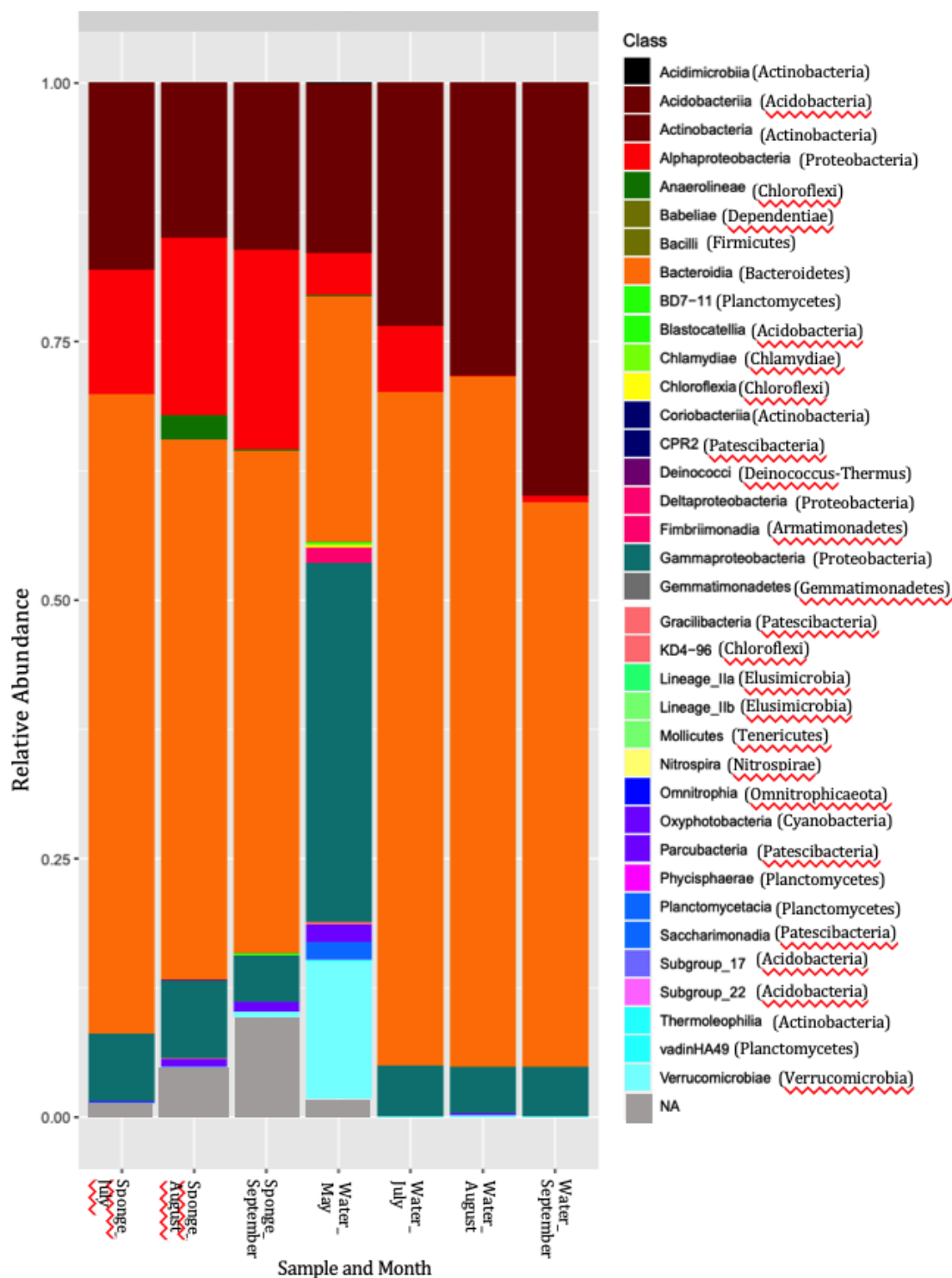


Figure 5: Bar graph of relative abundance of taxonomic hits at class level on Y-axis with sample and collection month information on X-axis. Sponge samples are on the left side of the chart, while water samples are on the right side. Phylum associated with each of the classes is listed on the key in parentheses.

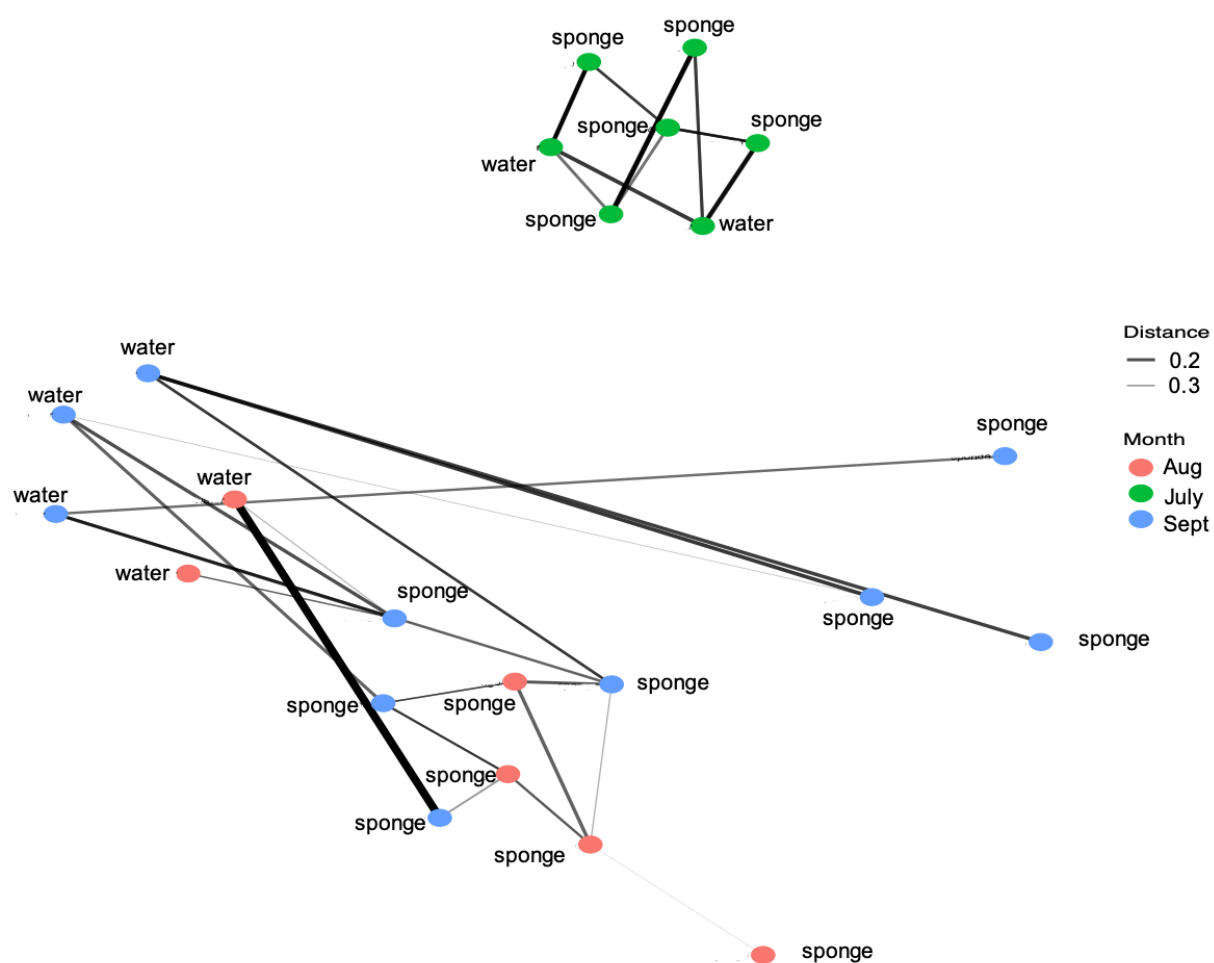


Figure 6: The network representation of the similarity in microbiome profile between the sponges and river water samples collected for preliminary data. Network is based on Bray-Curtis distance, length and thickness of lines indicate similarity between samples. The thicker and shorter the lines are the more similar the samples. Two separate networks were detected here with July samples (green dots) for water and sponge different from the August and September (red and blue) samples, indicating they are significantly different.

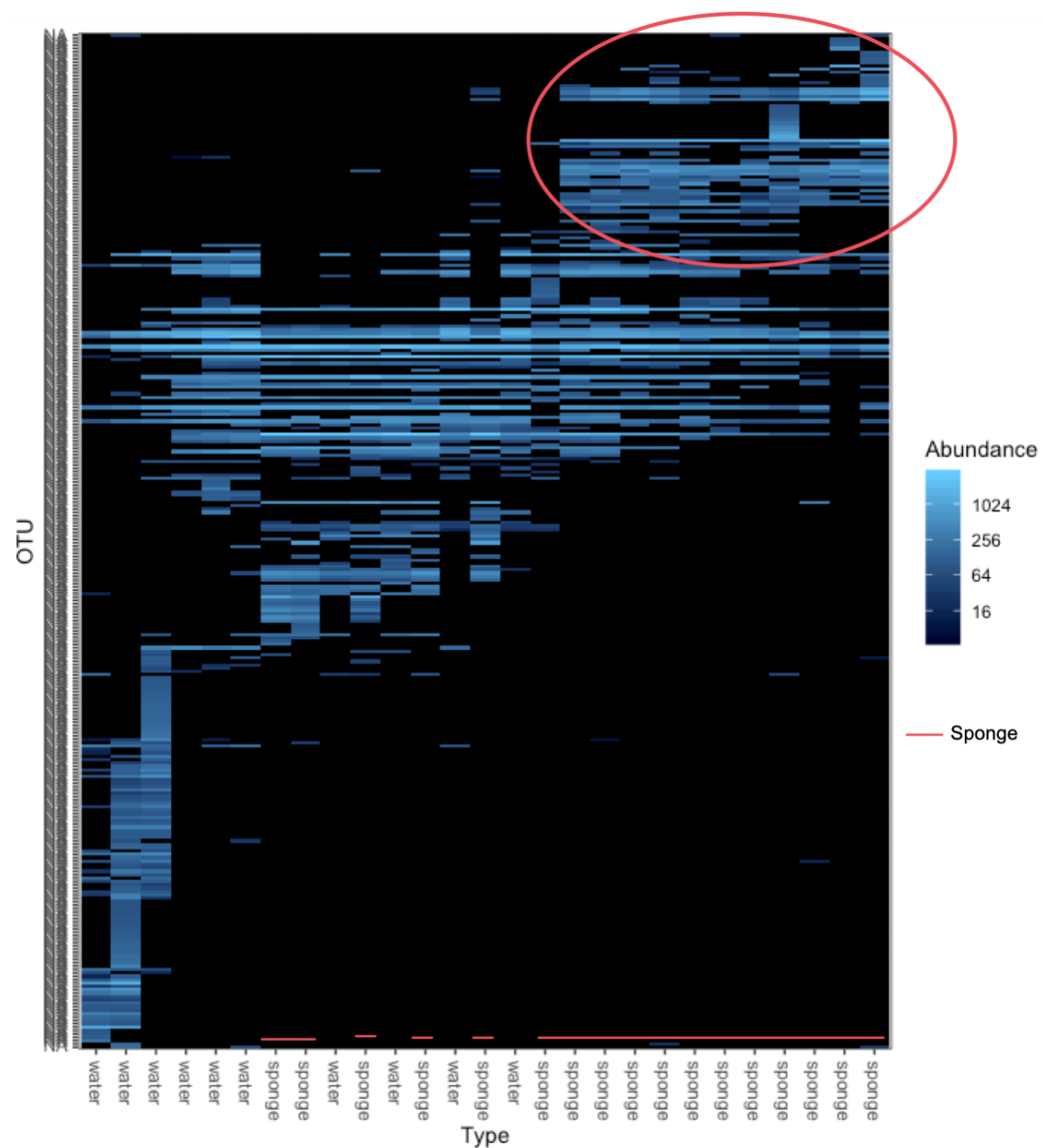


Figure 7: Heat map displaying the abundance of the top 300 ASVs at the phylum level for all sponge and water samples collected for preliminary data. The red oval represents ASVs that may be enriched or specific to sponges.

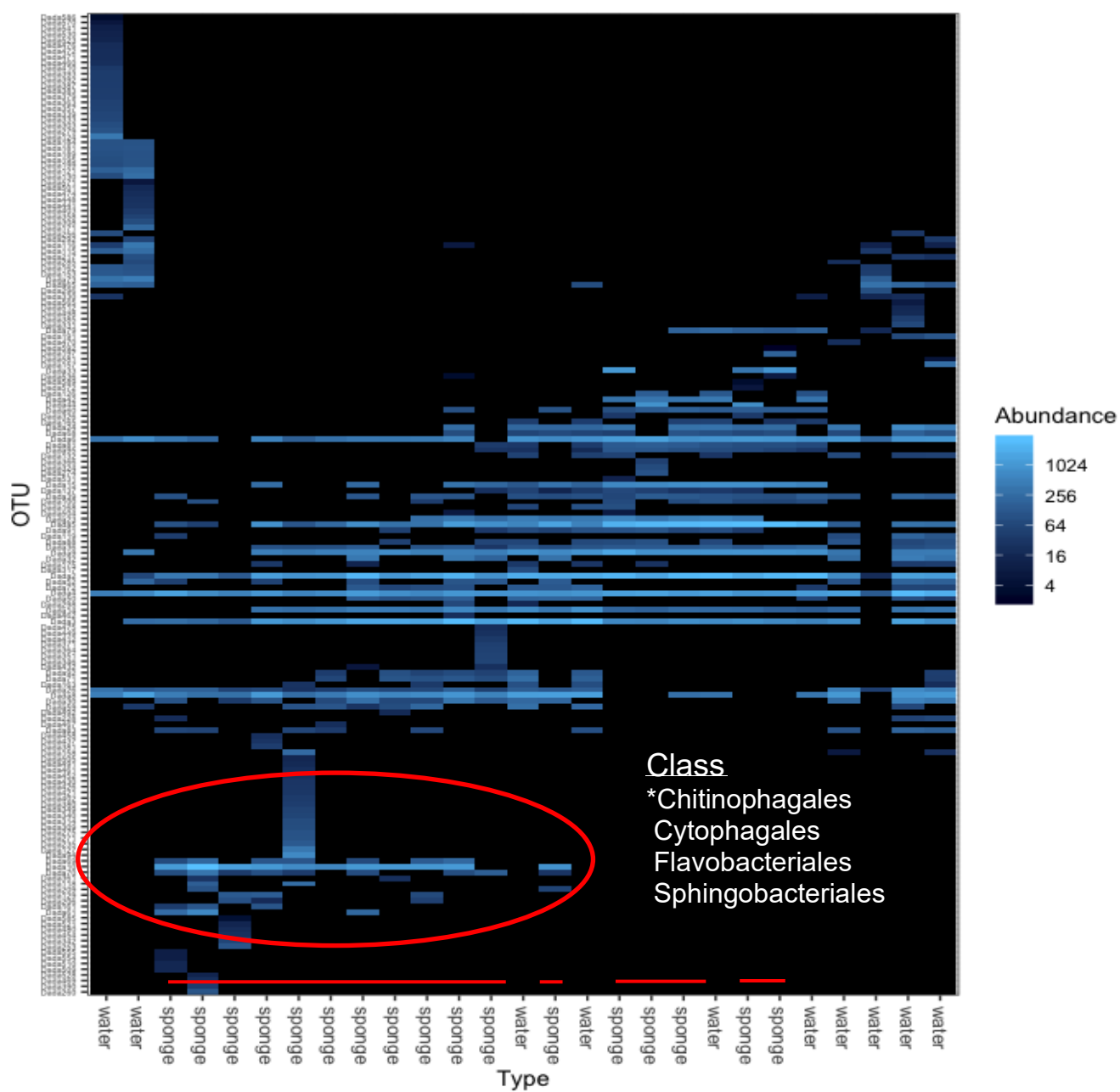


Figure 8: Heat map displaying the abundance of the ASVs among the phylum *Bacteroidetes* for all sponge and water samples. The red oval represents ASVs that may be enriched or specific to sponges. The classes that the circled ASVs comprise are listed with an asterisk marking the most abundant.

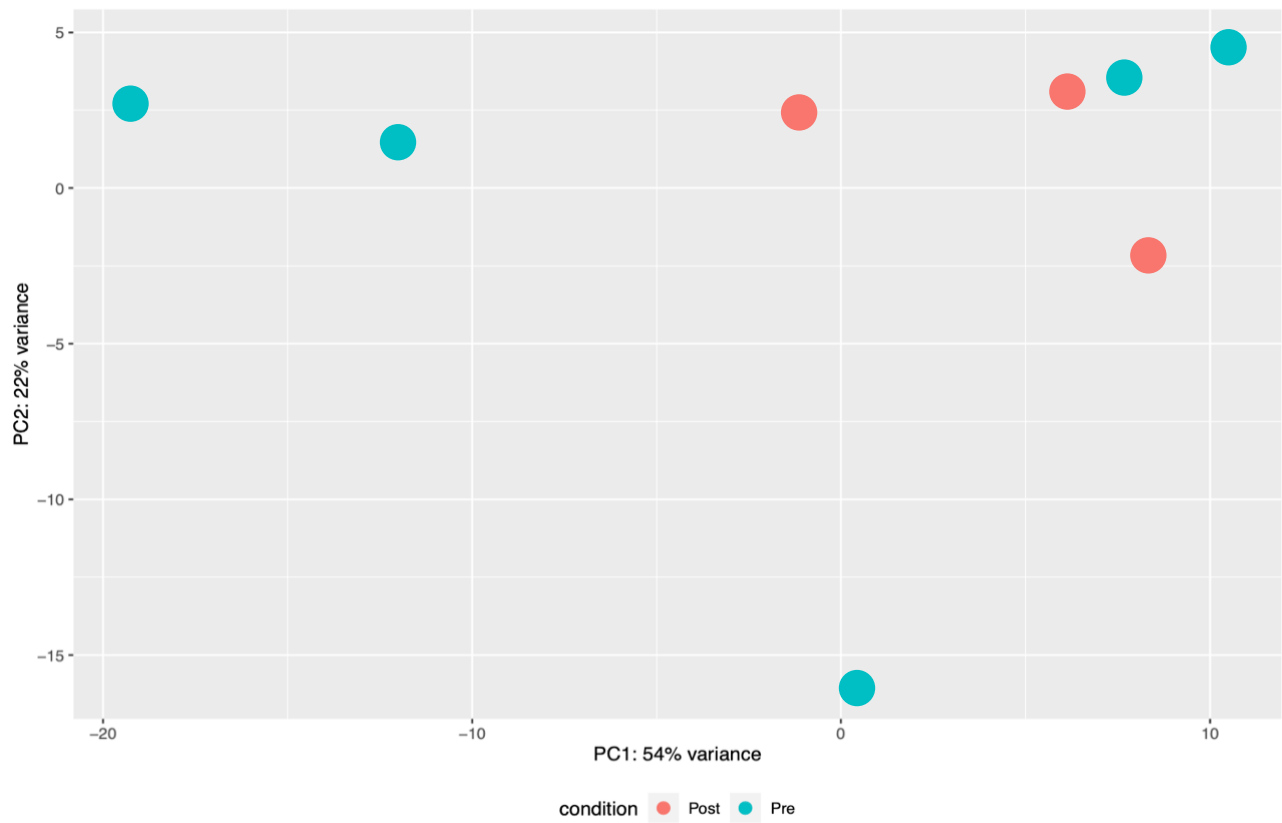


Figure 9: PCA plot of Pre (blue) and Post (red) Samples by organismal data, with the Y-axis, PC2, with a 22% variance and the X-axis, PC1, with a 54% variance.

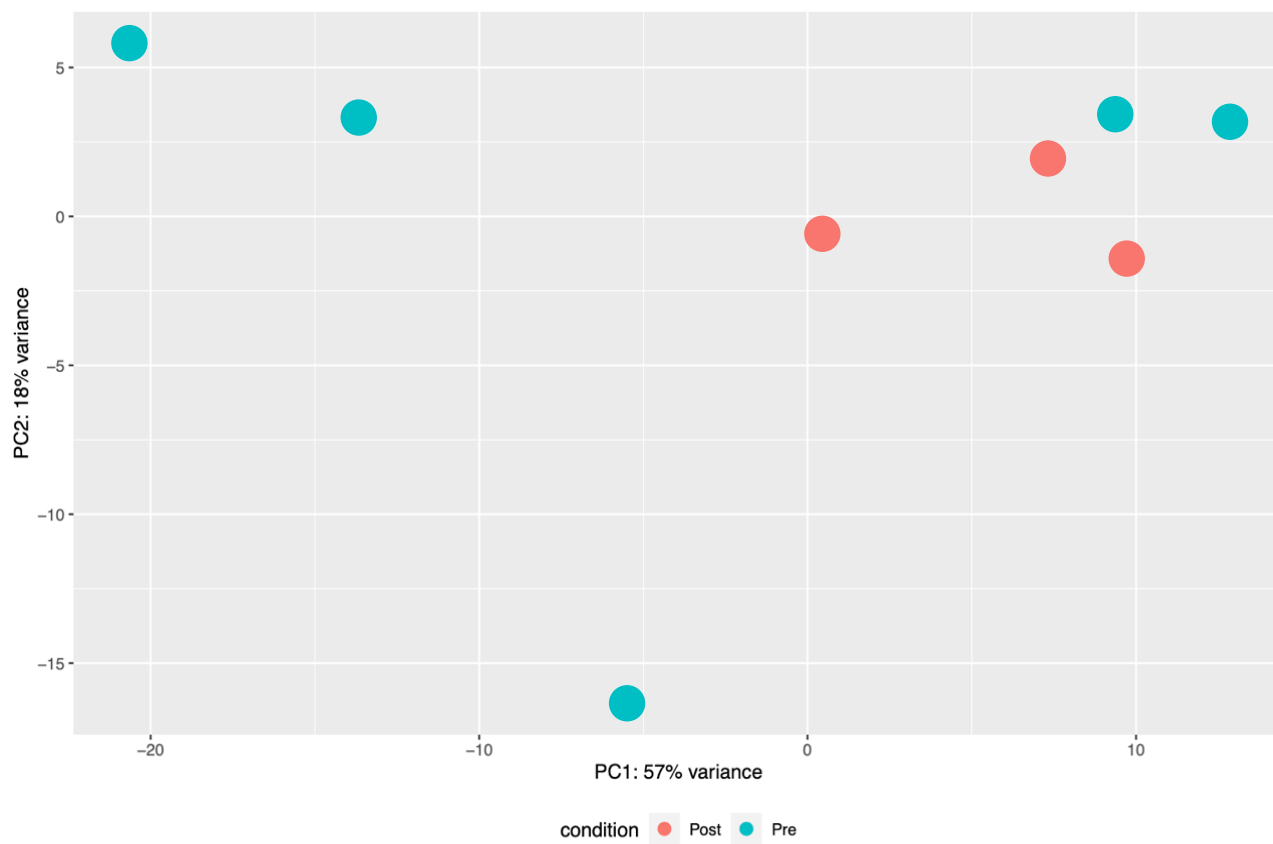


Figure 10: PCA plot of Pre (blue) and Post (red) Samples by functional data, with the Y-axis, PC2, with a 18% variance and the X-axis, PC1, with a 57% variance.

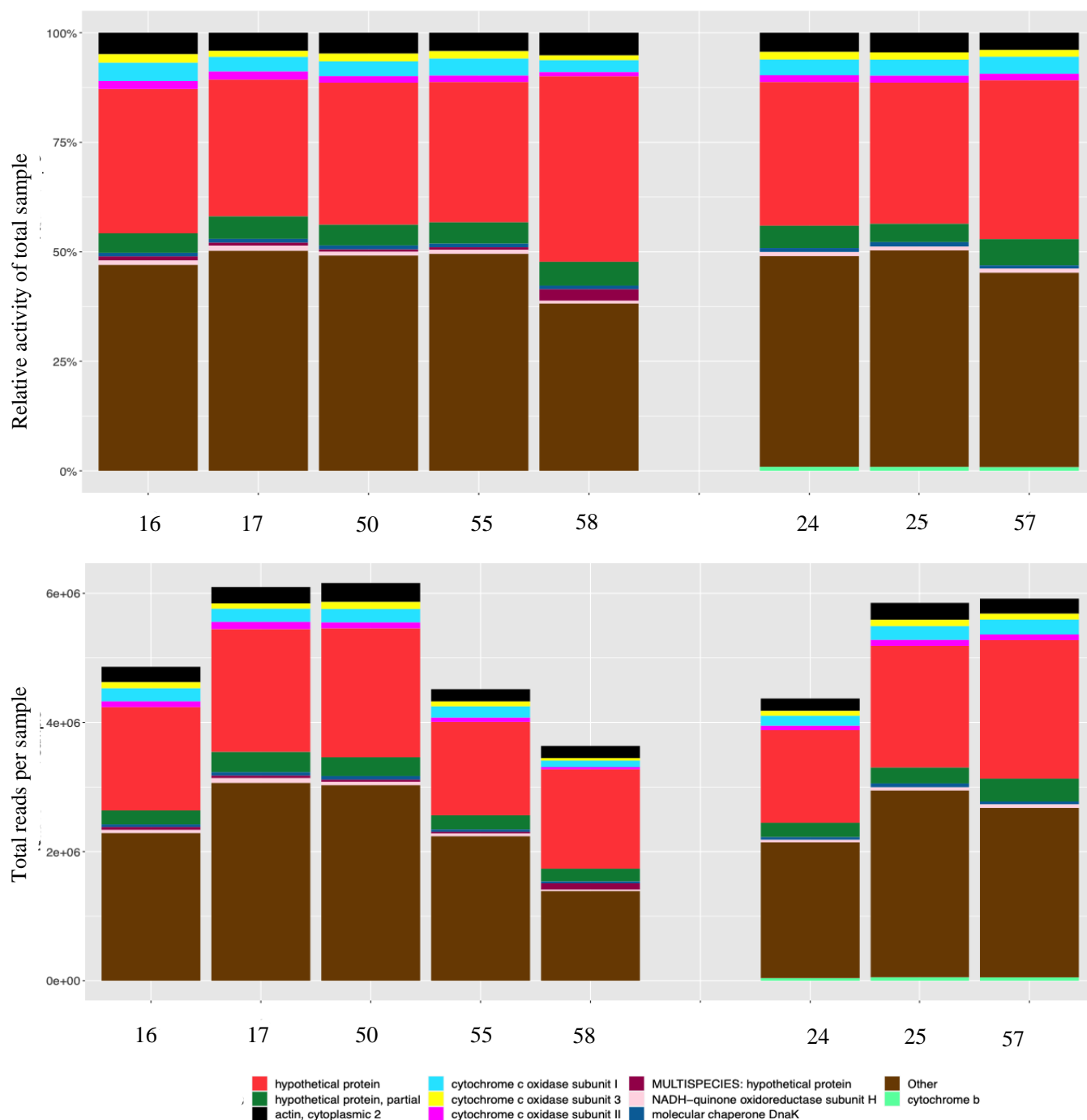


Figure 11: Relative activity graph of the top 10 functional data for each sample. The top bar graph is of relative activity of total sample while the bottom bar graph is total reads per sample. The samples are divided into Pre (left) and Post (right) with a color key located at the bottom.

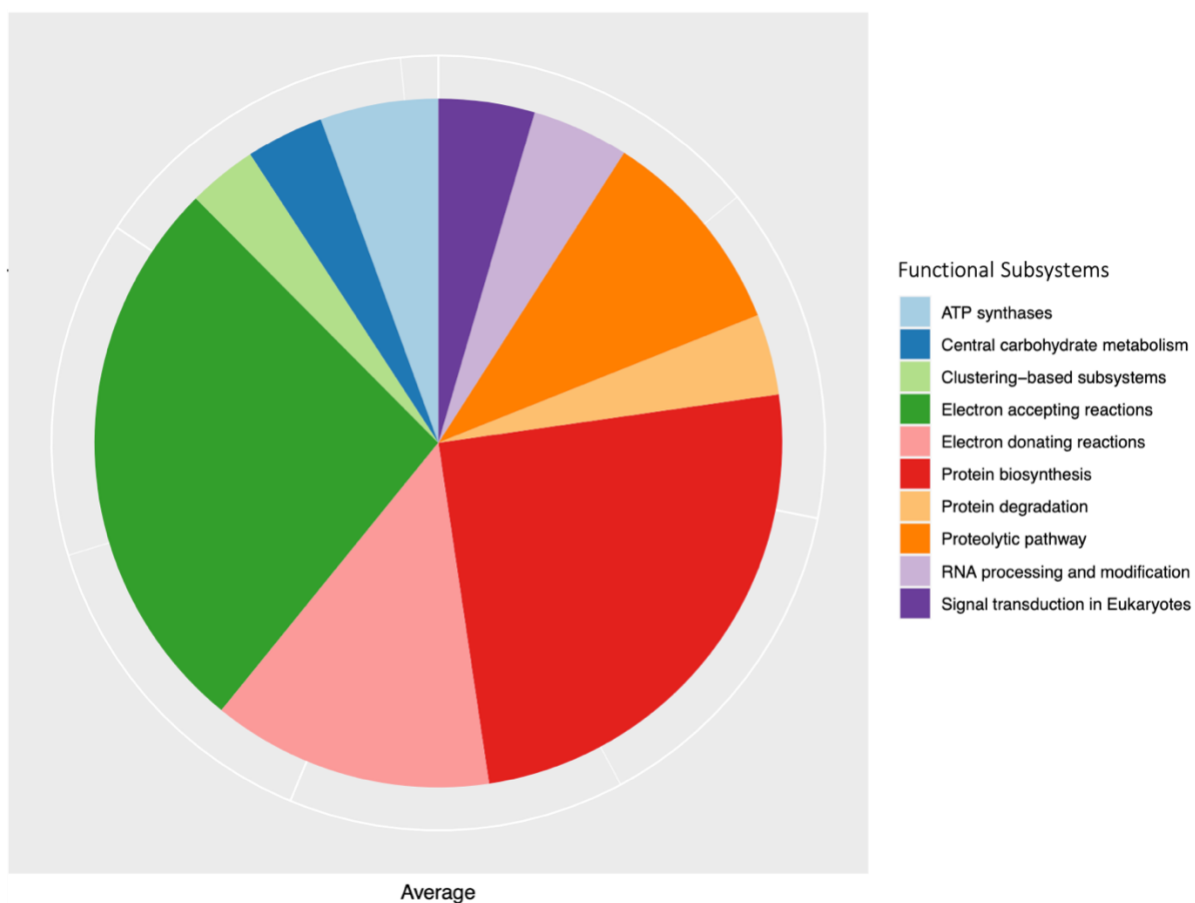


Figure 12: Pie chart representing the top 10 functional subsystems represented in the metatranscriptome samples by SEED.

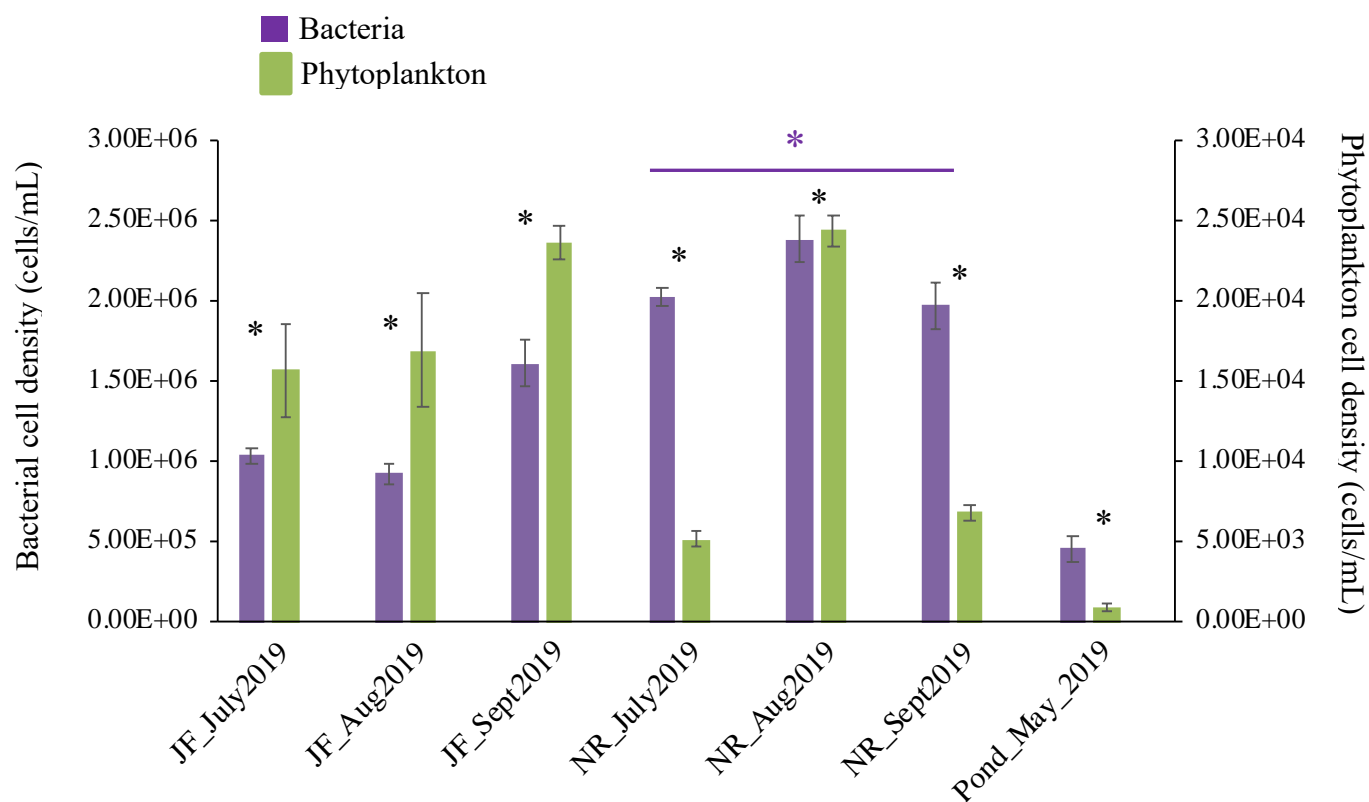


Figure 13: A bar chart showing the cell density (cells/mL) of Bacteria (purple) and Phytoplankton (green) via flow cytometry in water samples collected at Jacob Fork river (JF) and New river (NR) with corresponding months and years of collection.

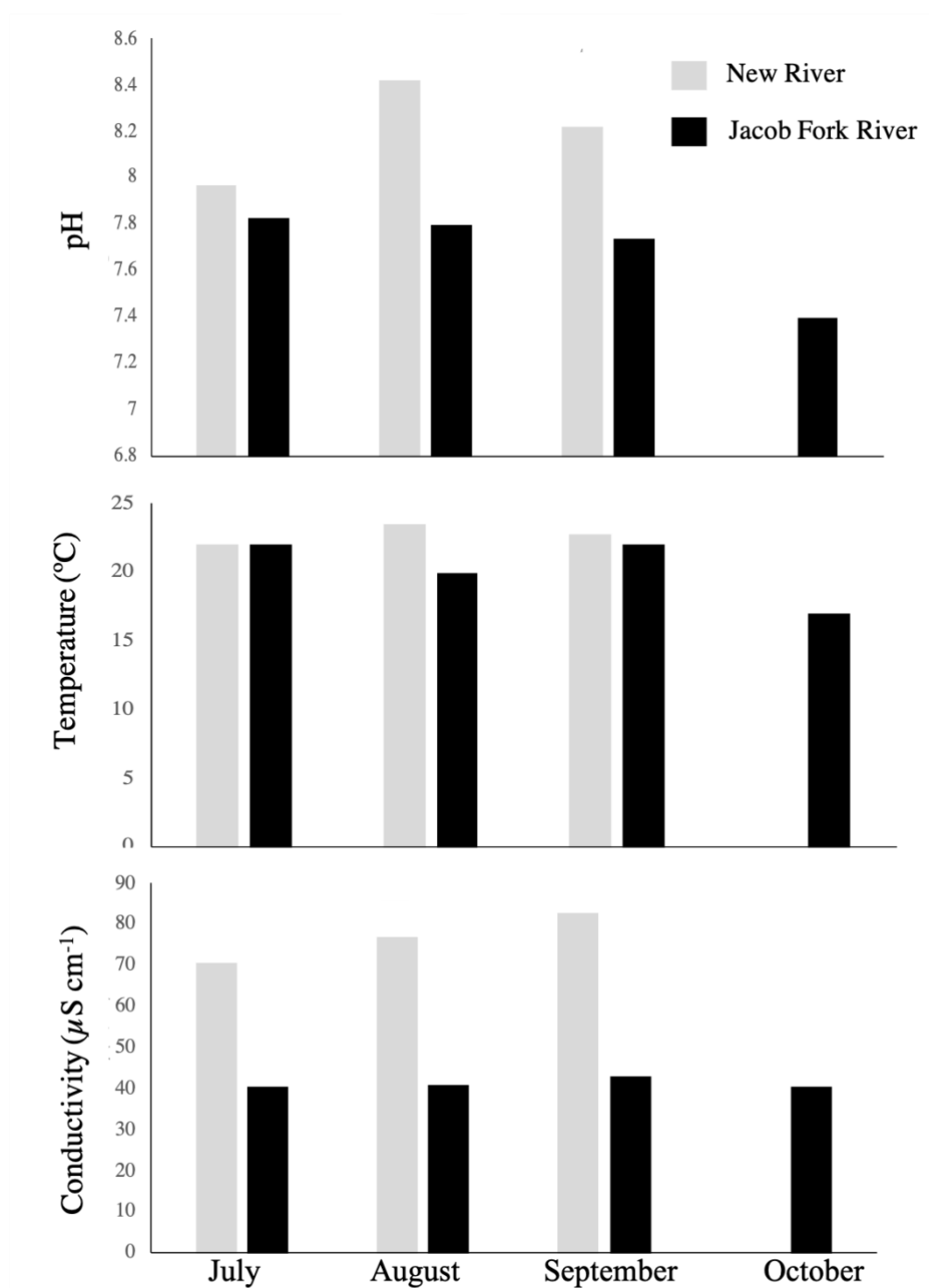


Figure 14: A series of bar charts showing different environmental factors collected on the Y-axis for each site, New river (grey) and Jacob Fork river (black), with the corresponding months (July-October) on the X-axis. From top to bottom: pH, temperature, and conductivity.

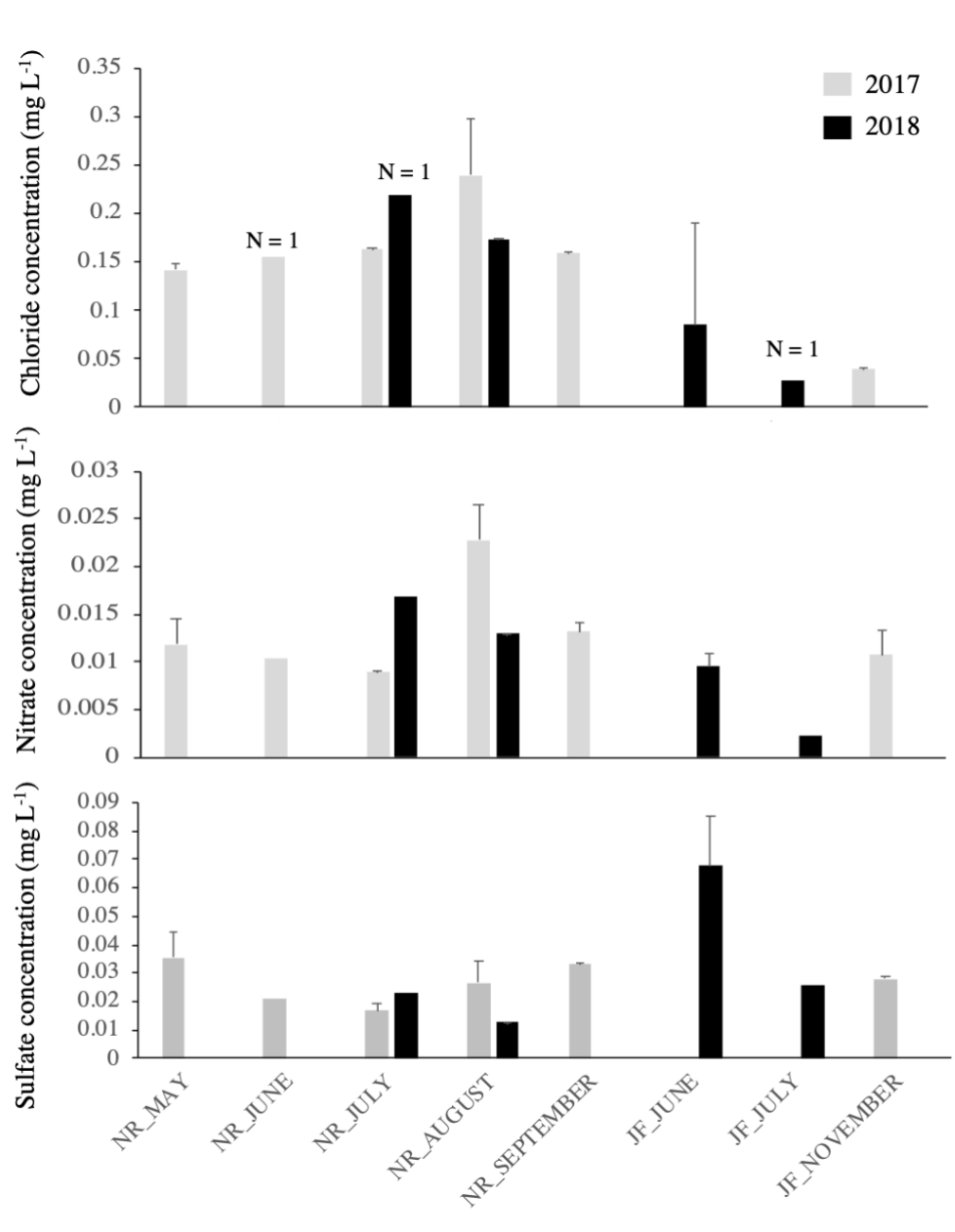


Figure 15: A series of bar charts showing different nutrient concentrations (mg L⁻¹) in collected water samples on the Y-axis, from top to bottom: Chloride, Nitrate, and Sulfate with the corresponding collection months and locations on the X-axis with NR-New river and JF-Jacob Fork river. The collection years are represented by grey bars (2017) and black bars (2018).

Table 1: Major cell types found in gemmules and mature sponge bodies

Cell Types	Function
Archeocytes	Cellular differentiation; Role in nutrient transport; Ingestion and digestion of food particles; Role in sexual reproduction
Thesocytes	Resting state of archeocytes in the gemmule
Choanocytes	Collar cells; Generate water flow
Sclerocytes	Spicule making cells
Exopinacocytes	Outer epithelial cells
Endopinacocytes	Inner epithelial cells
Basopinacocytes	Basal epithelial cells; Positions and anchors sclerocytes
Spongocytes	Active in collagen biosynthesis
Collagenocytes	Active in collagen biosynthesis
Lophocytes	Active in collagen biosynthesis
Pinacocytes	Pavement cells; Encloses the mesohyl
Transport cells	Transport sclerocytes to their location

Table 2: Qualitative data for each of the samples used in the study, such as the sample ID number, year and month in which they were collected, pre or post gemmule formation, and the location of collection site.

Sample_ID	Year	Pre_Post	Month	Collection_site
16	2018	Pre	July	New river
17	2018	Pre	July	New river
24	2018	Post	August	New river
25	2018	Post	August	New river
50	2019	Pre	July	New river
55	2019	Pre	August	New river
57	2019	Post	September	New river
58	2019	Pre	September	New river

Table 3: The loss of 16S reads during the trimming process. The original raw reads for each sample, read counts after filtering, read counts after denoised for forward and reverse reads, and nonchimeric reads.

Sample_ ID	Input_Reads	Filtered_ Reads	Denoised_F	Denoised_R	Merged_Reads	Nonchim_ Reads
CF11	46585	10769	10033	10098	7902	7813
CF12	88385	32441	29358	29373	20170	19716
CF13	78567	36294	33289	32890	21273	20795
CF14	73395	33401	32323	32284	22128	21414
CF15	50258	24180	23074	23285	17389	16958
CF16	65129	29235	28044	27888	20855	20448
CF17	76310	31211	30081	29671	21821	21292
CF18	95249	32067	30875	30576	20519	20117
CF19	71818	34712	33570	33431	21507	20739
CF20	77343	31184	30237	29937	19597	18719
CF21	36194	18858	18159	18126	11248	11069
CF22	38926	19580	18563	18575	13852	13751
CF23	47019	22044	20863	20721	14114	13744
CF24	52029	27632	26537	26424	16460	15928
CF26	69827	31118	29694	29419	19776	19367
CF27	69628	31285	29731	29559	19847	19401
CF28	59333	33011	31211	31041	18910	18295
CF29	76080	43502	42035	41900	24824	24089
CF30	76211	35093	33800	33530	21338	20644
CF31	46890	23981	22771	22227	11606	11310
CF32	76719	38033	36654	36571	22344	21970
CF40	45830	23656	22731	22651	12489	12103
CF41	79619	50765	49599	49389	19250	17635
CF42	38340	20866	20059	20019	11176	10700
CF43	76839	30794	28804	28888	20439	19581
CF44	76391	34319	32619	32776	23599	22353
CF45	62189	22116	22116	21978	15526	14520

Table 4: The loss of RNA reads during the quality trimming process. The original raw reads for each sample, read counts after PEAR merged paired reads, and the final read count after Trimmomatic removed low-quality reads.

Sample_ID	Raw_Reads	Post_Merge	Final_Read_Count
16	47,708,555	39,059,383	35,517,425
17	52,779,588	43,326,124	39,353,480
24	37,446,129	30,297,596	27,372,921
25	50,833,021	41,473,488	37,731,589
50	54,321,279	44,313,614	40,118,836
55	46,919,546	35,739,660	32,703,117
57	52,442,778	44,851,409	40,647,766
58	41,752,015	38,174,036	33,097,311

Table 5: Index table of Shannon and Simpson diversity between Pre and Post gemmule samples for organisms and functional results.

Organism Results		
	Pre	Post
Shannon diversity index	6.12	6.18
Simpson diversity index	0.98	0.98
Functional Results		
	Pre	Post
Shannon diversity index	4.65	4.69
Simpson diversity index	0.87	0.88

Table 6: Organisms that were significantly different in abundance between the pre and post gemmule sponge samples. Differential abundance was calculated using DESeq2 and the table includes mean (X), log2 fold change, indicating if the organisms increased or decreased in abundance, the error associated with the fold change (lfcSE), as well as the statistic (stat), *p*-value, and adjusted *p*-value.

Organism Name	baseMean	PreMean	PostMean	log2Fold Change	lfcSE	stat	pvalue	padj
<i>Kitasatospora cheerisanensis</i>	178.35	369.44	63.69	-2.53	0.34	-7.39	1.49E-13	1.10E-09
<i>Flavobacterium psychrophilum</i>	767.79	528.37	911.44	0.79	0.13	5.99	2.12E-09	7.82E-06
<i>Flavobacterium cauense</i>	353.09	224.50	430.24	0.95	0.16	5.76	8.60E-09	2.11E-05
<i>Bacillus soli</i>	188.34	400.60	60.98	-2.72	0.54	-5.00	5.86E-07	1.08E-03
<i>Sphingomonas melonis</i>	97.10	36.45	133.50	1.86	0.40	4.60	4.25E-06	6.27E-03
<i>Polynucleobacter asymbioticus</i>	2290.98	3348.48	1656.48	-1.01	0.23	-4.49	7.09E-06	8.72E-03
<i>Streptomyces milbemycinicus</i>	47.85	75.13	31.49	-1.28	0.29	-4.41	1.05E-05	1.11E-02
<i>Flavobacterium filum</i>	702.96	461.86	847.62	0.88	0.20	4.37	1.23E-05	1.14E-02
<i>Polynucleobacter sp.</i>	22264.68	31491.05	16728.86	-0.91	0.21	-4.28	1.88E-05	1.54E-02
<i>Pseudoglutamicibacter albus</i>	29.30	55.73	13.44	-2.04	0.48	-4.24	2.20E-05	1.62E-02
<i>Flavobacterium aquatile</i>	3146.72	1701.89	4013.62	1.24	0.30	4.16	3.15E-05	1.92E-02
<i>Polynucleobacter necessarius</i>	1174.49	1691.93	864.03	-0.97	0.24	-4.07	4.65E-05	1.92E-02
<i>Flavobacterium sasangense</i>	603.10	363.48	746.88	1.04	0.25	4.10	4.12E-05	1.92E-02
<i>Flavobacterium suncheonense</i>	363.99	271.51	419.47	0.63	0.15	4.14	3.41E-05	1.92E-02
<i>Sphingomonas taxi</i>	298.17	179.16	369.57	1.04	0.25	4.18	2.95E-05	1.92E-02
<i>Promicromonosporaceae bacterium</i>	269.82	384.84	200.81	-0.93	0.23	-4.07	4.69E-05	1.92E-02
<i>Sulfurimonas denitrificans</i>	209.86	271.01	173.16	-0.64	0.16	-4.10	4.06E-05	1.92E-02
<i>Pseudoxanthomonas spadix</i>	99.50	56.16	125.50	1.17	0.29	4.10	4.18E-05	1.92E-02
<i>Polynucleobacter wuianus</i>	2730.88	3992.19	1974.10	-1.02	0.25	-4.01	6.06E-05	2.23E-02
<i>Herbidospora daliensis</i>	72.19	102.90	53.76	-0.92	0.23	-4.02	5.79E-05	2.23E-02
<i>Flavobacterium saliperosum</i>	319.35	229.11	373.50	0.71	0.18	3.98	6.78E-05	2.38E-02
<i>Polynucleobacter</i>	3885.36	5615.76	2847.12	-0.98	0.25	-3.97	7.28E-05	2.44E-02
<i>Phormidium sp.</i>	955.29	1132.17	849.16	-0.42	0.11	-3.89	1.02E-04	3.27E-02
<i>Polynucleobacter sinensis</i>	1148.34	1581.13	888.66	-0.83	0.22	-3.84	1.23E-04	3.77E-02
<i>Unclassified Nitrosomonadales</i>	530.29	750.10	398.40	-0.91	0.24	-3.83	1.31E-04	3.85E-02
<i>Xenorhabdus khoisaniae</i>	76.55	179.38	14.86	-3.58	0.95	-3.78	1.60E-04	4.54E-02

Table 7: Functions that were significantly different in abundance between the pre and post gemmule sponge samples. Differential abundance was calculated using DESeq2 and the table includes mean (X), log2 fold change, indicating if the functions increased or decreased in abundance, the error associated with the fold change (lfcSE), as well as the statistic (stat), *p*-value, and adjusted *p*-value.

Functions	baseMean	PreMean	PostMean	log2Fold Change	lfcSE	stat	pvalue	padj
uroporphyrin-III methyltransferase	38.07	88.18	8.00	-3.42	0.45	-7.54	4.59E-14	3.40E-10
catechol 2,3-dioxygenase	330.24	141.29	443.61	1.65	0.27	6.09	1.11E-09	4.11E-06
bifunctional o-acetylhomoserine/ o-acetylserine sulphydrylase	70.63	118.03	42.20	-1.49	0.29	-5.05	4.43E-07	1.09E-03
porin family protein	255.87	156.20	315.66	1.02	0.22	4.71	2.47E-06	4.58E-03
DUF4293 domain-containing protein	121.12	64.45	155.13	1.28	0.28	4.60	4.32E-06	6.40E-03
disulfide bond formation protein B	93.44	39.16	126.00	1.69	0.37	4.52	6.26E-06	7.73E-03
MULTISPECIES: porin	383.67	545.81	286.39	-0.93	0.21	-4.46	8.06E-06	8.39E-03
Hsp70 family protein	23.04	42.13	11.59	-1.82	0.41	-4.44	9.06E-06	8.39E-03
type III glutamate--ammonia ligase	62.39	34.05	79.39	1.24	0.29	4.25	2.17E-05	1.78E-02
hydrogenase expression protein, partial	28.18	55.62	11.71	-2.32	0.57	-4.04	5.39E-05	3.63E-02
pilus assembly protein	22.96	7.02	32.53	2.23	0.55	4.05	5.11E-05	3.63E-02
elongation factor G, partial	234.31	114.09	306.44	1.42	0.36	4.00	6.32E-05	3.90E-02

Table 8: Functions subsystems using SEED that were significantly different in abundance between the pre and post gemmule sponge samples. Differential abundance was calculated using DESeq2 and the table includes mean (X), log2 fold change, indicating if the subsystems increased or decreased in abundance, the error associated with the fold change (lfcSE), as well as the statistic (stat), *p*-value, and adjusted *p*-value.

Function Subsystems	baseMean	PreMean	PostMean	log2Fold Change	lfcSE	stat	pvalue	padj
Motility and Chemotaxis	9664.44	8456.79	10389.02	-0.30	0.13	-2.32	2.04E-02	5.72E-01
OTHER	33854692.19	30797651.1	35688916.85	-0.21	0.29	-0.74	4.59E-01	9.71E-01
Respiration	787010.00	804586.93	776463.84	0.051	0.10	0.54	5.90E-01	9.71E-01
Cofactors, Vitamins, Prosthetic Groups, Pigments	74716.78	76855.51	73433.54	0.07	0.06	1.08	2.82E-01	9.71E-01
Cell Wall and Capsule	20212.54	19408.16	20695.16	-0.09	0.10	-0.98	3.28E-01	9.71E-01
Virulence	10933.64	11213.52	10765.72	0.06	0.12	0.48	6.35E-01	9.71E-01
Photosynthesis	6732.09	7199.09	6451.89	0.16	0.30	0.53	5.99E-01	9.71E-01
Phages, Prophages, Transposable Elements, Plasmids	4550.34	4270.72	4718.11	-0.14	0.21	-0.69	4.90E-01	9.71E-01
Sulfur Metabolism	2268.71	2168.71	2328.71	-0.10	0.14	-0.73	4.65E-01	9.71E-01
Iron Acquisition and Metabolism	1107.96	1143.57	1086.59	0.07	0.12	0.62	5.38E-01	9.71E-01
Phages, Prophages, Transposable Elements	880.20	707.66	983.73	-0.48	0.72	-0.66	5.08E-01	9.71E-01
Dormancy and Sporulation	70.00	31.07	93.35	-1.59	0.93	-1.71	8.80E-02	9.71E-01

Table 9: Functions that were significantly different in abundance between the pre and post gemmule sponge samples from two genera (Polynucleobacter and Flavobacterium) that were significantly different and abundant in Table 5. Differential abundance was calculated using DESeq2 and the table includes mean (X), log2 fold change, indicating if the functions increased or decreased in abundance, the error associated with the fold change (lfcSE), as well as the statistic (stat), *p*-value, and adjusted *p*-value.

Organism: Polynucleobacter								
Function	BaseMean	PreMean	PostMean	log2FoldChange	lfcSE	stat	pvalue	padj
magnesium chelatase subunit H	29.45	10.74	40.67	1.88	0.34	5.55	2.87E-08	2.12E-05
light-harvesting protein chlorophyllide a reductase iron protein subunit X	479.88	229.57	630.06	1.46	0.30	4.86	1.16E-06	4.27E-04
hydroxymethylglutaryl-CoA lyase	18.20	8.66	23.92	1.52	0.42	3.62	2.91E-04	3.58E-02
photosynthetic reaction center subunit L	21.88	36.08	13.36	-1.42	0.38	-3.77	1.62E-04	3.58E-02
photosynthetic reaction center subunit M	18.63	8.03	25.00	1.58	0.43	3.64	2.72E-04	3.58E-02
transaldolase	28.84	14.21	37.62	1.40	0.38	3.68	2.36E-04	3.58E-02
	59.41	37.27	72.69	0.93	0.26	3.58	3.43E-04	3.62E-02
Organism: Flavobacterium								
Function	BaseMean	PreMean	PostMean	log2FoldChange	lfcSE	stat	pvalue	padj
fibronectin type III domain-containing protein, partial	66.18	132.94	26.13	-2.30	0.42	-5.51	3.65E-08	9.42E-06
acyl-CoA desaturase	113.24	166.69	81.17	-1.07	0.21	-5.04	4.62E-07	5.96E-05
L-threonine 3-dehydrogenase	182.48	266.18	132.27	-1.04	0.23	-4.55	5.24E-06	4.51E-04
hypothetical protein	7982.82	9801.24	6891.77	-0.51	0.12	-4.34	1.43E-05	9.22E-04
4-hydroxyphenylpyruvate dioxygenase	49.30	72.15	35.60	-1.04	0.25	-4.22	2.43E-05	1.25E-03
NADH-quinone oxidoreductase subunit N	1868.23	2685.51	1377.87	-0.97	0.23	-4.17	2.99E-05	1.29E-03
beta-glucosidase	44.23	66.20	31.05	-1.12	0.28	-4.08	4.53E-05	1.67E-03
alkaline phosphatase family protein	110.27	150.17	86.33	-0.82	0.21	-4.00	6.32E-05	2.04E-03
fumarylacetoacetase	121.12	172.37	90.37	-0.96	0.24	-3.95	7.84E-05	2.25E-03
DUF11 domain-containing protein	25.25	42.17	15.10	-1.49	0.38	-3.91	9.31E-05	2.40E-03
proton-translocating NADH-quinone oxidoreductase chain N	134.00	196.30	96.63	-1.06	0.28	-3.85	1.19E-04	2.79E-03
DUF3347 domain-containing protein	26.76	41.67	17.82	-1.23	0.33	-3.74	1.87E-04	4.03E-03
cell division protein	575.06	755.24	466.96	-0.70	0.19	-3.63	2.80E-04	5.15E-03
RagB/SusD family nutrient uptake outer membrane protein	256.25	340.05	205.98	-0.72	0.20	-3.65	2.61E-04	5.15E-03
aconitate hydratase	147.85	204.82	113.66	-0.88	0.24	-3.61	3.05E-04	5.25E-03
MULTISPECIES: hypothetical protein	294.77	390.34	237.42	-0.73	0.21	-3.43	6.06E-04	9.77E-03
MULTISPECIES: ornithine--oxo-acid transaminase	309.37	402.29	253.62	-0.68	0.20	-3.36	7.82E-04	1.19E-02
ATP:cob(I)alamin adenosyltransferase	54.99	78.03	41.16	-0.95	0.29	-3.25	1.15E-03	1.63E-02
alpha-glucosidase	31.97	47.01	22.94	-1.02	0.32	-3.24	1.20E-03	1.63E-02
beta-mannosidase	39.35	56.16	29.27	-1.00	0.31	-3.20	1.38E-03	1.78E-02
RNA-binding protein	225.23	292.08	185.13	-0.66	0.21	-3.09	2.02E-03	2.48E-02
aminotransferase	22.55	33.12	16.21	-1.08	0.35	-3.04	2.37E-03	2.78E-02
small GTP-binding protein, Ras family	22.60	33.53	16.05	-1.06	0.36	-2.98	2.89E-03	3.25E-02
ankyrin repeat domain-containing protein	45.18	61.73	35.25	-0.85	0.29	-2.96	3.10E-03	3.33E-02
T9SS C-terminal target domain-containing protein	1199.97	1401.43	1079.09	-0.37	0.13	-2.82	4.84E-03	5.00E-02

Appendix 1.

Sponge Germination Protocol

Overview:

Gemmules collected from parental sponge in the field have the ability to be hatched in the laboratory. In order to do so they must be isolated and washed so they are in pristine condition upon storage in 4°C. Gemmules are typically stored for an extended period of time, prior to hatching. Gemmules take about 4 to 7 days to start hatching and attaching themselves to the substrate in the well plates. They typically continue to grow in size for 7-10 days before starting to desiccate.

Collection:

- Collect sponge tissue with visible gemmules with a sterile razor blade
- Tissue is then stored in 50 mL falcon conical tube of filtered river water or cold Strekal's media covered with foil
- Place tube containing sample on ice until returned to campus
- Store in fridge (4°C) still covered with foil to block out light for a year or more

Strekal's Medium:

- An aqueous solution enabling gemmules attachment (Strekal and McDiffett, 1974).
- 10X Strekal's Medium contains 0.9mM of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5mM CaCO_3 , 0.1 mM $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ and 0.1 mM KCl
- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
 - $\frac{246.47g}{\text{mol}} * \frac{1 \text{ mol}}{1000 \text{ mmol}} * \frac{0.9 \text{ mmol}}{L} * 1L = 0.222g$
- $\text{CaCO}_3 \cdot 2\text{H}_2\text{O}$
 - $\frac{100.086g}{\text{mol}} * \frac{1 \text{ mol}}{1000 \text{ mmol}} * \frac{0.5 \text{ mmol}}{L} * 1L = 0.05g$
- $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$
 - $\frac{284.20g}{\text{mol}} * \frac{1 \text{ mol}}{1000 \text{ mmol}} * \frac{0.1 \text{ mmol}}{L} * 1L = 0.028g$
- KCl
 - $\frac{74.5513g}{\text{mol}} * \frac{1 \text{ mol}}{1000 \text{ mmol}} * \frac{0.1 \text{ mmol}}{L} * 1L = 0.007g$

Separating Gemmules From Maternal Tissue:

- Most steps were done following the 'Pick, Wash and Hatch Gemmules Handbook'
- Important to note all following steps should be done on ice and keeping the Strekal's Medium cold during the procedure with minimum light exposure (I dimmed the room as the dissecting microscope produced plenty of light)
- Take a small portion of the sponge tissue and place it into a small petri dish containing cold Strekal's Medium
- Using a dissecting microscope and teasing needles, carefully removal all maternal tissue from the gemmules.
 - Be careful not to pop the gemmules-they won't be useful afterwards
- A disposable pipette can be used to transfer clean gemmules into a new falcon conical tube of Strekal's solution on ice to insure they don't get too hot by the microscope light
- Left over maternal tissue can either be disposed of or stored for later use

- Continue to repeat this process until all maternal tissue has been sorted through and all gemmules removed
- (Gemmules may be stored at this stage if needed-be sure to label as unwashed if you do)

Washing Gemmules:

- Prepare a hydrogen peroxide solution in a 15mL Falcon conical tube by diluting 400 μ L of 30% hydrogen peroxide in 13.6 mL (Handbook says 500 μ L in 13mL- but upon email the author says she uses a lower percentage to wash the gemmules)
- Gather all the clean gemmules into about 1mL of Strekal's solution (by dumping and pipetting out excess solution) and pipette the combination into the hydrogen peroxide solution tube
 - Adding the 1mL of Strekal will permit the hydrogen peroxide to reach a concentration of 1% (or less)
- Invert the 15mL tube every minute for a duration of 3 minutes
 - This will permit removal of algae and to weaken gemmules membrane
 - Little bubbles should appear
- Help the gemmules settle to the bottom of the tube by softly tapping the tube
 - Some of them will not settle because of the bubbles, don't worry and continue
- Remove the used solution by inverting the tube above a petri dish and rapidly pick up the few gemmules from the dish and put them back into the tube
- Fill the tube with new cold Strekal's medium and invert it immediately several times
- Change the solution again using the same inverting method and replace with new medium
- Incubate gemmules for 3 minutes in the new solution, inverting the tube every minute
 - All gemmules which are still floating can be removed at this step
 - As they are empty and contain no cells in them
- Repeat the last step 6 to 8 more times
 - You may do more if you feel it's needed- but do no less as you want to be sure all the peroxide is removed from the gemmule coat
- After that the gemmules are washed and ready to use. They can also be stored at 4°C at this time for later use
 - Please keep in mind 4°C is the fridge, not the freezer

Hatch Gemmules:

- Remove gemmules and Strekal's from fridge and reach room temperature
 - May proceed to next steps as this takes awhile
- Open an 8-well plate and put 1 large or 2 small cover glasses in each well
- Add 1-2 mL of Strekal's Medium in each well (or until the cover glass is covered)
- Remove any air between the well and cover-glass with a pipette tip
- Once gemmules and Strekal's reach room temperature, place 3-4 gemmules in each well

- Try to make sure the gemmules don't group together so they don't grow into one another
- Store gemmules in a dark, room temperature place for 2-3 days without disrupting them
 - I used a labeled cabinet so there was no risk of them being exposed to light in these beginning stages
- Handbook says to change the solution every 24 hours
 - I never changed the solution (afraid to suck up the gemmules or disrupt their attachment) and they still continued to grow

Vita

Taylor Ann Strobe was born in Knob Noster, Missouri to Anthony and Tracy Strobe. She was raised in Hamilton, Illinois by Anthony Strobe. She graduated from Nevada High School in Missouri in 2014. The following fall semester, she entered at Texas A&M University of Galveston to study Marine Biology and Marine Fisheries, and in May 2018 she was awarded the Bachelor of Science degree in dual majors. In the fall of 2018, she continued progress toward a Master of Science degree at the Appalachian State University in Biology with a concentration in Cell and Molecular. The M.S. was awarded in August 2020. Following the M.S. degree Taylor Strobe will be continuing on to receive her Ph.D. in the Interdisciplinary Graduate Program in Biomedical Sciences at University of Kansas Medical Center in Kansas City, Kansas.