A Culture-Independent Approach To The Sponge Microbiome

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SUMMARY

Sponges contain many diverse symbionts whose functional potential has yet to be determined. Sponges are also important sources of biotechnologically relevant biomolecules, often used for the treatment of diseases like cancer, or source of novel antibiotics. Being that sponges are important for biotechnology, development of sustainable mariculture practices is important not only for keeping the sponge alive, but also keeping their microbial consortia intact. Sequencing of the microbial consortia of marine sponges has been ongoing, not just to find novel molecules, but to also understand how the sponge lives in its environment and how their microbial consortia influence their health. Recent advances in metagenomics, the study of the complete genetic makeup of a complex community, has brought to light some of the inherent functions of sponge microbiota such as nutrient cycling and waste management. Previous work has also turned to metagenomic analysis to determine the producers of novel enzymes and natural products. Once thought to be produced by the sponge itself, new studies show that many of these products are likely of microbial origin, given their chemical structure, and their low yields from the sponge.

The diverse groups of microbes, both prokaryotic and eukaryotic, exhibited changes over a period in a mariculture setup and this result depends on the sequencing approach taken. DNA versus RNA-based sequencing yields different groups of microbes, with the RNA-based sequencing likely picking up active microbial communities. Shallow shotgun metagenomic sequencing has revealed the functional potential of biotechnologically relevant sponges and given an in-depth look at not only the bacterial and archaeal communities but also microeukaryotes. There may even be the potential for using sponges as sources of eDNA in other studies based on shotgun sequencing result.

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LIST OF ABBREVIATIONS

ASVs	Amplicon sequence variants
CTAB	Cetyltrimethyl ammonium bromide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
GC	Guanine-cytosine
GIGA	Global Invertebrate Genome Alliance
HMA	High microbial abundance
IMTA	Integrated Multitrophic Aquaculture
KO	KEGG Orthology
LMA	Low microbial abundance
MG-RAST	Metagenomics Rapid Annotation using Subsystems Technology
NCBI	National Center for Biotechnological Information
NGS	Next generation sequencing
NOG	Non-supervised Orthologous Groups
OTUs	Observed taxonomic units
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
rRNA	Ribosomal RNA

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1.0 INTRODUCTION

1.1 GENERAL OVERVIEW OF SPONGES

The phylum Porifera is spread throughout the world (Soest et al. 2012). These organisms are part of an ancient phylum, estimated to have branched from a common metazoan ancestor in the Pre-Cambrian period, or roughly 680MYA (Davidson and Erwin 2009). The phylum *Porifera* can be divided into 5 classes, Demospongia, Calcarea, Hexactinellida, Homoscleromorpha, and the Stromatoporoidea (Hooper et al. 2002), encompassing an extinct group of sponges from the fossil record (Stock 2001). Most sponges belong to the class Demospongia, which compose an estimated 81% of all sponges worldwide (Morrow and Cárdenas 2015). Sponges reproduce with both asexual and sexual reproduction. The forms of asexual reproduction include budding, being broken off, or in the case of freshwater sponges, by the formation of gemmules. Sexual reproduction in sponges involves the broadcast spawning of eggs and sperm into the water. In its larval form, the sponge is motile and eventually settles onto a hard substrate and begins to grow. Sponges are effective filter feeders, able to filter 24m^3 of water per 1kg of sponge tissue daily (Riisgård et al. 1993; Kennedy et al. 2007).

1.1.1 Sponge Body Plan

Sponges have a unique body plan allowing them to feed on particulate matter and bacteria and excrete waste, making them effective filter feeders and a sink for carbon (Pile et al. 1997). Particularly, sponges are a source of particulate matter for reef organisms the conversion of dissolved organic matter (DOM) in reef ecosystems (Goeij et al. 2013). Water is brought in with incurrent pores, or ostia. The sponge consumes DOM and particulate organic matter (POM), which includes bacteria (Pita et al. 2018; Bart et al. 2021; Bart et al. 2021), while the waste products are expelled from an out-current pore called an oscula (Figure 1.1). Along with an extreme filtering capacity, sponges are able to discern between food bacteria and symbionts from the water column (Wilkinson et al. 1984; Wehrl et al. 2007).

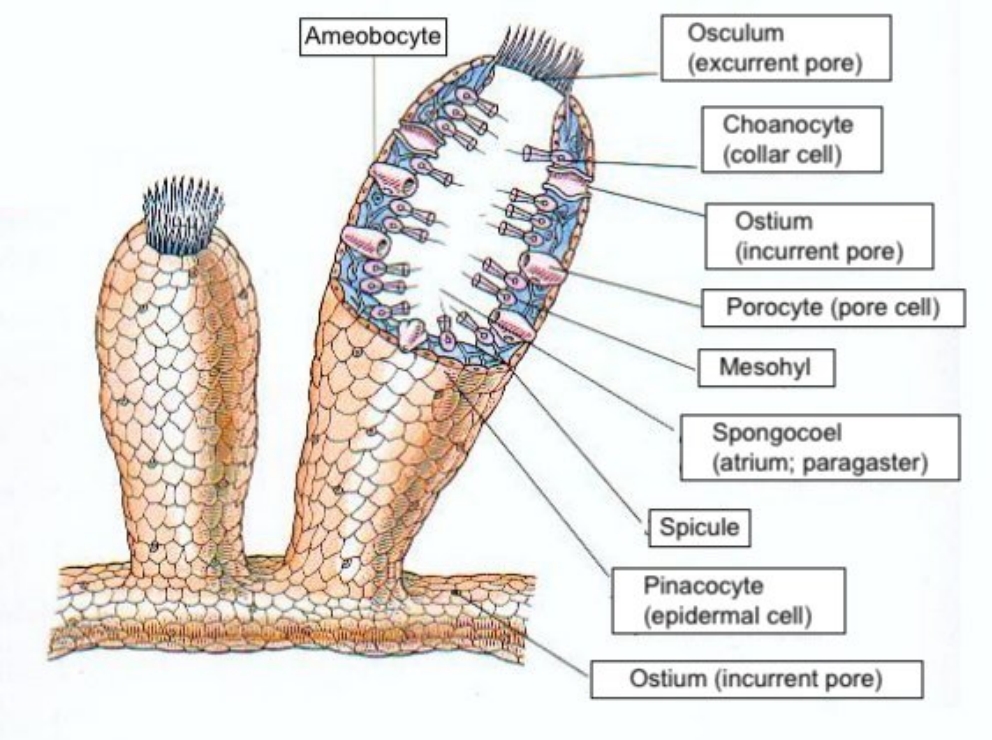


Figure 1.1-Anatomy of a sponge. Most sponges are made up of siliceous or calcareous spicules forming a sort of skeleton of the sponge. Spongin tissue forms around the spicules. The sponge itself contains two layers-the external covering or pinacoderm and the mesohyl. Within these layers are pores. Smaller pores up the sides of the sponge form the incurrent pore or ostia and the larger pores form excurrent pores or oscula. (Adapted from http://universe-review.ca/I10-82-sponge2.jpg,)

1.1.2 General Overview of Marine Sponges

Marine sponges are of interest for this research since they filter 24m^3 of water per kilogram of sponge in seawater per day (Kennedy et al. 2007), concentrating ambient microbes into their tissues to be consumed or potentially assimilated into their mesohyl, and are a host to a number of symbionts. These symbionts are important to the overall health of the sponge (Hentschel et al. 2006; Hentschel et al. 2012). Work by Bruck, Reed, and McCarthy (Brück et al. 2012), has shown that *Discodermia dissoluta* is abundant with microorganisms and have found similar results with populations of Alpha-

and Gammaproteobacteria in the sponge tissue between culture-dependent and restriction fragment length polymorphism and 16S rRNA clone libraries.

Marine sponges are commonly used for natural product research, given their ability to fight off infections. However, it is thought that many of the “sponge-derived” natural products may actually be of microbial origin, specifically host-associated microorganisms (Hochmuth and Piel 2009). The presence of these microorganisms within the sponge makes them a target for metagenomic research. Isolation of the gene or genes of interest and cloning into a cultivable vector is essential large-scale production, and there are numerous microbes within sponges, meaning there may be multiple homologs coding for the same gene (Hochmuth and Piel 2009).

1.1.3 Genomic and Metagenomic Understanding of the Host

The first sponge genome to be sequenced was the Great Barrier Reef sponge, *Amphimedon queenslandica* which was published in 2010 (Srivastava et al. 2010). It was found to contain 27,972 contigs and was assembled into 13,398 scaffolds. Its GC content was 37.5% with an overall length of 166.7Mb. Within the genome, 24,116 proteins were detected. It is important to highlight that there were prokaryotic genes found within the *A. queenslandica* genome (Conaco et al. 2016) indicating an intrinsic relationship with the sponge and potential vertical transmission of symbionts. The initial discovery of this occurred in the Srivastava paper in 2010, where sponge embryos were used for sequencing the *A. queenslandica* genome and during the process of analyzing the genome results, an alphaproteobacterial genome was detected (Srivastava et al. 2010; Conaco et al. 2016). A similar study was conducted on *Ianthella basta* and confirmed with fluorescence in-situ hybridization (FISH) that microbes were attached to gametes (Engelberts et al. 2022).

Low microbial abundance sponges tend to have microbiomes similar to the surrounding seawater, but still have diverse communities. High microbial abundance sponges are

dominated by Chloroflexi clades and tend to contain Candidatus Poribacteria. In addition, Proteobacteria are also a dominant phylum in HMA sponges.

Until recently, only *Amphimedon queenslandica* had a complete genome available. However, through efforts of the Global Invertebrate Genomics Alliance (GIGA) (Lopez et al. 2014; Voolstra et al. 2017) and independent labs, in 2016 there are now two more sponge genomes, *Stylissa carteri* and *Xestospongia testudinaria* (Ryu et al. 2016) and current efforts through projects like the Aquatic Symbiosis Project (McKenna et al. 2024) which contains 55 annotated porifera genomes.

1.2 MICROBIAL ECOLOGY

Microbiology is the study of microbial organisms-bacteria, fungi, protists, and viruses, or colloquially, microbes, and how they interact in their environment. Before the use of ribosomal RNA, or rRNA (Woese and Fox 1977; Olsen and Woese 1993),most of the information available on microorganisms was derived from pure culture or microscopy. The application of molecular techniques for microbial ecology was either expensive, challenging, or non-existent. Early marine microbiology was dependent on culturing bacteria, but the limitations of this method were apparent, especially with the advent of sequencing. The “great plate count anomaly” coined by microbiologists Staley and Konopka (Staley and Konopka 1985), defines cultivable bacteria as making up only a small percentage of the total bacterial diversity. These species reflect those that can grow under laboratory conditions, but they may not have much importance in the environment (Staley and Konopka 1985). Previously, microbiology and microbial ecology focused heavily on culturable microorganisms (Handelsman et al. 1998; Handelsman 2004; Riesenfeld et al. 2004) and those able to be maintained in laboratory stocks (Staley and Konopka 1985). It was not until the past few decades with different molecular techniques and new sequencing technologies that large swaths of microbial communities are able to be studied (Pascoal et al. 2021), and with metagenomic-based guidance, it could be possible to cultivate some of these organisms (Lewis et al. 2021; Liu et al. 2022).

With sponges in particular, microbial ecology has enabled the understanding of how sponge microbial symbionts contribute sponge health, nutrient cycling, defense, and habitat adaptation. Sponges are important components in reef and deep-sea ecosystems through the recycling of organic carbon (Webster and Thomas 2016). A 2022 study (Hanz et al. 2022) revealed that high microbial abundance (HMA) sponges are able to use dissolved resources associated with the base of the food web, while low microbial abundance (LMA) contain nutrient recycling pathways similar to organisms higher in the food web. Sponge health is greatly influenced by its associated symbionts providing chemical defense from predation and regulation of the host immune system and how it responds to environmental change and disease (Fan et al. 2013; Pita et al. 2018; Taylor et al. 2021; Pérez-Llano et al. 2023),

1.2.1 Metagenomics, Sequencing Technology, and the Intersection of Microbial Ecology

Metagenomics is the cultivation-independent analysis of mixed microbial genomic information from environmental samples (Handelsman 2004; DeLong 2009; Gilbert et al. 2010b; Gilbert and Dupont 2011). Molecular biological techniques (cloning, PCR, sequencing, etc.) make it possible to find uncultivable microbes. Metagenomics is a rapidly developing field and is gaining importance in microbial ecology as it reveals unexpected genes, phylogenies, and functions from the environment (New and Brito 2020; Lema et al. 2023). Sequence based metagenomic studies look at DNA sequences, mostly the 16S small subunit (SSU) rRNA gene but can include other markers like the 18S rRNA gene, internal transcribed spacer, and even the large ribosomal subunits, to determine taxonomy. With 16S rRNA gene sequencing, this approach involves selecting one of the many hypervariable regions, usually V1-V2, V3, V4, V4-V5, or V6, and designing universal primers. Many regions are more conserved, noted by the darker shapes (Figure 1.2) and those are generally more suitable for metabarcoding studies. Microbial metabarcoding studies are not true metagenomics as only single genes are analyzed, whereas with true metagenomics, the entirety of the

genomes in a sample are considered, giving a more extensive view of community structure and function (Handelsman 2004; Gilbert and Dupont 2011; New and Brito 2020).

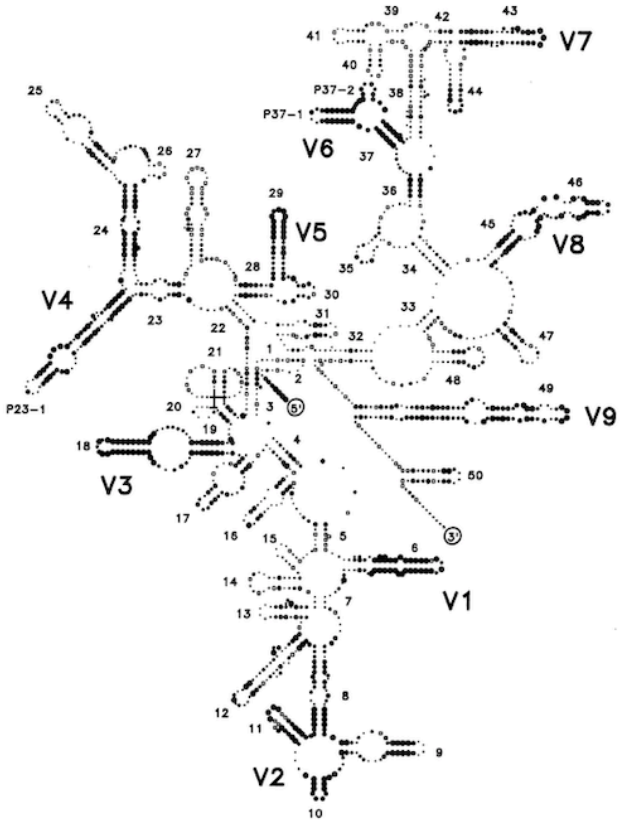


Figure 1.2-Structure of prokaryotic small subunit RNA (Neefs et al. 1993). Darker areas on the structure show high amounts of conservation. These areas are often the basis for universal sequencing primers and allows for the amplification of multiple species of bacteria and in some cases, archaea. The lighter areas show the amount of variability in the RNA SSU molecule.

Function-based metagenomics screenings consist of cloning metagenomic DNA into a host, generally a cosmid or fosmid due to the size of the insert, which will then express that gene or genes. Metatranscriptomics focuses on what genes are being transcribed in the organism and can be utilized to determine up and down regulation of a suite of genes. Similar techniques can be used to determine the gene composition in an environmental sample. Prior to next generation sequencing (NGS), for a metagenomics

study, technologies such as denaturing gradient gel electrophoresis (DGGE) (Muyzer and Smalla 1998; Schäfer and Muyzer 2001) and generating clone libraries for functional screening (Sousa et al. 2020; Lema et al. 2023; Santos-Pereira et al. 2023). Sequencing technology used for metagenomic studies include the Illumina platforms and just prior to Illumina, 454 pyrosequencing, though in more recent years , pyrosequencing has largely been discontinued (Heather and Chain 2016; Slatko et al. 2018; Giani et al. 2020). Newer technologies include Oxford Nanopore Technologies platforms and PacBio, both of which produce long read sequences as opposed to Illumina's short reads and up and coming Illumina competitor, Singular Genomics and Elemental Biosciences (Eisenstein 2023). . These "next generation DNA sequencing" (NGS) technologies, as they are referred, are important advancements in molecular biology as one can generate gigabytes and terabytes of genomic data (Caporaso et al. 2012; Berger and Yu 2023).

1.2.1.1 Illumina

Another common sequencing technology is Illumina, in which enzymatic amplification occurs on a glass surface, but the sequence length is small, usually about 150bp (Mardis 2011), but as technology improves, it will be able to produce longer reads. Multiplex identifiers are also used on the Illumina platform. Illumina is readily used by various microbiome projects, such as the Earth Microbiome Project (Gilbert et al. 2010a; Gilbert et al. 2014) and Sponge Microbiome Project (Thomas et al. 2016), as it generates millions of short reads for these high-throughput community studies (Figure 1.3).

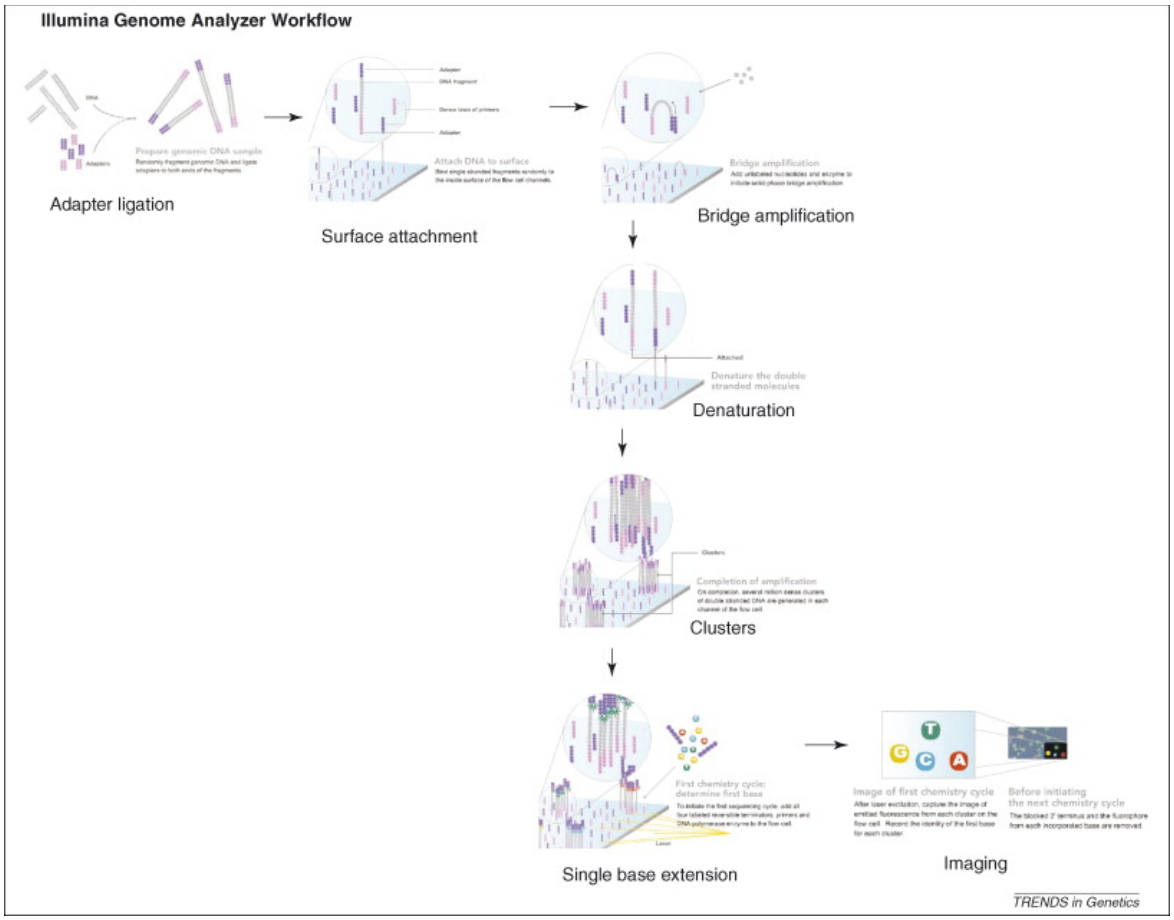


Figure 1.3-Illumina workflow from (Mardis 2008). Illumina sequencing, or sequencing by synthesis, first works by taking amplicons (or sheared genomic DNA for WGS) and attaching adapters to allow for attachment to the glass surface of the flow cell. DNA is denatured and undergoes a bridge amplification to attach to the surface. It is then amplified into two strands, the forward and reverse. The molecules are then denatured and clustered. Following this step is the chemistry to determine the sequence. Each nucleotide is represented by a wavelength of light.

Through NGS, it is now possible to view microbes in the 'rare biosphere' (Sogin et al. 2006) coined in 2006, a group of uncultivable, rarely occurring microbes. This new appreciation for unknown microbes may yield clues into the effects of environmental change (Sogin et al. 2006; Bell et al. 2010) and unearth new novel metabolic pathways essential for organismal survival (Handelsman et al. 1998; Desai et al. 2013; Bhushan et al. 2017).

1.2.3 Microbial Studies and Metagenomics

Diverse collections of microbes encode for many biochemical functions that may be beneficial to its environment and to neighboring microbes. Bacterial populations are focused on because they are the predominant group in an ecosystem, dubbed the “microbiome” and have comprehensively documented phylogenetic datasets and classification systems (Petrosino et al. 2009). With many diverse functions, microbes are suitable for research in natural products, which are chemicals produced by living organisms.

Metagenomic studies convey a multitude of information. Very recently, metagenomic techniques were applied to the Human Microbiome Project (HMP), a project aimed at determining the species of bacteria in biomes within the human body and the various functions these bacteria perform. Techniques from the HMP (Lazarevic et al. 2009) have inspired the Earth Microbiome project (EMP), which aimed at defining the microbial species and functions within various environments, and the most recent the Sponge Microbiome Project, a more niche project aimed at determining the functions and taxonomy of microorganisms within sponges.

1.3 DEMAND FOR NOVEL COMPOUNDS

There is a continuing demand for novel compounds (Schmeisser et al. 2007), particularly for the pharmaceutical (Laport et al. 2009; Bhimba et al. 2013; Ng et al. 2013; Santos-Gandelman et al. 2014; Schmitt et al. 2016; Gürlek et al. 2020) and agricultural industries (Chowdhary et al. 2018; Ali et al. 2019; Dotaniya et al. 2019; Sahu et al. 2019), and bioremediation (Bains et al. 2019; Bhatt 2019; Gangola et al. 2019). Currently, there are few patents filed for molecules derived from metagenomic libraries, due mostly to genes not being detected, or not yet assigned a function (Schmeisser et al. 2007). Metagenomic studies are not only for determining microbial species and function but have shown promise in the field of natural product discovery. If the chemical diversity produced by cultivable microorganisms is any indication of the potential of

uncultivable microbes, then there are many molecules yet to be discovered (Gillespie et al. 2002). Examples from one of the most studied uncultivable microbes, *Candidatus Entotheonella sp*. are seen in Figure 1.4. The various functions of microbes in the environment have a purpose for the microbe and for human interest (Li and Qin 2005). While still an emerging field, metagenomic studies have revealed novel distributions of genes, metabolism, and unpredicted phylogenies (Rinke et al. 2013; Raguideau et al. 2021) within various biomes (Rappé and Giovannoni 2003; Gilbert et al. 2010b; Caro-Quintero and Konstantinidis 2012, p.; Barra et al. 2017; Keller-Costa et al. 2022).

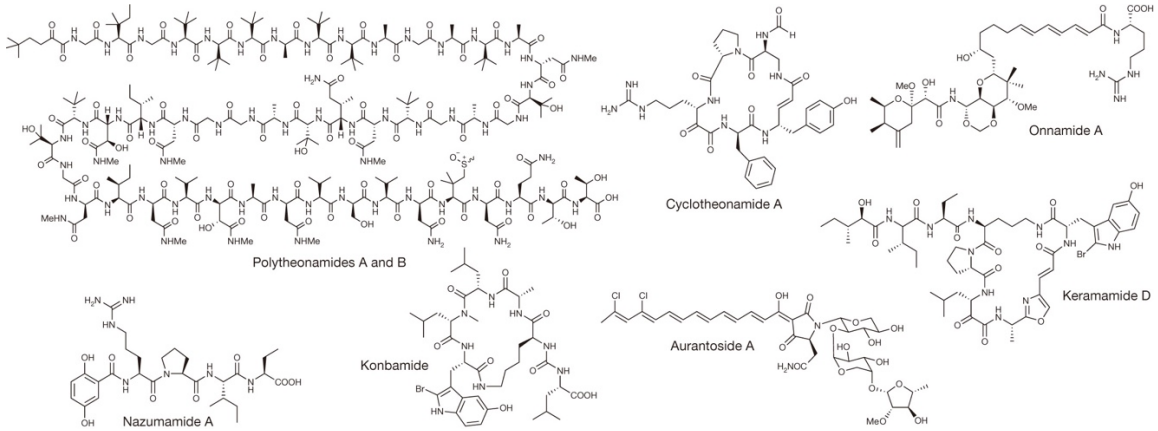


Figure 1.4-Compounds derived from the sponge-associated bacterium *Candidatus Entotheonella.* *Candidatus Entotheonella*, an uncultivable symbiont of *Theonella swinhoei*, but also found in other sponge species (Lackner et al. 2017). Many of these compounds display activities such as antifungal (Schmidt et al. 2000; Mori et al. 2018) and anti-cancer activity (Gunasekera et al. 1990; Bubb et al. 1995; Brück et al. 2008). The figure is from Wilson et al. 2014 (Wilson et al. 2014) .

1.3.1 Natural Products and the Intersection of Metagenomics

Natural products have been a useful tool in medicine for millennia with the earliest examples being produced from plant oils in ancient civilizations (Cragg and Newman 2005; Dias et al. 2012). Through the serendipitous discovery of penicillin (Fleming 1929) from mold and further discovery of streptomycin from soil-dwelling bacteria (Schatz et al. 1944), the use of microbes to produce natural products escalated (Dias et al. 2012). Natural products are chemicals produced from natural sources and bioactive

compounds are components of natural products, usually derived from food items, that have actions within the body that are deemed beneficial to the overall health of an organism (*NCI Dictionary of Cancer Terms* 2011; *Bioactive Compound - an overview | ScienceDirect Topics* [no date]). However, these intense efforts led not only to the development of new drugs, but also to the development of drug resistance, thus creating a need for more products (Lefevre et al. 2008; Roemer et al. 2011). A caveat is that the original pool of cultivable microbes is dwindling, meaning the next step is to derive products from the uncultivable (Lefevre et al. 2008), or finding ways to re-tool and re-purpose old drugs. Previous culture work using marine sponges has shown that the pool of cultivable microbes may actually be an artifact of the culturing technique and not the organism (Olson et al. 2000; Kennedy et al. 2007; Joint et al. 2010; Lewis et al. 2021; Jung et al. 2022; Rodrigues and de Carvalho 2022). This observation opened the door for metagenomic studies aimed at the discovery and development of new natural products.

1.3.1.1 Natural product research in marine sponges

Several sponge natural product studies have focused on polyketide synthases (PKS) (Piel et al. 2004; Schirmer et al. 2005; Kaluzhnaya et al. 2012; Della Sala et al. 2013; Trindade-Silva et al. 2013; Ueoka et al. 2015; Borchert et al. 2016; Kurnia et al. 2017; Rodríguez-Berríos et al. 2023), which are common molecules. Research has shown that PKS genes are almost absent in metazoans, suggesting a microbial origin (Hochmuth and Piel 2009). A well-studied sponge-derived drug, discodermalide, is derived from the deep water marine sponge *Discodermia dissoluta* (Gunasekera et al. 1990), and shows activity in the treatment of cancer and as an antifungal. The search for the biosynthetic pathway for discodermalide has yet to be detected (Shaw et al. 2006) with most focusing on the development of less toxic analogs (Guo et al. 2020) and synthesis (Marshall and Johns 1998; Smith and Freeze 2008; D and Varala 2022). A bacterium of interest is *Candidatus Entotheonella*, originally isolated from the sponge, *Theonella swinhoei* (Wilson et al. 2014). This unique bacterium produces several different compounds, namely, polyketides and peptides (Schmidt et al. 2000; Wilson et al. 2014), and is found in many sponge species (Brück et al. 2008; Peters et al. 2023). It

was found heavily associated with *D. dissoluta*, but more genomic information is needed to determine if *Ca. Entotheonella* is a potential producer, but it has been identified to carry a gene cluster that produces calyculin,A, a cytotoxic phosphatase inhibitor, derived from *Discodermia calyx* (Wakimoto et al. 2014).

Other examples include the cytotoxic macrolide, tedanolide from *Tedania ignis* (Schmitz et al. 1984), which stops the cell cycle in the DNA synthesis phase (S-phase), making it a potential molecule for developing cancer treatments. Currently, no further clinical work has been noted. Another group molecules derived from *T. ignis* include groups of diketopiperazines from a *Micrococcus sp.* isolated from *T. ignis* (Stierle et al. 1988a). Diketopiperazines are produced by several organisms like bacteria and fungi and are being explored for their use against biofilm formation and antibiotic activity against methicillin-resistant *Staphylococcus aureus* and several other activities (Borthwick 2012; de Carvalho and Abraham 2012).

1.4 DIFFICULTY OF USING METAGENOMICS FOR NATURAL PRODUCT RESEARCH

The inherent difficulty of using metagenomics to determine bacterial composition and function is that many bacteria contain more than one pathway for a specific product, which makes amplicon sequencing based approaches less than ideal since it will not distinguish novel genes and pathways a due to the limits of homologies to the primer (Piel et al. 2004; Uchiyama and Miyazaki 2009). As stated by Olsen and Woese (Olsen and Woese 1993), many genetic changes will produce similar phenotypes, in this case, similar pathways. The functional metagenomic approach relies on the construction of metagenomic libraries and molecular cloning of the gene or genes of interest (Uchiyama and Miyazaki 2009; Lam et al. 2015). This approach allows for the cloning of molecular pathways into expression vectors and screening for function (Selvin et al. 2012; Popovic et al. 2015; Ufarté et al. 2016; Popovic et al. 2017) as seen in Figure 1.5. Selection of positive colonies can then be performed. Some examples of this include selecting for particular enzymes, such as proteases (Morris et al. 2012), amylases (Nair et al. 2017), lipases (Henne et al. 2000), etc. (Morris and Marchesi 2016; L. Robinson et al. 2021;

Jeilu et al. 2022)., by supplementing culture media with lactose-free milk powder, starch, or tributyrin respectfully (Morris and Marchesi 2016). Screening for positive clones involves monitoring for zones of clearing on culture media and adding specific dyes like iodine to starch supplemented media. Minimal shearing of DNA is also required for functional metagenomic studies since shearing can lead to false diversity and chimeric sequences (Schmeisser et al. 2007). Large biosynthetic gene clusters (BGCs) are more complicated to complete due to their size, often over 100kbp and may contain a high GC content compared to smaller gene clusters. Some current work utilizes methods such as transformation associated recombination (TAR) which in a 2014 study, allowed the expression of a 67-kb BGC from a marine bacterium, *Saccharomonospora* sp., to be expressed in *Streptomyces coelicolor* and produce the antibiotics, taromycin A (Yamanaka et al. 2014). Other methods include lambda-Red which in 2018 was used to insert a 26kbp gene cluster for thiolactomycin derived from the marine bacterium *Salinispora pacifica* into *Salinispora tropica*, which does not contain this BGC (Zhang et al. 2018). In the case of anticancer based screenings, high throughput cell culture assays are performed with the prospective compounds or extracts and analyzed via microplate reader looking at viability (Kallifatidis et al. 2013; Karan et al. 2020) and cytotoxicity assays (ter Haar et al. 1996; Zheng et al. 2006; Samirana et al. 2021).

An added difficulty to metagenomic DNA extraction is the physical size of the microbes. In many cases, the microbes within a given environment are smaller and problematic to lyse, to the point that many labs have their own procedure for DNA extraction (Schmeisser et al. 2007). Another method for determining the total community composition and composition of functional genes within an environmental sample is through shotgun sequencing. DNA is simply extracted and prepared depending on the requirements of the sequencing technology (Illumina, 454, Oxford Nanopore, etc). This approach is not always best for functional metagenomics as it will only tend to show the functional potential in a sample rather than the active genes. Follow up utilizing a functional library or metatranscriptomics can verify if the identified genes are active (Moitinho-Silva et al. 2017; Fiore et al. 2020).

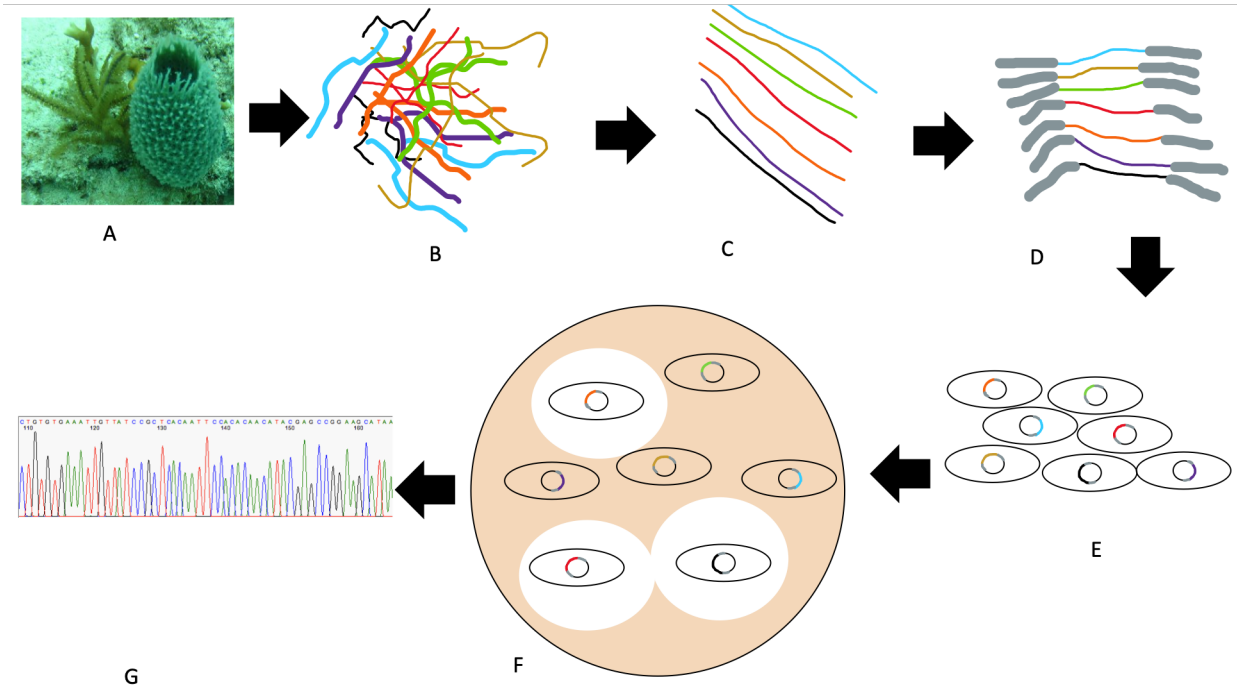


Figure 1.5-The schematic for the creation of functional metagenomic libraries. The sample is first collected (A), and DNA is extracted from it (B). The DNA is then sheared (C), ligated into a vector (D), and transformed into a microbial host by heat shock or electroporation (E). The host libraries are then plated onto a screening media containing antibiotics (F). Positive libraries are selected, grown, and PCR amplified, sequenced, and annotated (G). Chromatogram image adapted from the University of Michigan Medical School Biomedical Research Core Facilities website https://brcf.medicine.umich.edu/cores/advanced-genomics/faqs/sanger-sequencing-faqs/interpretation-of-sequencing-chromatograms/.

1.4.1 Limits of Molecular Cloning for Natural Products

A method around this is to clone the desired pathway or pathways into a host, usually *Escherichia coli* (Li et al. 2009; Uchiyama and Miyazaki 2009), but recent work has also utilized *Streptomyces coelicolor* and *Saccharomyces cereviseae* for larger pathways. A cloning vector, such as a bacterial artificial chromosome (BAC), can have up to 200kb inserted (Li et al. 2009) or a fosmid is used. Large insert libraries are needed to study

functional analysis as many of the genes and pathways are quite large (Schmeisser et al. 2007). However, maintenance of large insert libraries remains a challenge. Toxic genes can prevent the host from maintaining the expression vector (Saïda 2007). Toxic genes can decrease the fitness of the host, such as *E. coli* (Kimelman et al. 2012), and result in reduced uptake and expressionx of the gene of interest. Therefore, the use of other hosts such as yeasts (Joska et al. 2014) and *Streptomyces coelicolor* (Nechitaylo et al. 2014) and the creation/design of newer vectors that can retain toxic genes have been proposed and tested with some success (Motamedi et al. 1995; Sevillano et al. 2013; Aubry et al. 2019). A more modern approach is to utilize newer technologies in synthetic biology (McCarty and Ledesma-Amaro 2019; Palazzotto et al. 2019). Some natural products are toxic or a large burden to some bacteria (Kimelman et al. 2012) and when they build up in a clone, it can end up killing the cell or have reduced growth. By incorporating the product into the genome or splitting the product to divide intermediate parts to a co-culture or consortia and reduce the toxic effects (McCarty and Ledesma-Amaro 2019).

1.5 MARICULTURE OF SPONGES

A big draw in sponge biology is the ability to maintain them in mariculture. One reason for this is for an increase in biomass (van Treeck et al. 2003). Many useful sponge-derived compounds are present only in small quantities and until it is synthesized for large scale use, the alternative is to have available biomass (Maslin et al. 2021). Mariculture of sponges can be one of many things: Sponges suspended on a rope, an offshore ‘farm’, and inland based mariculture facilities. The big drawback to pulling the sponge from its natural environment would be that it is difficult to mimic the exact conditions for optimal growth of the sponge, thus causing a potential loss of biomass. In addition, changes in the microbiome can occur overtime (Mohamed et al. 2008) affecting the amount of product a sponge might make.

1.5.1 Sponge on a rope or mesh-bag mariculture

Sponges suspended on a rope in the sea are a promising form of mariculture as the sponge is still kept in their original environment but are closely monitored in a controlled

area via nylon rope suspension. The ropes are attached to horizontal or vertical frames and suspended in the water column (Barthel 1986). Studies have shown that this method of cultivation has a high survival rate, good growth rate (Voogd 2007), and no significant effects on the amount of bioactive compounds present (de Voogd 2007). Similarly, sponges can also be grown in mesh bags. In a 2013 study, the marine sponge, *Discodermia dissoluta,* was cultured in mesh bags to view growth rate and measure the amounts of discodermalide, an antitumor compound derived from *D. dissolute* (Gunasekera et al. 1990), was present. Normally, *D. dissoluta* is found in the deeper ocean (>30m), but a population exists in Colombia that can be found in shallower waters (Ruiz and Zea 2012). Results from the experiment show that there was in increase in growth rate of the sponge in the mesh bags and a moderate rise in the amount of discodermalide production (Ruiz et al. 2013).

1.5.2 Inland mariculture of sponges

Using integrated multitrophic aquaculture (IMTA) may be a solution to keeping a supply of organisms for food or biotechnology. Traditional aquaculture which generates high amounts of waste (Dauda et al. 2019) which enters the environment causing pollution (Carballeira Braña et al. 2021). With growing demand for certain products, particularly fish, IMTA is considered a strong alternative to traditional methods where instead of a monoculture of an organism, multiple organisms are kept together (Gökalp et al. 2019; Gökalp et al. 2021), almost mimicking the natural environment.

1.5.3 Alternative Method to Mariculture

While mariculture in any form is a viable way to get biomass, it can often be costly and take up space depending on the method. Alternative ways being tried to generate more biomass have been to grow the sponge cells in cell culture (Conkling et al. 2019; Urban-Gedamke et al. 2021; Hesp et al. 2023). The generation of sponge cell lines has been challenging (Pomponi et al. 1998; Grasela et al. 2012; Schippers et al. 2012) until recently where optimized media has been used to generate sponge cell lines and keep them alive through multiple passages. In the study by Conkling, the optimization of cell culture media with media M199 and M1 with a suite of amino acids yielded viable cells

until the fifth passage (Conkling et al. 2019). A further study used a more optimized M1 media which yielded viable cells until the fifth passage, with the exception of *Geodia baretti* cells, which started decreasing in numbers at the 19th passage, but survived to the 25th passage (Hesp et al. 2023). Finally, the maintenance of sponge cells in a 3D culture has also been proposed, wherein, sponge cells are encapsulated in permeable gel microdroplets (Urban-Gedamke et al. 2021). Research in this area is currently ongoing.

1.6 ABOUT THE SPONGES USED IN THIS STUDY

1.6.1 *Tedania ignis*

Tedania ignis, or the mangrove fire sponge, is a tropical species of sponge native to southern Florida and the Caribbean Islands (Jouett et al. 2015). It is noted by its distinct bright orange coloration and is frequently found attached to the roots of the red mangrove tree. An important feature of *T. ignis* is that is elicits a strong skin reaction when touched (Yaffee and Stargardter 1963). *T. ignis* has potential as an important sponge for biotechnology, containing a multitude of potential pharmaceutically relevant products such as diketopiperazines from an associated *Micrococcus* sp. (Stierle et al. 1988b) and tedanol (Costantino et al. 2009), an anti-inflammatory compound. A chemical of interest from *T. ignis* is tedanolide, a cytotoxic macrolide and potent anti-cancer molecule (Schmitz et al. 1984; Taylor 2008). Currently the producer/producers of this molecule have yet to be identified. Based on the structure of the molecule and low concentration within a sponge, tedanolide is likely microbial in origin (Schmidtz et al. 1983; Stierle et al. 1988a; Taylor 2008; Costantino et al. 2009).

1.6.2 *Cinachyrella keukenthali*

In a 2014 study conducted by Cuvelier et al. (Cuvelier et al. 2014), it was found that a species of *Cinachyrella keukenthali* collected in Fort Lauderdale, FL split into two distinct microbiomes. Initially with this study, it was thought that one *Cinachyrella* taxon was collected, however, based on the microbiome profiles of the collected sponges, it

was determined that there were two collected, albeit not the initial intention of the study. One group contained a microbiome dominated by Alphaproteobacteria and had a lower diversity of microorganisms. The other group contained more of the rare microbiota and a higher diversity of microorganisms. Between the two, there were 631 shared operational taxonomic units (OTUs) with 12 OTUs belonging to archaeal taxa and 627 belonging to bacterial taxa. Earlier studies of the microbiome of *Cinachyrella*, and its relatives *Cinachyra*, were performed in the Indo-Pacific (Cleary et al. 2018; Cleary et al. 2019; Riuitzler and Smith [no date]), making the work of Cuvelier et al. (Cuvelier et al. 2014) the first to be performed in the western Atlantic. It suggests that microbiome signatures could be another way of identifying sponge taxa,

1.6.3 *Hymeniacidon perlevis*

Hymeniacidon perlevis is a common intertidal sponge found throughout Europe. It is noted by its bright red-orange color and known by its common name, the crumb-of-bread sponge. *H. perlevis* contains high levels of cyanobacteria (Alex et al. 2012) and produces hymenialdisine (Meijer et al. 2000), an anti-cancer drug and the compound norharman (Zheng et al. 2006), another potent cytotoxic molecule. Hymenialdisine is produced by *H. perleve*, but also by other species of sponge (Song et al. 2011; White et al. 2012). It is determined that this molecule is made by the host as it and its derivates have been found localized to sponge cells rather than its symbionts (Song et al. 2011; Feng et al. 2013). Norharman is produced by the symbionts in *H.perleve* and other microbial symbionts in other organisms (Zheng et al. 2006).

1.6.4 *Halichondria panicea*

Halichondria panicea is a common intertidal sponge found throughout Europe and the United Kingdom (de Voogd et al. 2023). Sponges identified as *H. panicea* have been documented in the Pacific Ocean, but it is yet to be determined if those sponges are actually *H,panicea* or a case of misidentification (Erpenbeck 2004). It is considered a low microbial abundance (LMA) sponge as only a few microbial species are found in its tissues (Wichels et al. 2006) and the density of these microbes within its tissues are low,

as shown by electron microscopy and metataxonomic studies (Mu et al. 1998; Wichels et al. 2006; Giles et al. 2013). It was also found that *Halichondria panicea* harbors high levels of Alphaproteobacteria, particularly a species of belonging to the *Roseobacter* group. While this fluctuates seasonally, it still tends to be the dominant bacterial groups in *H. panicea*. A recent project in Iceland named the dominant *Roseobacter* as *Candidatus Halichondribacter symbioticus* (Knobloch et al. 2019)*. Halichondria panicea* is known to harbor several species of Actinobacteria that produce antimicrobials. A 2010 study (Schneemann et al. 2010) detected the actinobacterial isolates via 16S rRNA gene sequencing. The isolated species showed activity against *Escherichia. coli* K-12, *Staphylococcus lentus*, *Bacillus subtilis*, and *Candida glabrata*. 122 different substances were identified from the metabolites produced by these Actinobacteria and were verified with NMR, HPLC-MS, and UV. Further work on *H. panicea* and another sponge from this thesis, *Hymeniacidon perlevis*, explored the activity of cultivable bacteria against multi-drug resistant *Staphylococcus aureus* (Rodriguez Jimenez et al. 2021).

1.7 AIMS AND OBJECTIVES

The overarching aim of this thesis is to understand the microbiome and microeukaryome of marine sponges, how the microbiome shifts in a mariculture setup, and explore the taxonomic make-up and functional potential of sponge microbial communities utilizing shallow shotgun metagenomics.
The main aims and hypotheses of this project are

1. **To determine the changes in the microbiome of marine sponges using both DNA-based and RNA-based amplicon sequencing-** I hypothesize that current DNA focused sequencing methods do not fully capture the microbial structure in sponges and likely picks up dormant microbial constituents rather than the active community. Using RNA-based amplicon sequencing should reveal the active constituents in the community.
   This project aims to identify changes in the community structure of *Tedania ignis* kept in a sustained aquaculture system over time by utilizing metabarcoding of the 16S rRNA gene and identifying total and active components in the sponge.
2. **To determine the microeukaryotic structure of sponges using both DNA-based and RNA-based amplicon sequencing.** Microeukaryotic structure in sponges is poorly understood and due to host reads, can be cumbersome in removing host sequences pre-extraction or bioinformatically. The aim of this study is to provide a profile of microeukaryotic organisms (fungi and protists) and emphasize the challenges of amplicon-based screenings for microeukaryotes in a host-symbiont system.
3. **To conduct metagenomic profiling of the sponge, *Cinachyrella keukenthali*.**-*Cinachyrella keukenthali* is an important sponge for the Global Invertebrate Genome Alliance (GIGA) as it remains compact through its life and has the potential as a source for bioactive compounds. The aim of this chapter is to provide a taxonomic and functional profile of in *C. keukenthali* using an assembly-independent approach with MG-RAST.

4. **To profile the metagenome of *Hymeniacidon perlevis*-***H. perlevis*** produces a suite of bioactive compounds which may be microbially derived. The aim of this study was to provide a taxonomic and functional profile and identify important functions and taxa and connect the identified functions with associated taxonomic groups in the metagenome of *H. perlevis*.
5. **To profile the metagenome of *Halichondria panicea*-***H. panicea*** is host to microbial communities that have been shown to produce new bioactive compounds. The aim of this study was to determine the taxonomic structure and functional potential of the *H. panicea* metagenome, via shallow shotgun sequencing, and connect important functional groups identified in MG-RAST with specific taxonomic groups within *H, panicea*..

2.0 GENERAL MATERIALS AND METHODS

2.1 DNA EXTRACTION

2.1.1 PowerLyzer Powersoil Kit

A piece of sponge, roughly 2-3cm3, was ‘cut and squeezed’ in 2-3mL L-buffer (0.1M Tris, 0.1M EDTA, 0.1M NaCl, pH 8.0). Samples were transferred to 1.5mL tubes and spun down. The L-buffer was pipetted off and the pellet was resuspended in Bead Solution from the QIAGEN PowerLyzer kit (Qiagen, Inc., Hilden, DE) and transferred to bead tubes. Solution C1 was added, and the samples were vortexed and heated at 65°C for 10 minutes. Samples were then bead-beat 10 for ten minutes on a vortex adapter. Samples were then spun for 1 minute at 13000X. Supernatant was transferred to a new tube and Solution C2 was added and briefly vortexed. The samples were kept at 4°C for 5 minutes before being spun again. Supernatant was transferred to another tube and Solution C3 was added. Samples was mixed and kept at 4°C for 5 minutes again. Samples was spun and supernatant was transferred to a new tube and Solution C4 was added and gently inverted to mix. Sample was then filtered through a spin column and the supernatant was disposed of. Solution C5 was added and the samples were spun again. Another spin was necessary to make sure all of solution C5 was removed and the column dry. Solution C6 (10mM Tris-Cl, pH 8) was added to the column. The column was kept at room temperature for 10 minutes before being spun. DNA was quality checked on a C1000 Nanodrop Spectrometer (ThermoFisher, Waltham, MA)., diluted to 10ng/μL and send for sequencing.

2.1.2 DNEasy

25 mg of sponge tissue was cut and soaked in 1mL of 1X TE while equilibrating to room temperature. Sample was pelleted and the TE was removed. Buffer ATL was added to the tube. 20μL of QIAGEN (Qiagen, Inc., Hilden, DE) Proteinase K and RNAse A (10μg/mL) were added and the sample was briefly vortexed. Samples were incubated at 56°C until they looked completely lysed. Samples were vortexed occasionally during the

incubation to speed lysis. Buffer AL was added post-incubation and mixed, followed by 100% molecular grade ethanol and thoroughly mixed. Sample was transferred to a spin column and centrifuged at 8000rpm for 1 minute. Flow-through was discarded. Buffer AW1 was added, and the tube was placed onto a new flow-through tube. Samples were centrifuged at 8000rpm for 1 minute and the flow through was discarded. This was repeated with buffer AW2. The samples were then p laced onto a sterile 1.5mL tube and 200µL of buffer EB (no included in Kit) was added to the column. Sample sat for 10 minutes at room temperature before being centrifuged at 8000rpm.

2.1.3 CTAB

Samples were added to a 2mL tube containing 1X TE buffer and allowed to come to room temperature. Briefly, samples were vortexed and centrifuged at highest speed for 5 minutes to pellet sponge. The Tris-EDTA was discarded to begin the extraction. 500µL of warm (55°C) CTAB was added to the tube and the tissue was macerated with a pellet pestle. 1µL of β-mercaptoethanol was added to each tube followed by 40µL of QIAGEN (QIAGEN, INC., HILDEN, DE) Proteinase K. The solution was brought up to 1mL with the addition of more CTAB. The samples were kept on a heat block at 65°C for 1-2 hours, or until the sample looked completely lysed. Samples were pelleted and the supernatant was transferred to a fresh tube. 1 volume of chloroform was added and mixed until the samples looked emulsified. Samples were spun at 9000rpm for 10 minutes. The top layer was transferred to a fresh tube. 500µL of 1X TE was added to the sample tube, mixed and pelleted, and extracted as previous, and the resulting top layer was combined with the first. 1 volume of room temperature isopropanol was added to the sample and kept overnight at -20°C. Samples were pelleted and resuspended in 1mL of 1X TE. RNAse A (10µg/mL) was added to the samples and incubated at 37°C for 30 minutes. Samples were precipitated with 1/10 3M sodium acetate (pH 5) and 2.5 volumes of cold molecular grade ethanol. Samples were centrifuged and the ethanol was pulled off. Samples were rinsed twice with 70% RT molecular grade ethanol and centrifuged. Ethanol was removed and the samples were kept on the bench top for 30

minutes to dry and were resuspended in QIAGEN (Qiagen, Inc., Hilden, DE) buffer EB. Samples extracted by CTAB and DNEasy were then combined and quantified.

2.2 RNA EXTRACTION AND CLEANUP

Sponge tissue was kept stored at -80°C until extraction. A pestle and mortar were cleaned, autoclaved, and stored in at -80°C until extraction. An approximately 1-2cm^3 sample of sponge was placed into the pestle and mortar in a container of dry ice. Liquid nitrogen was poured into the pestle and mortar and the snap frozen tissue was ground into a fine powder before being transferred into a sterile 1.5mL tube. Total RNA was extracted using the RNEasy Kit (Qiagen, Inc., Hilden, DE) with QIAShredder tubes (per manufacturer's instructions). RNA concentration was checked using the Qubit BR RNA kit (ThermoFisher Scientific, Waltham, MA). Total RNA was treated for genomic DNA contamination using the TURBO-free DNase kit (ThermoFisher Scientific, Waltham, MA). A PCR was run to check for complete inactivation of the DNase enzyme using 515f/806R primers. Complementary DNA was created using the RETROSCRIPT Kit (ThermoFisher Scientific, Waltham, MA) with the heat activation step and random hexamers per the manufacturer's instructions.

2.3 DNA CLEANUP AND SEQUENCING

The samples for metagenomic sequencing were cleaned using the GELase kit (Illumina, Inc., Madison WI). Briefly, the samples were quantified using a Qubit Broad Range DNA kit (ThermoFisher, Waltham, MA). DNA was loaded onto a 2% LMP TAE gel and run for one hour. High molecular weight bands were cut out and transferred to tared tubes. Samples were placed at 70°C for 3 minutes or until the gel fully dissolved. Samples were tempered at 45°C and the appropriate volume of GELase enzyme was added, depending on the weight of the gel. Samples that were not previously treated (ie, the PowerLyzer samples) had 10µg/mL of RNAse A added during the digestion. The digestion was at least one hour. To purify the TAE and remaining enzyme out, the sample underwent an ethanol precipitation. Sodium acetate (0.1M) was added to the

samples, followed by 2.5 volume of ice-cold 200 proof molecular grade ethanol. Samples were inverted to mix and kept at -80°C for 20 minutes. Samples were centrifuged at maximum speed and the ethanol was pipetted off. Samples were washed twice with RT 70% molecular grade ethanol and resuspended in QIAGEN (Qiagen, Inc., Hilden, DE) buffer EB (10mM Tris-Cl, pH 8.5).

2.4 SHOTGUN METAGENOMIC LIBRARY PREPARATION

500ng of sample (some less than that for samples done via PowerLyzer) was sheared on a 15µL tube on a Covaris instrument to 350bp. Sheared samples were pooled to a volume less than 32µL. Samples were prepared using the NextFlex Rapid DNA-kit for Illumina. Fragmented DNA was added to an end repair and adenylation buffer with enzyme and incubated at 22°C for 20 minutes, followed by 20 minutes at 72°C. Following this, adapters were ligated onto the adenylated DNA using ligase enzyme provided by the kit. Samples were incubated at 22°C for 15 minutes. 50µL of room temperature AMPure beads were added to the samples and incubated at RT for 5 minutes. Tubes were transferred to a magnetic plate and held for 5 minutes or until the supernatant was clear. Supernatant was discarded. 200µL of fresh 80% ethanol was added to the samples, incubated for 30 seconds, and removed. This was done twice. Samples were allowed to dry for 5 minutes at room temperature. Samples were resuspended in 52µL of resuspension buffer provided by the kit and mixed and incubated at room temperature for 5 minutes. Samples were replaced onto the magnetic stand for 5 minutes and 50µL of the clear sample was transferred to a fresh tube.

To size select, 27µL of AMPure beads were added to the tubes and incubated at room temperature for 5 minutes. Tubes were then moved onto the magnetic stand and allowed to sit for 5 minutes. Clear supernatant was transferred to a fresh tube. 8µL of AMPure beds were added to the tube and mixed. Samples were incubated at RT for 5 minutes then placed onto the magnetic bead stand for 5 minutes. Supernatant was discarded. Samples were washed twice with 200µL of 80% ethanol following the same

procedure from earlier. 22µL of resuspension buffer from the kit was added and the samples sat for 5 minutes at RT before being moved to the magnetic stand for 5 minutes. Clear sample was transferred to a fresh tube.

2.4.1 Quality Check

2µL of sample was diluted 1:20 following directions from the QIAseq NGS Library Quantification (Qiagen, Inc., Hilden, DE) protocol for Illumina as another method of quantifying libraries prior to pooling. The qPCR method was performed as the libraries were PCR-free. This method is recommended by Illumina (Illumina DNA PCR-Free Library Prep Reference Guide (1000000086922). [no date]) and further by Bronner and Quail for PCR-free libraries or those less strict library sizes to detect the number of molecules containing the adapter sequence to bind to the chip (Bronner and Quail 2019). Working dilutions of 1:2000 and 1:20000 were made from the 1:20 sample. Five 10-fold dilutions of a DNA standard provided by the kit was also performed. The SYBRGreen master mix provided by the kit was brought to room temperature and briefly vortexed and centrifuged. Triplicate reactions were made for each sample. Sample layout followed the plate format recommended by the kit. Samples were run with qPCR using a LightCycler96. PCR protocol for the Light cycler is as follows: 10min at 95°C followed by 15seconds at 95°C, 30 seconds 60°C, 120 seconds at 72°C for 35 cycles. C_q values were plugged into an Excel sheet provided by QIAGEN (Qiagen, Inc., Hilden, DE) for this kit.

2.4.2 MiSeq Cluster Check

To check that quantification was accurate and that libraries would cluster appropriately, samples were normalized to 1nM and pooled. 1N NaOH solution was diluted to 0.2N with laboratory grade water. 20µL of this solution was added to 20µL of pooled library. Sample was vortexed and incubated for 5 minutes at room temperature. 990µL of chilled HT1 was added bring the library to 20pmol. PhiX was also denatured this way. PhiX and the sample were combined and placed into a MiSeq Nano cartridge and run.

2.4.3 NextSeq500

For the NextSeq500 run, 20µL of normalized library and 20µL of 0.2N NaOH were combined and mixed to denature the library. Library was centrifuged at 290g for 1 minute. 20µL of 200mM Tris-CL pH 7 (or RSB) was added, vortexed and centrifuged again. The library was diluted to 20pM in 940µL of HT1, vortexed, and centrifuged at 280g for 1 minute. Library was again diluted to 1.8pM. A 1.8pM PhiX spike-in was also done. Library was loaded onto a NextSeq 500 high output 350 cycle cartridge.

2.5 METAGENOMIC ANALYSIS

Raw FASTQ files were first quality checked using FastQC (Andrews 2010). Reads were then trimmed using Trim Galore! (Krueger [no date]) and phiX contaminants were removed using Bowtie2 (Langmead and Salzberg 2012).

2.6 AMPLICON ANALYSIS

2.6.1 16S Ribosomal RNA Amplicons

Before sequence analysis, the SILVA128 database was formatted for mothur. Briefly, the database was downloaded using wget and opened in ARB. The search field in ARB was set to ARB_color which was then set to 1. The equal sign was unclicked to indicate not equal. Search was clicked followed by “Mark Listed Unmark rest”. Search and query were closed. The file was then exported. Within export, the settings were marked, Filter set to none and compression to no. A full FASTA file was generated. Before fully saving, an EFT file was made. A custom file created by Pat Schloss was input to save the FASTA file with. Mothur was used to further process the SILVA128 (Quast et al. 2013) sequences. The script pcr.seq was used to modify the sequences to the V4 hypervariable region using 515F/806R (Caporaso et al. 2011) oligos in a text file. The align file was degapped so it could be used in the classification step.

Amplicons were analysed in mothur (v1.39.5) (Schloss et al. 2009). Firstly, contigs were joined using the make.contigs script and the rename function within to change the name of the sample from the MiSeq output into the shorter sample ID. A summary of the joined sequences was generated. 5015797 sequences were found pre-quality checking. Sequences were then screened using the screen.seqs script to remove homopolymers (maxhomop=7) and filter out samples longer than 300 bp. From here, the unique.seqs script was run and then summarized. This found a total of 4064682 sequences of which 602679 were unique. Sequences were aligned to the SILVA128 (Quast et al. 2013) alignment generated earlier. The same number of sequences were found using the summary.seqs. Sequences were screened to span the v4 hypervariable region starting at 13862 and ending at 23444. Summary.seqs found a total of 3844468 sequences of which 560713 were unique. Sequences were then filtered using filter.seqs. The filtered alignment was 578 bp, 49422 columns were removed, and the number of sequences used to construct the filter was 560713. Unique.seqs were run again. Sequences were pre-clustered using the pre.cluster script. UCHIME(Edgar et al. 2011) was run to check for chimeras and then subsequently removed. Sequences were classified using the SILVA128 (Quast et al. 2013) taxonomy and FASTA files. Unknown, mitochondria, and chloroplast sequences were removed. Sequences were then run through the cluster.split script with a taxonomy level set to 4 and a cutoff of 0.15. OTUs were classified and a shared file was generated. The shared file and consensus taxonomy file were then imported into R (R Core Team 2021) to run in phyloseq (McMurdie et al. 2013).

3.0 THE TOTAL AND ACTIVE MICROBIAL COMMUNITY DYNAMICS OF THE SPONGE *TEDANIA IGNIS* IN A SUSTAINED MARICULTURE SETUP OVER TIME

3.1 INTRODUCTION

Sponges are unique filter feeding organisms. They have the ability to filter approximately 24m^3 of seawater per kilogram of tissue per day (Reiswig 1974; Kennedy et al. 2007; Leys et al. 2011), concentrating ambient microbes into their tissue where they are either consumed for energy or incorporated into the mesohyl and become symbionts. These symbionts are important to the overall health of the sponge (Hentschel et al. 2006). The information on what these organisms do within the sponge is limited and is recently becoming more available. What is known is that these organisms contribute to nutrient cycling within the sponge and produce secondary metabolites which can reduce predation (Pawlik et al. 2002) and act to prevent disease within the sponge (Engel and Pawlik 2000; Kelly et al. 2005).

Marine sponges are sources of biotechnologically relevant compounds, many of which may be of microbial origin,but have yet to be identified. For example, the compound discodermalide, from the Caribbean deep sea sponge, *Discodermia dissoluta,* is a potent bioactive molecule found in low quantities in the sponge (about 0.002% w/w) (Gunasekera et al. 1990). So far, the producer has yet to be identified. *Tedania ignis*, a mangrove-associated sponge, has known cytotoxic compounds such as tedanolide (Schmitz et al. 1984), but its exact origin has not been determined. It is argued that many of these molecules are likely of microbial origin given their low presence within the sponge (Schmidtz et al. 1983; Hochmuth and Piel 2009). Traditionally, large amounts of the metabolite of interest could be obtained by growing the producing organism and extracting the desired compound. However, more than 90% of known bacteria in sponges are unculturable, or in some circumstances, grow and produce as a consortia (Joint et al. 2010). Knowing the microbial make up of these sponges, through such projects as the global sponge microbiome project (Thomas et al. 2016), is vital to not only understanding the potential interactions within the host, but also may yield clues in deciphering natural product producers, particularly with newer, high throughput sequencing datasets utilizing

techniques such as RNASeq (Johansen et al. 2010) , functional metagenomic screening (He et al. 2013; Ufarté et al. 2016), and even technologies such as the iChip (Kali 2015; Ledford 2015) currently utilized by NovoBiotic Pharmaceuticals.

Another important aspect is the ability to produce enough biomass of sponge whether it be for pharmaceutical purposes or for experiments. Obtaining enough biomass of sponge, or recalcitrant species of bacteria within the spogne, is crucial to getting enough of the desired product as most natural products are present in low amounts (Belarbi et al. 2003). Given the supply issue, other methods are needed to get either enough sponge material such as mariculture, or synthesis of identified compounds (Mickel et al. 2004; Sipkema et al. 2005; Smith and Lee 2007; Smith and Lee 2007). Monitoring how microbiota change overtime is important for reproducibility in experiments as seen in two mariculture experiments by Mohamed and colleagues where microbiota were monitored over a specified time in *Ircinia strobilina* and *Mycale laxissima* (Mohamed et al. 2008b; Mohamed et al. 2008a). These studies found that microbial communities in sponges show a strong change at first, becoming more diverse, but eventually stabilize after a time. In 2008, (Mohamed et al. 2008b) it was further determined that patterns of small molecules extracted from the sponge, while showing some minor changes, generally remained stable overall.

Keeping sponges in mariculture can be a solution to the supply problem with natural products. In 2011, Bergman et al kept three species of demosponges in a mariculture setup to evaluate changes in their microbial communities as most natural products are hypothesized to come from the microbial symbionts rather than the host. This study showed poor growth for two of the sponges and fluctuations in microbial communities. For one sponge species, *Negombata magnifica,* the mariculture setup seemed appropriate and it thrived (Bergman et al. 2011).

Further, mariculture is a useful tool in sponge biotechnology applications. Keeping sponges in a mariculture setup as a co-culture with other organisms to remove metabolic wastes (Gökalp et al. 2019) and can be useful in improving water quality and converted

dissolved wastes into particulate material for other creatures to consume, in a process called the “sponge loop” (Gökalp et al. 2021). Mariculture can be variable for different sponge species, however, as some do not tolerate the setup as well as others. Fluctuations in microbial communities can affect health, growth, and ultimately, the target natural product (Bergman et al. 2011).

Genomic DNA is commonly used for microbiome studies due to the quickness and ease of extraction. DNA is a very stable molecule, as DNases are quickly denatured by many chaotropic salts and heat. A major issue with this method, is that there is no distinction between the live and dead members of a community. Rarer species may be missed, due to reasons such as primer bias (Ghyselinck et al. 2013; Nearing et al. 2021; He et al. 2023), the number of PCR cycles (Kool et al. 2023), annealing temperature, and collection/extraction bias (Sergeant et al. 2012). We are left unable to determine the functional members of a community. Live and dead bacteria can be stained with amine-reactive dyes like those found in conventional live/dead assay kits (Perfetto et al. 2010). There are, however, some existing methods to remove DNA from dead organisms, such as selectively removing dead bacterial cells via ethidium monoazide (EMA) and propidium monoazide (PMA), which intercalate into cells with compromised membranes (Nocker and Camper 2006), but does not permeate intercellular DNA. These methods are them followed up with light exposure which downstream, inhibits PCR of the dead bacterial DNA, however, quantitative results on complex communities have yielded variable results (Wang et al. 2021). Other methods include generating complementary DNA (cDNA) from RNA and generating amplicons as with traditional microbiome sequencing. This method has been previously (Kamke et al. 2010; Moitinho-Silva et al. 2014) but it is not common. As RNA transcription is an active process, it has the advantage of representing the active microbial community rather than just the total community, which could contain free DNA, dead bacteria, and not account for an increased RNA copy number. This can result in an overrepresentation of certain members of the microbiome and underrepresent rare taxa (De Vrieze et al. 2016). Ribosomal RNA copy numbers are variable between prokaryotic organisms and may demonstrate a survival strategy for these organisms in their

environment and may aid in recovery from perturbations in their environment (Klappenbach et al. 2000).

Tedania ignis is a mangrove-associated sponge found throughout the tropical Atlantic Ocean (Figure 1). It is characterized by bright orange coloration, volcano-shaped ocscula, encrusting growth pattern on mangrove roots, and severe skin reactions upon touching it. *Tedania ignis* is a source of tedanolide, a cytotoxic macrolide, in addition to precursor molecules such like diketopiperazines. Many of these molecules are thought to be of microbial origin given their structure and low yields from the sponge, with the amount of tedanolide initially isolated from *T. ignis* at about 4mg from approximately 586 lb (265.6kg) of wet sponge (Schmidtz et al. 1983), or about 0.02 micrograms.

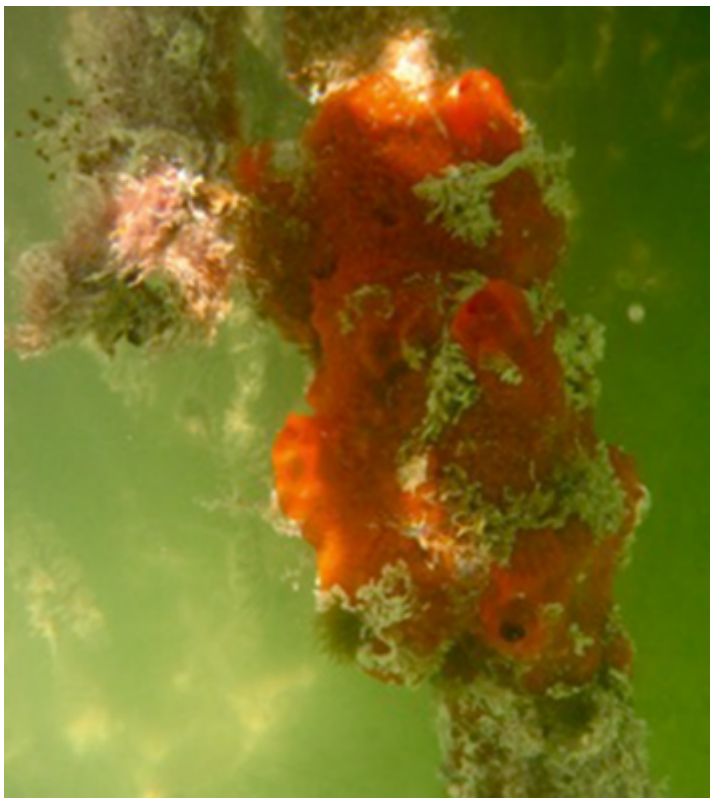


Figure 3.1-*Tedania ignis* collected from Summerland Key, FL attached to red mangrove root The material covering the sponge consisted of bryozoans and hydrozoans which were scraped off before sample was processed.

For this study, we sequenced the microbiota of *Tedania ignis* using the Illumina MiSeq platform to monitor changes due to mariculture and compare to the environmental samples. We will also show the utility of including cDNA for future microbiome analyses. The questions we hope to answer are whether sponge microbiota change over a short term in a sustained mariculture environment, providing a temporal analysis of sponge microbiota variability, and how variable these communities are utilizing total genomic DNA and RNA (as cDNA) to highlight the live communities of within the sponge.

3.2 MATERIALS AND METHODS

3.2.1 Sponge Collection

Specimens were collected under permit number D---C1L94300770.

Six *Tedania ignis* specimens were collected from Summerland Key, FL on two occasions via snorkelling. The specimens were cut from red mangrove prop roots and transported in ZipLoc™ bags back to Mote Tropical Research Lab, Sarasota, FL, USA. Sponges were temporarily stored in the mariculture setup there before processing. Environmental samples were processed within two hours of arriving back to Mote. Environmental refers to a sample that was frozen at the point or near to the point of sampling and not kept in the raceway under the experimental conditions. The “Environmental” sample was taken and frozen before the sponges were placed in the raceway. Samples were taken from each specimen, and immediately stored in 4mL cryotubes at -80°C. The remaining live tissue was kept in a raceway setup before being transported back to Harbor Branch Oceanographic Institute, Fort Pierce, FL, USA. These sponges died a week later and this event lead to a second collection being undertaken. No environmental sample was taken. Sponges were stored at Mote until transit back to Harbor Branch. Live tissue was allowed to acclimate to temperature conditions of the integral multi trophic aquaculture facility in another raceway setup (Figure 2) before tissue samples were taken and frozen. An extra sample was taken for pure culture experiments. Remaining pieces of tissue were frozen for further use. Ultimately four samples were used for community analysis. The temperature and salinity of the aquaculture setup were maintained at a constant

temperature of 26˚C and 30 practical salinity units (psu). A sample of each sponge was taken upon arrival to Harbor Branch. This first sampling is referred to as Timepoint_00. Further samples were collected at 7 days, 14 days, 28 days, and 56 days (Timepoint_07, Timepoint_14, Timepoint_28, and Timepoint_56, respectively) from each individual.

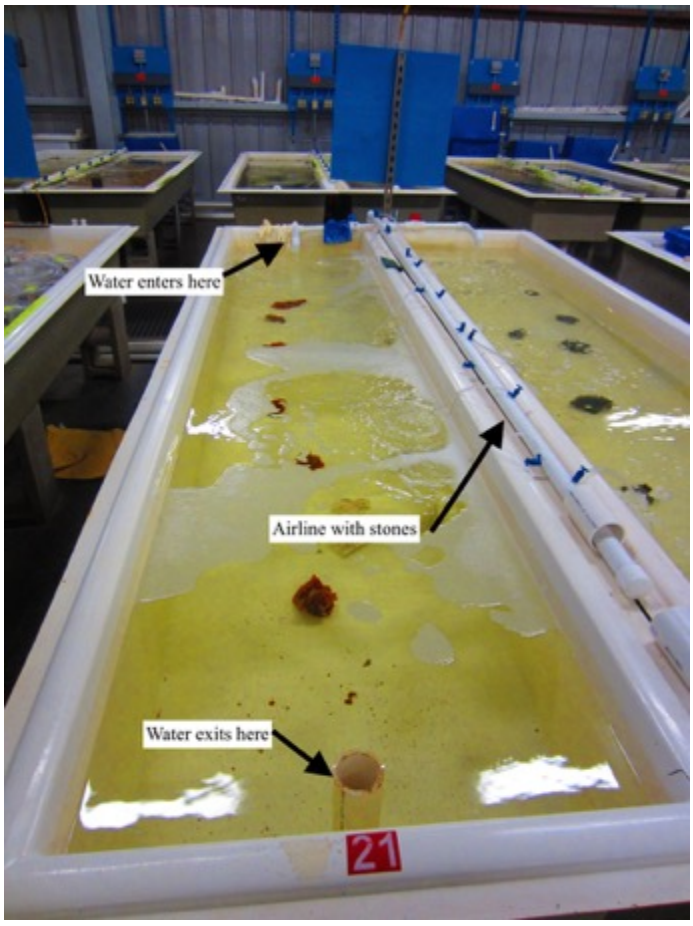


Figure 3.2- Raceway setup at the HBOI Integrated Mariculture Trophic Assessment building Sponges were kept at the same temperature and salinity for two months. Water was dripped in from a saltwater source outside of the building and allowed to flow out to remove waste products.

3.2.2 DNA Extraction

Samples were extracted using the PowerLyzer Powersoil Kit (Qiagen, Inc., Hilden, DE) with modifications (Supplementary Data) for DNA. An empty sample was extracted at the

same time to account for laboratory contamination. DNA in the air sample was undetectable via gel electrophoresis and failed to amplify in test PCR so it was excluded from sequencing. Samples were shipped to Halmos College of Natural and Marine Sciences Guy Harvey Oceanographic Campus of Nova Southeastern University on dry ice to be extracted for RNA and for sequencing. Total RNA was extracted using the RNEasy Kit (Qiagen, Inc., Hilden, DE) with QIAShredder tubes (Per manufacturer's instructions. RNA concentration was checked using the Qubit BR RNA kit (ThermoFisher Scientific, Waltham, MA). Total RNA was treated for genomic DNA contamination using the TURBO-free DNase kit (ThermoFisher Scientific, Waltham, MA). A PCR was run to check for complete inactivation of the DNase enzyme using 515f/806R primers. Complementary DNA was created using the RETROSCRIPT Kit (ThermoFisher Scientific, Waltham, MA) with the heat activation step and random hexamers per the manufacturer's instructions.

Resulting genomic DNA and complementary DNA were amplified using the V4 primers 515f/806r, which contain semi-conserved (Caporaso et al. 2011) with Illumina adaptors in Kapa Biosystems *Taq* Polymerase. PCR conditions were 95°C for 3 minutes, followed by 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and 72°C for 5 minutes as outlined from the Illumina 16S Metagenomic Sample Preparation protocol. The resulting PCR products were cleaned using AMPure XP beads. Briefly, AMPure XP beads were added to the 96 well PCR plate, gently pipetted up and down to mix and incubated at room temperature for 5 minutes. The 96 well plate was placed onto a magnetic stand and allowed to sit for 2 minutes or until the supernatant looked clear. The supernatant was discarded, and the beads were rinsed with fresh 80% v/v ethanol. The plate was placed onto the magnetic stand for 2 minutes and the ethanol was pipetted off. The process is repeated one time. The beads were allowed to air dry for 10 minutes. 10mM Tris (pH 8.5) was added to the PCR product, pipetted up and down to mix. The 96-well plate was incubated at room temperature for 2 minutes and replaced on the magnetic stand for another two minutes. The resulting supernatant was transferred to a clean 96 well plate. The cleaned PCR products were put through an index PCR where the multiplexing indexes were added. Briefly, the index primers were setup according to

the Illumina protocol. PCR protocol for the index primers was performed using Kapa Biosystems *Taq* polymerase and the PCR conditions were 95°C for 3 minutes, 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and 72°C for 5 minutes. Indexed PCR products were cleaned using the AMPure XP protocol.

3.2.4 Sequencing

Four sponge samples from each time point and the environmental samples from the first sampling were sequenced on a MiSeq using the v2 500 cycle (2x250 PE) kit. Raw MiSeq reads were deposited into the European Nucleotide Archive (ENA) under accession number PRJEB54665.

3.2.5 Data Analysis

Sequences were analysed in mothur (v.1.45.2). Sequences were joined using the make.contigs script and trimmed to 300bp using screen.seqs. Duplicate sequences were removed using unique.seqs and reads were rarefied to 10873 sequences/sample. Sequences were aligned using an aligned SILVA128 database and screened again. Sequences were pre-clustered and run through UCHIME (Edgar et al. 2011) to identify and remove chimeric sequences. A shared file was made and classified using SILVA v128. Data was subsequently imported into R (v4.0.5).

3.2.6 Statistical Analysis

The shared and taxonomy file were merged in phyloseq (McMurdie et al. 2013). OTUs with less than 10 associated reads were removed. The resulting phyloseq object was rarefied to the lowest abundance and split between the genomic DNA (gDNA) and the complementary DNA (cDNA) reads.

Alpha diversity was run on the whole table with the time points and nucleic acid type as variables. For the split data, only the time points were analysed. A one-way ANOVA was

run using the Simpson diversity index and the number of observed OTUs for each phyloseq object. A Tukey HSD test was run for the ANOVA output.
Beta diversity was run for each phyloseq object using Bray Curtis and Non-multidimensional scaling (NMDS). An ADONIS analysis and PERMUTEST from the R package vegan (Oksanen et al. 2017) were run.

3.3 RESULTS

The total number of reads pre-filtering was 3,684,081 with a maximum of 1,211,638 and a minimum of 10,873. Post singleton and low abundance filtering, the number of reads was reduced to 3,645,652 with a minimum of 10,743 and a maximum of 1,206,080.

3.3.1 Alpha Diversity Analysis

For the complete table, alpha diversity between the two nucleic acid types showed a high statistical significance with both the number of observed OTUs ($p<0.001$) and the Simpson diversity index ($p<0.001$) . As seen in Figure 3 the cDNA samples are shown to have more diversity and higher observed OTUs than the gDNA samples, perhaps with the cDNA samples picking up rarer, more active organisms, those with higher copy numbers of rRNA, or potentially excluding bacteria that could be dormant (Lennon and Jones 2011). When comparing the whole table by time point there was no significant difference in the ANOVA results for the observed OTUs ($p=0.122$) nor the Simpson diversity ($p=0.261$). The TUKEY test comparing the total (gDNA) to the active (cDNA) communities showed a p-value of 3e-07 for the observed OTUs and a value of 0 for the Simpson diversity. When tested using time, the p-value for both the observed OTUs and Simpson diversity showed no significance in any of the comparisons.

When comparing the time points for the total community (Figure 3.3) there is no significant difference between in the number of observed OTUs across time points ($p=0.073$), no difference in Simpson diversity ($p=0.203$). Regression analysis of the total community showed that changes in observed OTUs overall were unseen over time ($R^2=0.3405$, p-value=0.1521). Regression analysis showed no significant changes over time with Simpson diversity ($R^2=0.3240$, p-value = 0.1798) (Table 3.1). No significant differences

were found using the Tukey test comparing each time point for either the observed OTUs or the Simpson test.

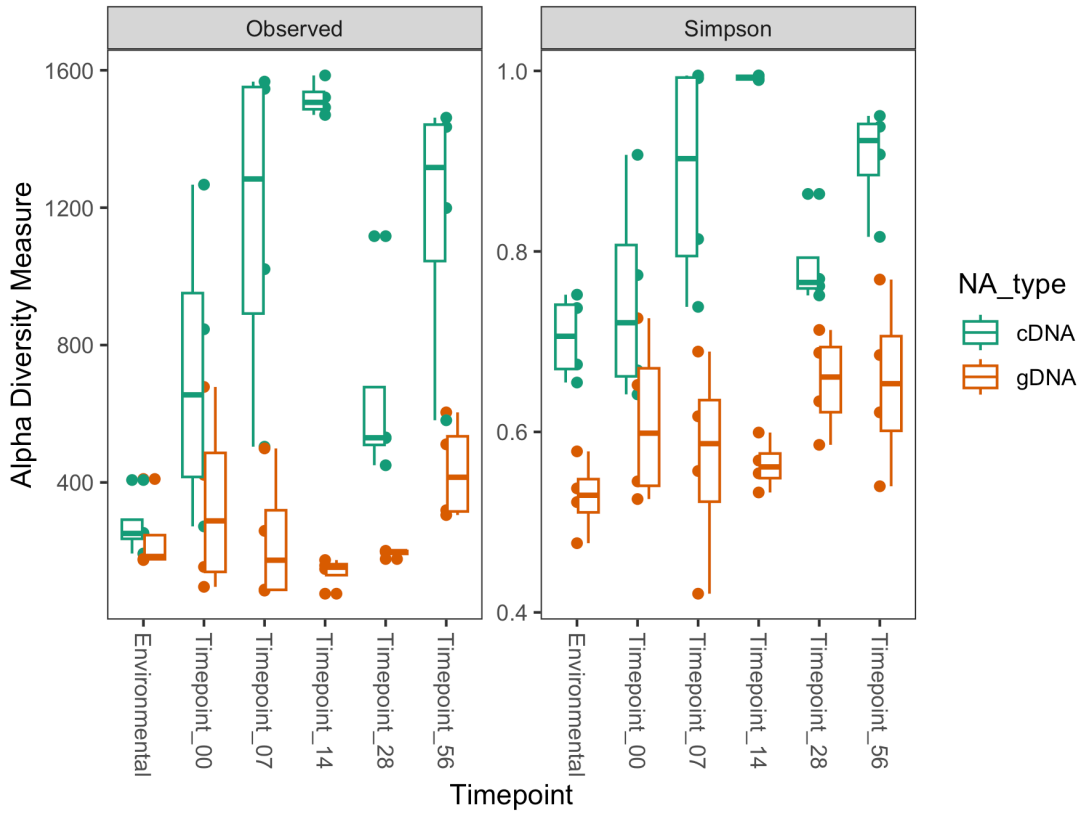


Figure 3.3-Alpha diversity boxplot of all samples coloured by nucleic acid type and plotted over time. An important component to note is that the cDNA samples show higher diversity and observed OTUs than that of the gDNA samples.

Table 3.1-Linear Regression Analysis of total DNA (gDNA) dataset based on timepoint-No significant differences were identified in the total community dataset.

Diversity Metric	**p-value**	**R^2 value**	**p-value**
Observed OTUs	0.073	0.3405	0.1521
Simpson	0.203	0.3240	0.1798

For the cDNA (Figure 3.3), there is a significant difference between the observed OTUs (p=0.000465) and a change in Simpson diversity across time points (p=0.000712).

Regression analysis shows a good fit for the observed OTUs for the active community samples (R^2=0.6492, p-value= 0.0011) overtime and for the Simpson diversity (R^2=0.6632, p-value = 0.0008) (Table 3.2). Given this significance, it is possible that the active communities have a strong influence on the overall results, leading to the significant changes for the whole dataset. Utilising the Tukey test, some significant differences were found between sample time points for observed OTUs and Simpson outlined in Tables 3.3 and 3.4.

Table 3.2-Linear Regression Analysis of the active (cDNA) dataset based on timepoint-Significant differences were found between the cDNA dataset, suggesting that there is more OTU abundance and diversity in the active community within the *T.ignis* microbiome than compared to the total DNA dataset in Table 1

Diversity Metric	**p-value**	**R^2 value**	**p-value**
Observed OTUs	0.000465	0.6492	0.0011
Simpson	0.000712	0.6632	0.0008

Table 3.3-Tukey Results of the complementary DNA Observed OTUs-Significant Tukey results for the observed OTUs ANOVA of the active community samples based on timepoint to identify which pairs of timepoints are different from each other in terms of observed OTUs.

Time points	**Difference**	**Lower**	**Upper**	**Adjusted p-value**
TP7-ENV	699.50	143.05	1255.95	0.0093
TP14-ENV	952.00	395.55	1508.45	0.00045
TP56-ENV	701.25	144.80	1257.70	0.0091
TP14-TP0	639.00	82.55	1195.45	0.019
TP28-TP14	-621.00	-1177.45	-64.55	0.024

Table 3.4-Tukey Results of the complementary DNA Simpson Diversity-Significant Tukey results for the Simpson diversity ANOVA of the active community samples based on timepoint identifying which pairs of timepoints show the most differences in diversity.

Time points	Difference	Lower	Upper	Adjusted p-value
TP7-ENV	1.98e-01	0.00048	0.40	0.049
TP14-ENV	3.11e-01	0.11	0.51	0.0011
TP56-ENV	1.98e-01	0.00055	0.40	0.049
TP14-TP0	2.63e-01	0.065	0.46	0.0058
TP28-TP14	-2.49e-01	-0.45	-0.051	0.0091

3.3.2 Beta Diversity Analysis

Beta diversity was calculated using the Bray-Curtis distance metric and non-multidimensional scaling (NMDS) (Figure 3.4). Comparing the samples overall, each nucleic acid type clusters together as seen in Figure 3.4, indicating a large-scale difference between the two nucleic acid types. This was quantified using ADONIS ($p=0.001$, $R^2=0.25781$). Further, a PERMUTEST showed a similar significance ($p=0.001$, permutations=999) (Table 3.5). However, the R^2 value is low, suggesting that there are other unaccounted factors for these differences in community structure. For each time point, overall, the environmental samples cluster together regardless of nucleic acid type, but further time points begin to deviate from each other. The ADONIS results show a significant difference in diversity between time points ($p=0.016$, $R^2=0.20935$) and further validated with PERMUTEST ($p=0.003$, permutations=999) (Table 3.6).

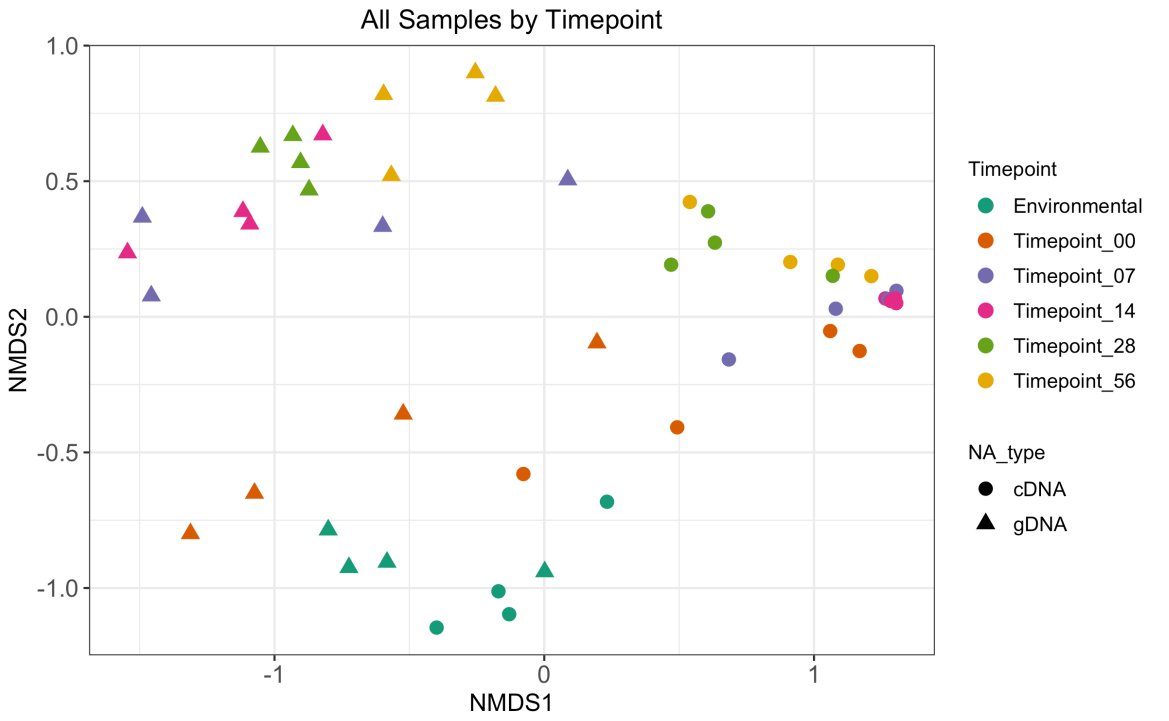


Figure 3.4- Non-multidimensional scaling (NMDS) plot of the Bray-Curtis beta diversity of the entire sampling run. Each sample type clusters by active and total communities in addition to the sub-clustering between time point.

Table 3.5-ADONIS and PERMUTEST results for nucleic acid types-Comparison of the beta diversity between the microbial communities of the different nucleic acid types using ADONIS and PERMUTEST

	R^2	**p-value**
ADONIS	0.25781	0.001
PERMUTEST	N/A	0.001

Table 3.6-ADONIS and PERMUTEST results for nucleic acid types-Comparison of the beta diversity between the microbial communities at different timepoints using ADONIS and PERMUTEST

	R^2	**p-value**
ADONIS	0.20935	0.016
PERMUTEST	N/A	0.003

For the gDNA sampled time points (Figure 3.5), the ADONIS results show a significant difference between the different time points (p=0.013, R^2=0.38451), but PERMUTEST shows no significant difference between the time points (p=0.346, permutations=999). The group dispersions are likely not different between the samples for the total community, affirming the ADONIS results and indicating that there is no significant difference based on sampling timepoint.

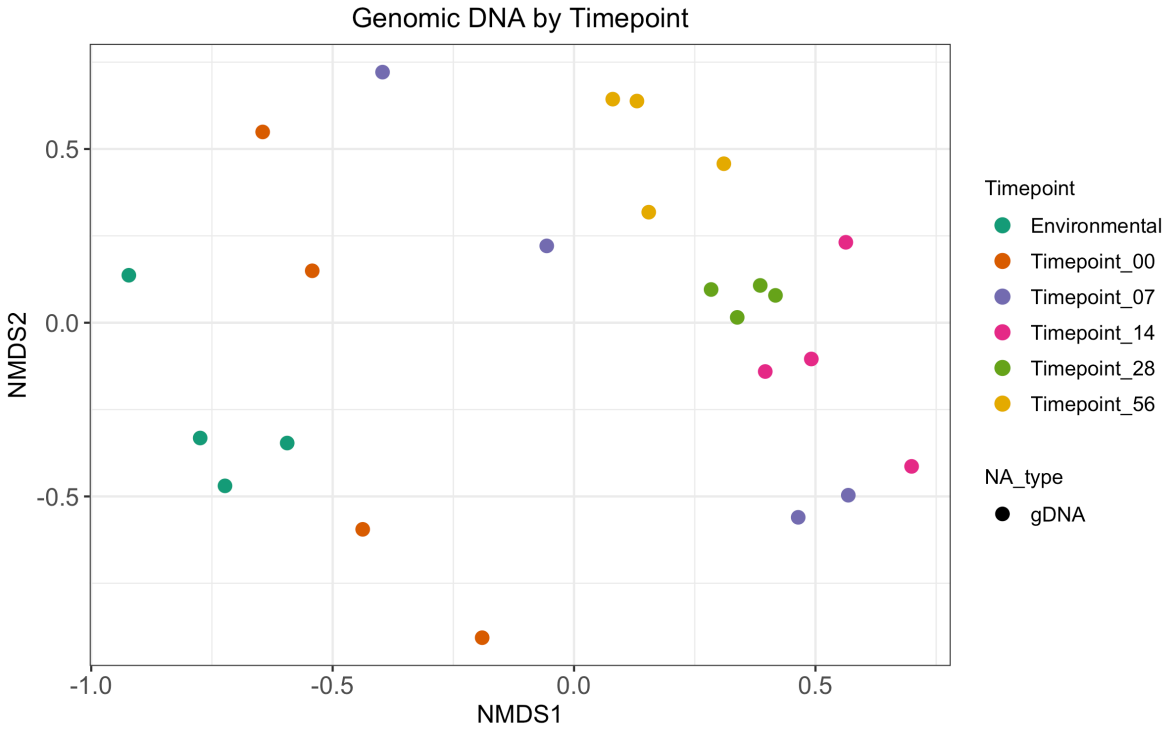


Figure 3.5- Non-multidimensional scaling (NMDS) plot of the Bray-Curtis total community beta diversity of the total community. Samples cluster together by each timepoint.

In the cDNA sampled time points, the ADONIS results show a significant difference in beta diversity (p=0.002, R^2=0.55546) and significance with PERMUTEST (p=0.015, permutations=999). Also of note is the higher R^2 indicating a better fit to the data. In Figure 3.6, the environmental samples cluster together, but beyond that, the remaining time points become non-homogenous. In contrast to the total community PERMUTEST result,

the result for the active communities is significant meaning that there may be other factors affecting the ADONIS results.

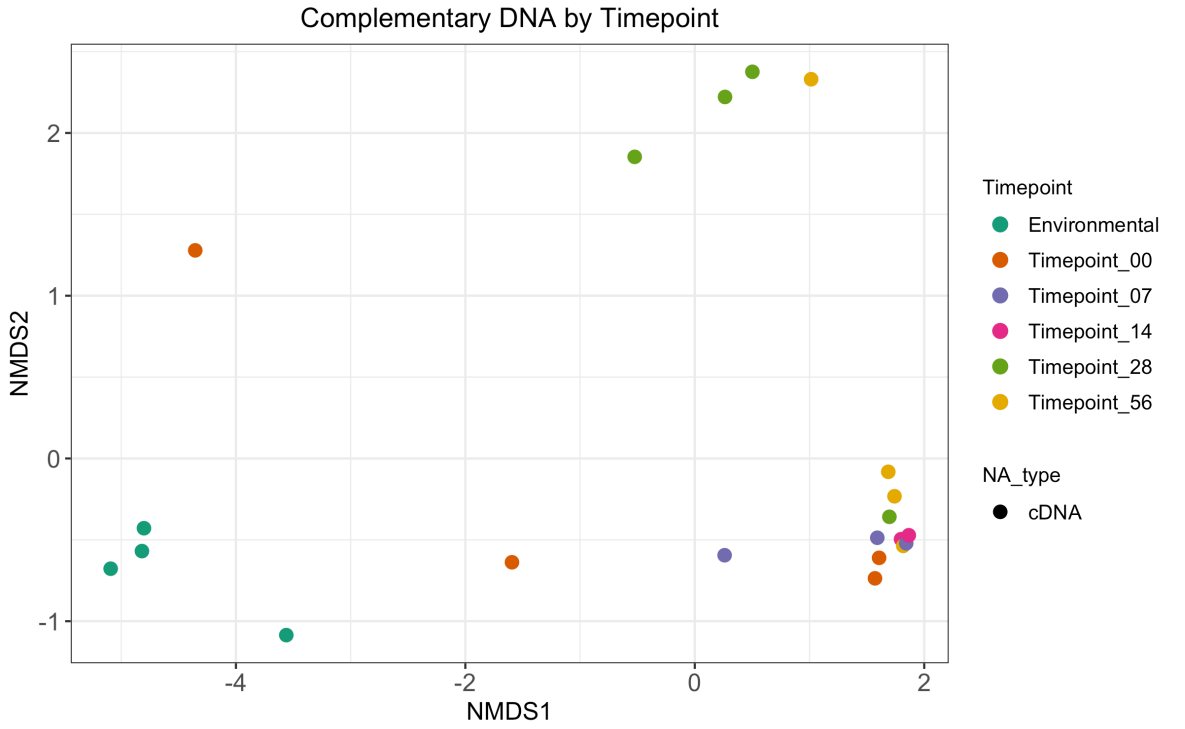


Figure 3.6- Non-multidimensional scaling (NMDS) plot of the Bray-Curtis beta diversity of the active community. Samples cluster together by each time point except for timepoint_00.

3.3.2 Taxonomic Profile of Abundant Genera

Taxonomic profiles of the sponge microbiota showed changes over time, particularly in the abundant (>0.02%) bacteria (Figure 3.7). Initial analysis included unclassified bacteria, but defining parts of the rarer community was difficult and unclassified bacteria are too broad to warrant discussion. Unclassified bacteria were removed (genus barplot with unclassified bacteria in Supplementary information-Figure 1). Between both community types and nearly all time points, there were two constant families- unclassified Proteobacteria and Oceanospiralles. The gDNA samples showed a mostly

constant group of prokaryotic symbionts over time, with relatively little change and a strong dominance of unclassified Proteobacteria. Later timepoints show a small set of growth of Desulfobacterales, and the archaeal family, Nitrosopumilaceae, but the community is mostly Proteobacteria. The active components showed many changes, in addition to a lower abundance of total prokaryotic organisms compared to that of the total community. While still dominated by unclassified Proteobacteria further timepoints show small shift in the groups of bacteria present, mainly the unclassified Proteobacteria, Rhodobacterales, Oceanospiralles Desulfobacterales, and Chromatiales.

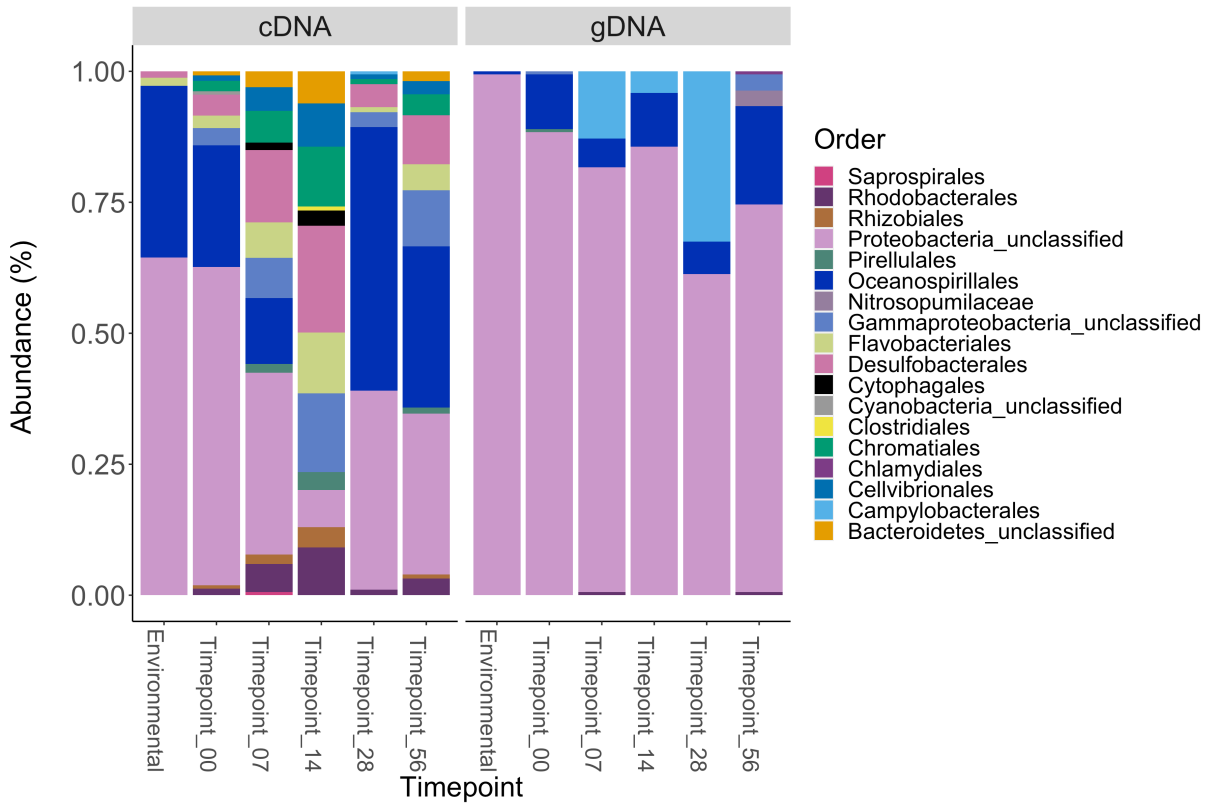


Figure 3.7- Taxonomic profile of abundant (>0.02%) orders in the cDNA and total gDNA communities by time point. Unclassfied bacteria and archaea were removed. Relative abundance is lower in the cDNA community, but there is more diversity. By sequencing the active community, extraneous DNA from dead or environmental DNA is excluded, allowing some of the more overlooked groups to be identified.

3.4 DISCUSSION

Abundant microbes across all samples consist mainly of unclassified Proteobacteria, and the order Oceanospiralles (Figure 3.7). Within Oceanospiralles is the bacterial species *Endozoicomonas*, a symbiotic marine microbe(Neave et al. 2016) associated with invertebrates like corals (Rua et al. 2014), ascidians (Schreiber et al. 2016), and sponges (Nishijima et al. 2013; Rua et al. 2014). *Endozoicomonas* has a potential role in nutrient cycling(Nishijima et al. 2013; Schreiber et al. 2016), DMSP breakdown and the production of antimicrobial compounds, however, this remains largely theoretical. This genus of bacteria is highly distinct within its invertebrate hosts and seems to be unique to marine invertebrates. Proteobacteria, or now known as Pseudomonata, encompass a large phylum of bacteria consisting of the alpha-, beta, gamma-, delta-, epsilon-(Campylobacterota), and zetaproteobacteria. Specific to the genomic DNA samples, there are abundant communities of unclassified Proteobacteria and Rhodobacteraceae. *Endozoicomonas* becomes more apparent through the other timepoints as do the Sulfurovum, and even the Gammaproteobacteria and the archaeal group *Nitrosopulmus* sp.. Exact causes for this are unknown, but possible explanation could be that an unaccounted factor, such as the seawater the sponges were stored in after sampleing, or the act of being transported and placed into a controlled raceway environment accounted for these differences (Kurm et al. 2019).

A bacterial order prevalent in the genomic DNA samples was Campylobacterales, another symbiont and opportunistic pathogen in many organisms. The dominant genus within Campylobacterales was *Sulfurovum* (Supplemental Figure 3.1S). *Sulfurovum* is a group of bacteria that uptake sulphur as an electron donor (Inagaki et al. 2004). It is facultatively anaerobic and found mostly in the deep sea (Ghosh and Dam 2009) but has been previously identified in anemones (Har et al. 2015). The reasoning behind these communities being so high in an otherwise “low microbial abundance” sponge is still unclear. From the cDNA samples, the abundant microbes consisted mainly of rarer and unclassified organisms. The Campylobacterales previously seen in the gDNA samples is no longer present in the cDNA samples. Other bacteria involved in the sulfur cycle are the Chromatiales (the purple sulfur oxidizing bacteria) and Desulfobacterales, which act as

sulfur-reducers. The environment *Tedania ignis* is native to is a mangrove swamp, characterized by anaerobic sediments and decaying organic material and important for sulfur cycling in marine environments (Nóbrega et al. 2022; Mo et al. 2023). The amount of dissolved oxygen in mangrove waters and sediments is generally lower than that of other aquatic environments and is more prone to hypoxia (Middelburg and Levin 2009). The mangrove environment itself may also have a role directly with associated sponges, particularly with the role of tannins and the presence of *T. ignis*. A 2009 study found an association with high tannin presence and recruitment of *T. ignis* larvae to red mangrove prop roots (Hunting et al. 2010).

When compared as one group, diversity and OTU counts did not show any significant differences. This is, perhaps, attributable to the genomic DNA “swamping” out the other groups of microbes that were detected in the cDNA samples, or even to biases in PCR of multiple groups of bacteria, wherein certain bacterial species can amplify more readily than others (Blazewicz et al. 2013; McLaren et al. 2019; Nearing et al. 2021; Silverman et al. 2021). Total genomic DNA tends not to discern between live and dead cells, so that is a possible reason behind the high similarity between samples.

Contrarily, the cDNA samples showed highly significant differences, indicating that more taxa are being picked up in addition to the common ones. The use of RNA, as cDNA, is one method for distinguishing the live bacteria from the dead since RNA is produced from actively transcribing cells. The method itself does suffer from several weaknessnes, namely, RNA is not stable making it difficult to use for some sample types and transcription and growth rates are not equated (Blazewicz et al. 2013; De Vrieze et al. 2016). This can be seen with regards to the Simpson diversity between nucleic acid types. The cDNA samples are overall much closer to one than the gDNA samples, which indicates a higher Simpson diversity. Over time, it seems that the communities are becoming more diverse. The exact cause is not fully discernable, but reasons for these changes could include environmental perturbations as mentioned earlier, or perhaps due to different energy resources or unaccounted shifts in water chemistry(Broman et al. 2017) could have contributed to these community changes.

There is separate clustering of cDNA to gDNA shows that there are large differences in diversity between the nucleic acid types. A potential driver could be that ribosomal RNA counts in bacteria are often correlated with cellular growth (Klappenbach et al. 2000). Perhaps, since the cDNA picks up the active components, the rarer community is picked up easily. This was previously seen in a study of anaerobic salt digesters where the patterns of microbial communities showed much higher divergence in RNA based sampling versus DNA based sampling(De Vrieze et al. 2016) and further seen in comparing communities of other sponge species(Kamke et al. 2010). Initially, there is some clustering with the gDNA and cDNA groups with regards to the environmental samples. The clustering by nucleic acid type is not seen in the timepoint_0 samples which were kept for two days and transported. The environmental samples show similar clustering patterns, but upon placement in mariculture, the communities between nucleic acid types begins to diverge. A divergence in the communities has previously been noted (Kamke et al. 2010).

The genomic DNA communities cluster among their own groups. As time moves forward in the mariculture setup, the individual sponges from each time point begin to cluster closer together, particularly those samples taken at the 14-day time point. Ultimately, while there may be some individual variation among the groups, there is only a small significant difference between each community type.

Initially, the environmental samples of the cDNA communities start out clustered together. As time passed in the mariculture setup, these groups began to diverge from each other. Group dispersion may be a factor in the results acquired for the ADONIS and PERMUTEST. Further analysis is needed to show if other factors may be in play affecting the results. There are differences in the way the samples are diverged, further backed up by the results of the PERMUTEST.

Previous studies have taken high microbial abundance sponges and followed them through mariculture. Early work by Mohamed et al. on the sponges *Ircinia strobilina*

(Mohamed et al. 2008b) and *Mycale laxissima* (Mohamed et al. 2008a) have shown that communities in sponges show a strong change upon entry into a mariculture setup, but do stabilize. Further even with strong pressures from eutrophication, a process where nutrients build up and can cause an algal bloom, sponge microbial communities show stability in mariculture (Baquiran and Conaco 2018). These studies (Mohamed et al. 2008a; Mohamed et al. 2008b), though, do not consider the active members of sponge microbial community and were based on genomic DNA sampling, DGGE, and DAPI staining and pure culture for analysis.

In addition to identifying the microbial community and understanding their structure and potential role within the sponge, is identifying the producers of natural products within the sponge. *Tedania ignis* produces an array of compounds, notably tedanolide, but also small molecules like diketopiperazines produced by an associated *Micrococcus* sp. (Stierle et al. 1988).. To further identify the presence of this bacterium, and others as potential natural product producers, further sequencing such as shotgun metagenomic sequencing can be done. Bacterial groups identified from a metagenome assembled genome can be put through algorithms like antismash (Medema et al. 2011) or Natural Product Domain Seeker (NPDoS) (Klau et al. 2022). Further, sponge tissue samples can be analysed with MALDI-IMS to identify localization of different compounds (Aichler and Walch 2015; Ueoka et al. 2015). To identify the overall chemical composition of the sponge, further work can include a profile of metabolites using technology like nuclear magnetic resonance (NMR) and/or liquid-chromatography mass spectrometry (LC-MS) (Kumar et al. 2012; Samirana et al. 2022; Zhang et al. 2022).

A more comprehensive set of studies needs to be performed, particularly to account for other variables such as pH, nutrient composition, and dissolved solids, etc., and how these factors could influence sponge microbial diversity. Further, it could change the group dispersions for beta diversity analysis. Low R^2 values were calculated for the beta dispersions of the sponge communities suggesting another variable or variables are

affecting the communities rather than just time or whether the community is the total community or the active community.

Other approaches to distinguishing the active community members amongst the total population would include live/dead staining of microorganisms in sponge tissue or depletion of dead bacteria using ethidium monoazide. In addition, a negative or a kit control should be included for sequencing. Due to no amplification and no quantifiable product on a Qubit fluorometer, the negative control was excluded from sequencing. What this study showed was an attempt to identify changes in microbiota of a sponge in a mariculture setup and begin a baseline for future studies.

SUPPLEMENTARY INFORMATION

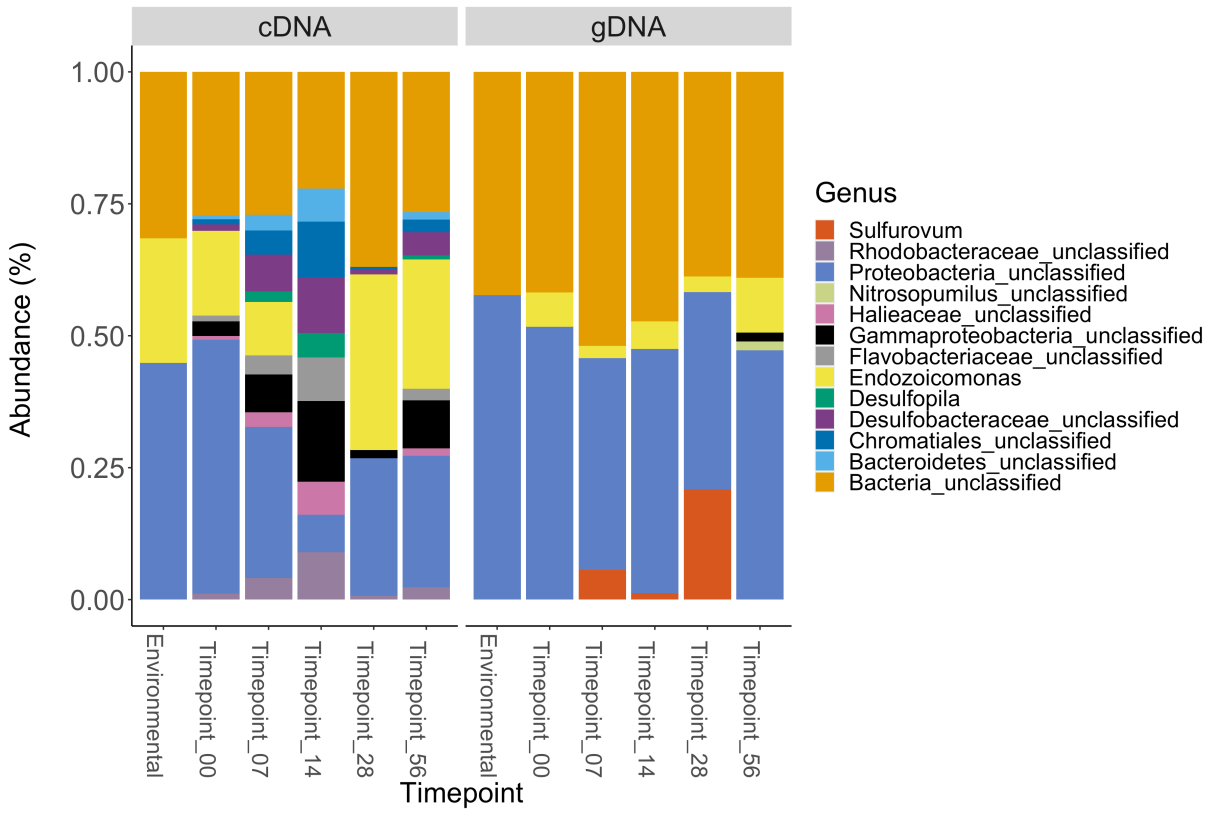


Figure 3.1S- Taxonomic profile of abundant (>0.02%) genera in the total and active communities by time point containing unclassified bacteria.

4.0 MICROEUKARYOTE COMMUNITY ANALYSIS OF THREE TROPICAL SPONGES USING HIGH THROUGHPUT SEQUENCING METHODS

4.1 INTRODUCTION

Sponges harbor diverse groups of prokaryotes and microeukaryotes based on sequencing, culture, and electron microscopy data. Most studies focus on the microbiome, bacteria and to a lesser extent archaea, by utilizing 16S ribosomal RNA gene amplicon sequencing and shotgun metagenomics. These amplicon studies, have yielded scores of information about bacterial and archaeal communities, at the exclusion of microeukaryotes, fungi and protists (Olm et al. 2019). Microeukaryotic community data over the years has started to increase, particularly as databases, like UNITE (Nilsson et al. 2019) and Warcup (Deshpande et al. 2016), begin to fill in with more data. (West et al. 2018).

Fungi, on their own, are important components to the ecology of many ecosystems, functioning to decompose detrital matter and producers of natural products (Holler et al. 1999; Höller et al. 2000; Edrada et al. 2002; Lin et al. 2003; Debbab et al. 2010; Wiese et al. 2011). In particular, new studies on the relationship of fungi within the sponge holobiont have shown they have various roles with regards to sponge health and disease (Pérez-Llano et al. 2023), and natural products (Selvakumar et al. 2020; Wang et al. 2021; Kaliaperumal et al. 2023). With respect to disease, fungi are often seen in plant diseases and to an extent, mammalian disease. However, in the marine environment, fungi have been implicated in causing a disease in Caribbean soft corals (Garzón-Ferreira and Zea 1992) and have been detected in coral and sponge disease lesions. Sponges are filter-feeders that pull in water from the surroundings into their ostium (pl. ostia) and expel the filtered water from the osculum (pl. oscula). Within their tissues are specialized cells that trap bacteria, detrital material, zoo- and phytoplankton,

and microscopic eukaryotes. If not consumed by the sponge, sometimes these organisms can be incorporated into the sponge tissue (Wehrl et al. 2007).

4.1.1 Electron Microscopy studies of sponges

Filamentous fungi have been identified in the Atlantic sponge *Dragmacidon reticulatum* using MALDI-TOF (Passarini et al. 2013). Previous electron microscopy studies of sponges have shown little to no yeasts or filamentous fungi, but there is evidence of endosymbiotic, maternally transmitted yeasts detected using electron microscopy in the genus *Chondrilla* (Figure 4.1; Maldonado et al. 2005).

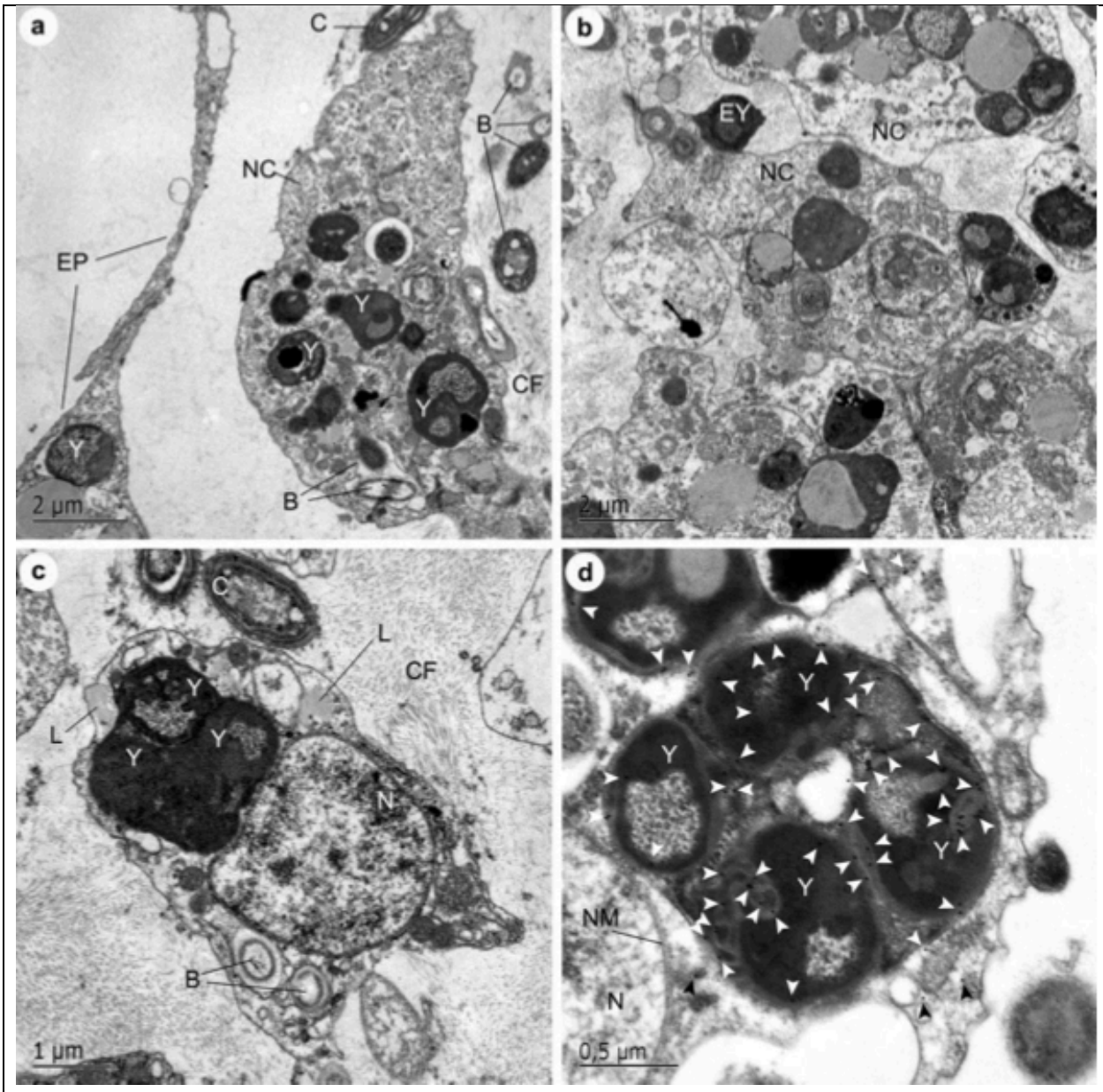


Figure 4.1 Yeasts in tissue of female sponges. In photo A, the boundaries of the sponge can be seen. In photo B, labelled, EY, is extracellular yeast. In photo C and D, yeasts can clearly be seen in the sponge cell. The boundaries in photo D, show the size and layout of the yeasts within sponge cells (Maldonado et al. 2005).

4.1.2 Pure culture studies of sponge fungal communities

In the same study (Passarini et al. 2013), pure cultures were made from two *Dragmacidon reticulatum* samples and ninety-five fungal isolates were identified using six culture media. In a similar vein, 81 fungal isolates were identified from the Atlantic/Mediterranean Sea sponge *Tethya aurentium*, with 21 genera identified using molecular methods (Wiese et al. 2011). Other studies include the isolation of non-marine fungi from sponges (Baker et al. 2009) and marine invertebrates leading to some speculation of laboratory contamination (Bringmann et al. 2003; Proksch et al. 2003; Maldonado et al. 2005). More diverse fungi tend to be detected from pure culture rather than molecular methods (Baker et al. 2009). This discrepancy comes down to conservation of genes between the host and the associated microeukaryotes. In amplicon sequencing, to determine community structure, sequencing primers are designed around conserved flanking regions in the genes of interest, such as 16S rRNA gene for prokaryotes, and 18S rRNA gene and ITS region for eukaryotes. This approach with 16S rRNA genes works well, since the 16S rRNA gene is well conserved in bacteria and archaea, and only a few mitochondrial or chloroplast sequences are amplified compared to the numbers of prokaryotes. In eukaryotes, the 18S rRNA gene and ITS region, this becomes more challenging as they can co-amplify host DNA, which tends to be more present than the microeukaryote populations (Nascimento-Silva et al. 2022). More recent approaches to overcoming non-target read contamination include the use of a peptide nucleic acid clamp (PNA) which binds to the host DNA and prevents its amplification during PCR. This technique has seen success with terrestrial plants (Lefèvre et al. 2020a; Mayer et al. 2021; Viotti et al. 2024), the rhodophyte *Porphyra umbilicalis* (Quigley et al. 2018), and in corals (Reigel et al. 2020).

4.1.3 Molecular methods to show fungal diversity - DGGE/Cloning 18S rRNA genes

Clone libraries were made by extracting DNA which was used for PCR on three 18S rRNA gene regions and 5 ITS gene regions used in either direct DGGE or as part of a nested PCR and then DGGE from *Suberites zeteki* and *Mycale armata* (Gao et al.

2008). Sixty-one positive clones were found and 46 contained fungal inserts from 23 species belonging to two phylum the Ascomycota (*Dothideomycetes et Chaetothyriomycetes incertae sedis, Capnodiales, Dothideales, Hypocreales, Eurotiales, Pleosporales*, and *Saccharomycetales)* and Basidiomycota (*Corticales, Polyporales, Agaricales*, and *Malasseziales*).

4.1.4 High Throughput Sequencing of Amplicon DNA

Difficulties in studying fungal groups, particularly in sponges, arise due to co-amplification of genes from the host DNA, regardless of the chosen marker genes. In an article utilizing the 18S rRNA genes (Naim et al. 2017), most of the sequences returned matched up to the host sponges. It was only after removing the host reads did diverse groups of fungi start to show through.

In a 2018 study by Nguyen and Thomas, of the Indo-Pacific sponges *Cymbastela concentrica*, *Scopalina* sp., and *Tedania adhelans*, three divisions of fungi were found which included 12 orders between all the sponges and seawater using the full length ITS region (ITS1-ITS4 primer). Non-fungal sequences were removed, but no indication of the percentage of non-fungal reads were noted (Nguyen and Thomas 2018). Nguyen and Thomas also showed that despite the large number of fungal reads identified, the number of observed fungal OTUs per sample was low (Table 4.1).

Table 4.1 Number of reads, observed fungal OTUs, expected fungal OTUs (Chao1), Coverage (Good's coverage) and Shannon's index in seawater and sponge samples at a 97% sequences similarity threshold (taken from Nguyen and Thomas[13]). Fungal reads for many samples only make up a small proportion of the total quality filtered reads. Following that, the OTU counts are also low indicating that only a few fungi are present in these sponges, regardless of abundance.

Sample name	Abbreviation	Total no. of quality-filtered reads	No. of fungal reads	Observed fungal OTUs	Expected OTUs (Chao1) ± SE (sub-sampled)	Good's coverage (sub-sampled)	Shannon's index (sub-sampled)
C. concentrica 1 2014	C_1_14	16,199	14,849	8	3 ± 0	1.00	0.09
C. concentrica 2 2014	C_2_14	3,026	1,120	4	3 ± 0	1.00	0.90
C. concentrica 3 2014	C_3_14	6,198	4,808	4	1 ± 0	1.00	0.00
C. concentrica 1 2016	C_1_16	6,578	269	24	28 ± 18	0.97	1.94
C. concentrica 2 2016	C_2_16	21,700	3,437	27	10 ± 3	0.99	0.63
C. concentrica 3 2016	C_3_16	9,483	320	30	44 ± 35	0.94	2.07
Scopalina sp. 1 2014	S_1_14	1,532	49	2	NA	NA	NA
Scopalina sp. 2 2014	S_2_14	7,071	12	3	NA	NA	NA
Scopalina sp. 3 2014	S_3_14	1,992	107	4	NA	NA	NA
Scopalina sp. 1 2016	S_1_16	4,859	444	29	27 ±15	0.97	1.33
Scopalina sp. 2 2016	S_2_16	3,281	206	32	NA	NA	NA
Scopalina sp. 3 2016	S_3_16	24,565	665	36	39 ± 50	0.96	1.38
T. anhelans 1 2014	T_1_14	681	681	2	2 ± 1	1.00	0.03
T. anhelans 2 2014	T_2_14	6,947	6,703	9	5 ± 4	1.00	0.96
T. anhelans 3 2014	T_3_14	64	8	3	NA	NA	NA
T. anhelans 1 2016	T_1_16	19,481	18,300	21	5 ± 4	1.00	1.21
T. anhelans 2 2016	T_2_16	16,379	6,947	27	9 ± 17	0.99	0.77
T. anhelans 3 2016	T_3_16	30,977	30,956	19	3 ± 2	1.00	0.72
Seawater 1 2014	SW_1_14	1,066	2	2	NA	NA	NA
Seawater 2 2014	SW_2_14	3,379	3,314	2	2 ± 1	1.00	0.03
Seawater 3 2014	SW_3_14	2,647	1,588	3	1 ± 0	1.00	0.00
Seawater 1 2016	SW_1_16	6,139	3,379	33	12 ± 8	1.00	2.05
Seawater 2 2016	SW_2_16	12,439	5,576	45	21 ± 9	0.98	2.37
Seawater 3 2016	SW_3_16	25,069	19,496	40	14 ± 18	0.99	1.24

Other groups utilized the 18S rRNA gene (Naim et al. 2017) to determine the fungal diversity in marine sponges. High levels of host tissue are detected from the sample and the community looks uniform. Removal of the co-amplified host shows remarkable diversity in the sponges. For this study, three sponges from the Mediterranean (*Axinella damicornis*, *A. verrucosa*, *Petrosia ficiformis*, and *Aplysina aerophoba)* and North Sea (*Halichondria panicea, Haliclona xena,* and *Suberites massa*) were collected and sequenced utilizing the V7-V8 hypervariable region of the 18S rRNA gene (Table 4.2).

Fungi were seen in greater diversity and 44 OTUs were detected overall within the sponges with most reads identifying as yeasts (Figure 4.2).

Table 4.2-Table from Naim et al. 2017 (Naim et al. 2017) detailing the number of sequences remaining post-extraction of fungal reads. Note the large number of quality-checked pyrosequencing reads compared to the actual number of fungal reads detected. The number of OTUs for most is limited to less than 10 with some exceptions, showing that fungi are not an abundant group within the sponge.

Sample name	Abbrev.	Sponge taxonomic order	Total no. of filtered reads	No. of fungal reads	Fungal reads		
					Observed OTUs	Expected OTUs	Coverage
North Sea water	**WNS**		6,613	1,050	69	94 ± 13	0.97
H. panicea 1	**P1**	*Halichondrida*	12,418	3	3	6 ± 4	0
H. panicea 2	**P2**	*Halichondrida*	8,389	149	15	29 ± 13	0.95
H. panicea 3	**P3**	*Halichondrida*	7,214	256	16	18 ± 3	0.98
H. xena 1	**X1**	*Haplosclerida*	3,728	22	5	6 ± 2	0.91
H. xena 2	**X2**	*Haplosclerida*	4,155	14	5	7 ± 3	0.79
H. xena 3	**X3**	*Haplosclerida*	5,381	15	5	5 ± 1	0.87
S. massa 1	**M1**	*Hadromerida*	14,265	25	8	18 ± 10	0.8
S. massa 2	**M2**	*Hadromerida*	19,955	27	9	17 ± 8	0.78
S. massa 3	**M3**	*Hadromerida*	10,961	11	9	16 ± 7	0.36
Med. Sea water	**WMS**		2,023	617	17	17 ± 1	1.00
P. ficiformis 1	**F1**	*Haplosclerida*	5,704	1,200	23	29 ± 7	1.00
P. ficiformis 2	**F2**	*Haplosclerida*	4,623	486	17	17 ± 0	1.00
P. ficiformis 3	**F3**	*Haplosclerida*	4,462	281	16	16 ± 0	1.00
A. aerophoba 1	**A1**	*Verongida*	14,900	7	4	5 ± 1	0.71
A. aerophob a 2	**A2**	*Verongida*	15,285	32	13	15 ± 2	0.84
A. aerophoba 3	**A3**	*Verongida*	17,472	21	21	11 ± 4	0.81
A. damicornis 1	**D1**	*Halichondrida*	14,900	0	0	–	–
A. damicornis 2	**D2**	*Halichondrida*	15,572	0	0	–	–
A. damicornis 3	**D3**	*Halichondrida*	17,472	0	0	–	–
A. verrucosa 1	**V1**	*Halichondrida*	13,583	0	0	–	–
A. verrucosa 2	**V2**	*Halichondrida*	13,701	0	0	–	–
A. verrucosa 3	**V3**	*Halichondrida*	130,365	1	1	1 ± 0	0

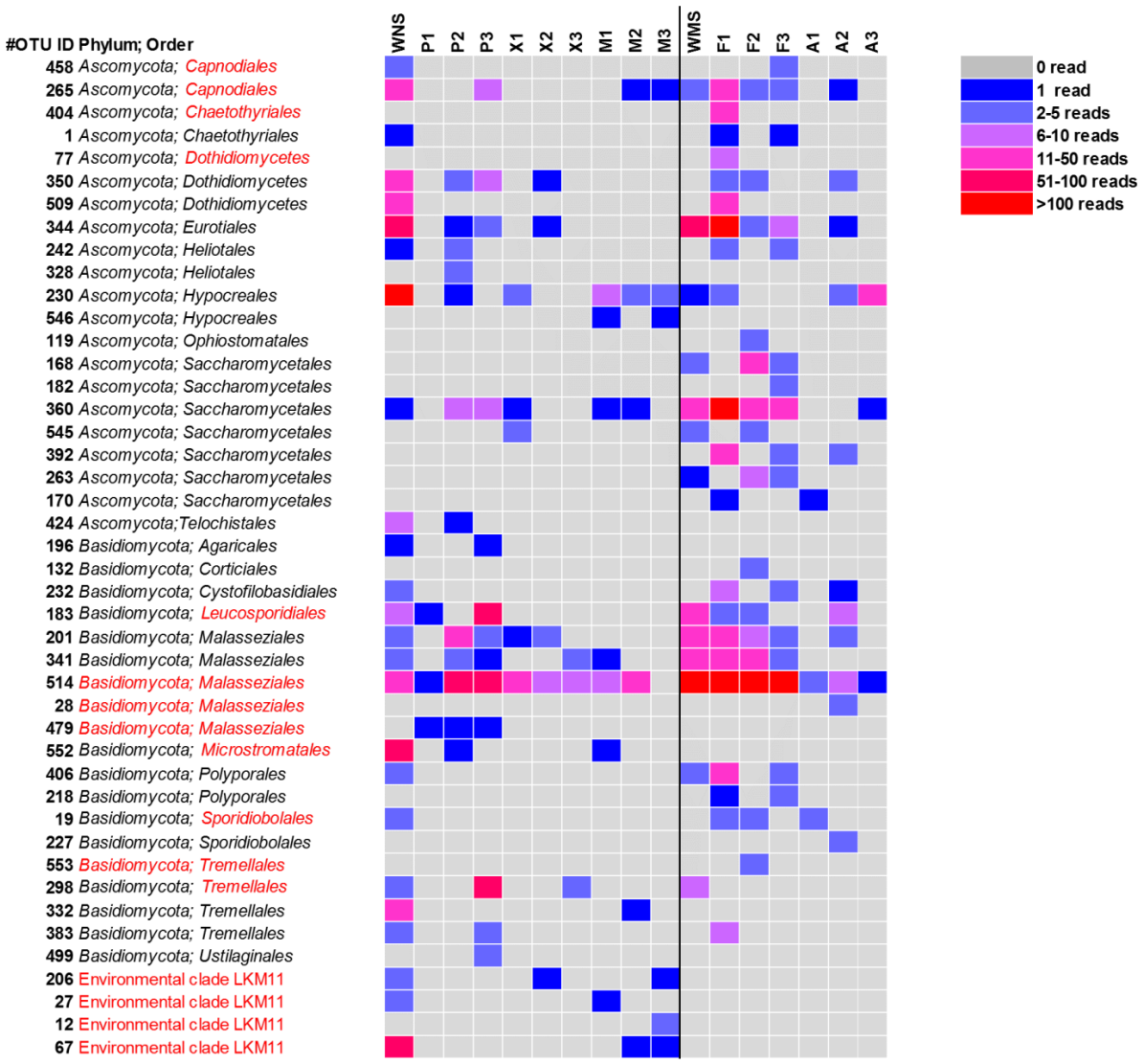


Figure 4.2-Heatmap of fungi found in each sponge sample from Naim et al. 2017 (Naim et al. 2017) The highest abundance of fungi is shown to be from class Malasseziales, a type of yeast. It should be noted that the majority of fungi identified in both sets of sponges have an occurrence of less than 10 OTUs.

In this study, the eukaryotic composition of three species of sponges will be presented using the internal transcribed spacer 2 (ITS2) gene. Two species are tropical sponges collected from marine aquariums as live rock hitchhikers and the other from the mangroves of Summerland Key, FL. The data generated will show the fungal

composition and the microeukaryotic composition of three tropical marine sponges and discuss the pitfalls of this approach for host-eukaryotic symbiont studies.

4.2 MATERIALS AND METHODS

4.2.1 Sponge Collection

4.2.1.1 *Collospongia auris*

The sample of sponge initially identified as *Collospongia auris* was collected from Bristol Aquarium (Bristol, UK). The sponge had hitchhiked on live rock and was removed to a holding tank until it could be collected. Upon arrival back to Cardiff University, the sponge was cut into four pieces with one piece immediately frozen. Only one sample was recovered from the Bristol Aquarium. The remaining pieces were placed into 2L beakers with air stones for an experiment. One week after arrival, the tissue on the beaker sponges was beginning to recede and appear necrosed. The sponges were sacrificed and frozen immediately. Samples were labelled as CU12 for the timepoint 0 sample. Sample 13 was collected a week later, and sample 14 was taken 14 days after the sponge became necrotic.

4.2.1.2 *Haliclona* sp.

Samples from two individuals of *Haliclona* sp. were obtained from two tanks at the Plymouth National Aquarium (Plymouth, UK) and transported back to Cardiff. Upon arrival, a 2.5cm piece of tissue was removed from each sponge and immediately frozen for further processing. The remaining sponge was placed into an aquarium and tethered to the live rock. Subsequent sampling occurred at one week, two weeks, and one month.

4.2.1.3 *Tedania ignis*

Tedania ignis was collected from the mangrove swamps around Summerland Key, FL, USA, near Mote Marine Lab. Upon arrival at Mote, a 2.5cm piece of tissue was immediately frozen from each specimen (environmental sample). The remaining sponges were kept in a holding raceway at Mote Marine Lab until transport to Harbor Branch Oceanographic Center, FL, USA. These sponges did not survive and

succumbed to extreme biofouling and tissue degradation. A second set of sponges were collected and transported back to Harbor Branch Oceanographic Center. A 2.5cm piece of tissue was immediately collected and frozen (Timepoint_0). The remaining sponges were kept in an open circulated raceway and subsequently collected at one week, two weeks, one month and two months.

4.2.2 Nucleic Acid Extraction and cDNA Synthesis

Samples were extracted using the PowerLyzer Powersoil Kit (Qiagen, Inc., Hilden, DE) for DNA. Frozen sponge tissue (3g) for DNA extraction were cut and squeezed in sterile petri dishes using 2mL of L-buffer (10 mM Tris-Cl, pH 8.0; 0.1 M EDTA, pH 8.0; 0.02M NaCl) (Russell and Sambrook 2006). The resulting slurry was transferred to a sterile 1.5mL centrifuge tube and spun at 12,000 x g for one minute. The supernatant was pipetted off and the resulting pellet was resuspended in Solution C1 and Bead Solution from the PowerLyzer kit. An empty sample was extracted at the same time to account for laboratory contamination. DNA in the air sample was undetectable via gel electrophoresis and failed to amplify in test PCR so it was excluded from sequencing.

Tedania ignis samples were shipped to Halmos College of Natural and Marine Sciences Guy Harvey Oceanographic Campus of Nova Southeastern University on dry ice to be extracted for RNA and for sequencing. *Haliclona sp.* and *Collospongia sp.* samples were extracted for RNA at Cardiff University. Total RNA was extracted using the RNEasy Kit (Qiagen, Inc., Hilden, DE)with QIAShredder tubes (Qiagen per manufacturer's instructions. RNA concentration was checked using the Qubit Broad Range (BR) RNA kit (Invitrogen). Total RNA was treated for genomic DNA contamination using the TURBO-free DNase kit (ThermoFisher Scientific). A PCR was run to check for complete inactivation of the DNase enzyme using 515f/806R primers. Complementary DNA was created using the RETROSCRIPT Kit (ThermoFisher Scientific) with the heat activation step and random hexamers per the manufacturer's instructions.

4.2.3 PCR and Sequencing Analysis

Genomic DNA and complementary DNA samples from *Collospongia auris* and *Haliclona* sp*.* were sent to Research and Test Laboratories (Lubbock,TX) for sequencing of the ITS2 region. *Tedania ignis* was sequenced at Nova Southeastern University (Dania Beach, FL) following the same library preparation method of RTL.

Data was analysed using DADA2 (Callahan et al. 2016) in R (R Core Team 2021). Primer sequences were input and reverse complemented. Sequences were pre-filtered for N regions. The primers and their reverse complements were removed using cutadapt (v2.4). The read files were quality filtered, but not trimmed and the error rate was calculated for the forward and reverse reads. Samples were dereplicated to reduce the computational workload for each read direction. Following dereplication, the reads were merged together and made into an amplicon sequence variant (ASV) table as ASV analysis can greater resolve sequences (Callahan et al. 2017). Chimeric sequences were removed using the consensus-based method during the merging step. Taxonomy was assigned using the UNITE (Nilsson et al. 2019) database (02-02-19) for all eukaryotes. The resulting taxonomy table and a transposed ASV table were exported into phyloseq. Bar graphs were generated using phyloseq (McMurdie et al. 2013) and ggplot2 (Wickham 2016, p.2). Feature IDs generated in DADA2 were the representative sequence. For further analysis purposes, the non-fungal reads, mainly host, were run using the blastn web interface with defaults using the nt database.

4.3 RESULTS

4.3.1 Confirmation of Host Sequences-*Collospongia auris*

Collospongia auris is not present in the eukaryotes FASTA file available through UNITE so reads were initially identified as another species of sponge. ASVs identified as the host were run through blastn web interface using the nr/nt database with default settings. These sponge species were not listed in the UNITE database so a secondary blastn search was necessary. These reads were identified as *Lendenfeldia chondrodes*,

a sponge now understood to have been misidentified as *Collospongia auris*. *L. chondrodes* and *C. auris* are both closely related sponges in that they both belong to the family Thorectidae, but each belong to different subfamilies with *L. chondrodes* belonging to the subfamily Phyllospongiinae (Ehlers 1870) and *C. auris* belonging to subfamily Thorectidae (Bergquist et al. 1990). According to Bergquist (Bergquist et al. 1990), these sponges both look similar and have similar morphologic structures. Differences could show up in their terpene content in future studies which might inform their taxonomy.

4.3.2 Top Taxa in *L. chondrodes*

Figures 4.3A and 4.3B show the top 20 genera present in *L. chondrodes* prefiltering for Porifera sequences (4.3A) and postfiltering (4.3B). Initially, 81 unique taxa were identified from the samples, however, post-filtering of poriferan host reads reduced the number of unique taxa to 65. Richness and observed OTUs in each sample are shown in Figures 5A and B. More observed OTUs were detected with complementary DNA samples, but the genomic DNA samples show more diversity in prefiltered samples after the removal of poriferan sequences (Figure 4.4A), which was performed for comparison purposes. In Figure 4.4B, the poriferan sequences were filtered resulting in a higher alpha diversity within the complementary DNA sequences, but lower observed OTUs. The number of OTUs were also affected in these samples (Supplementary Table 1).

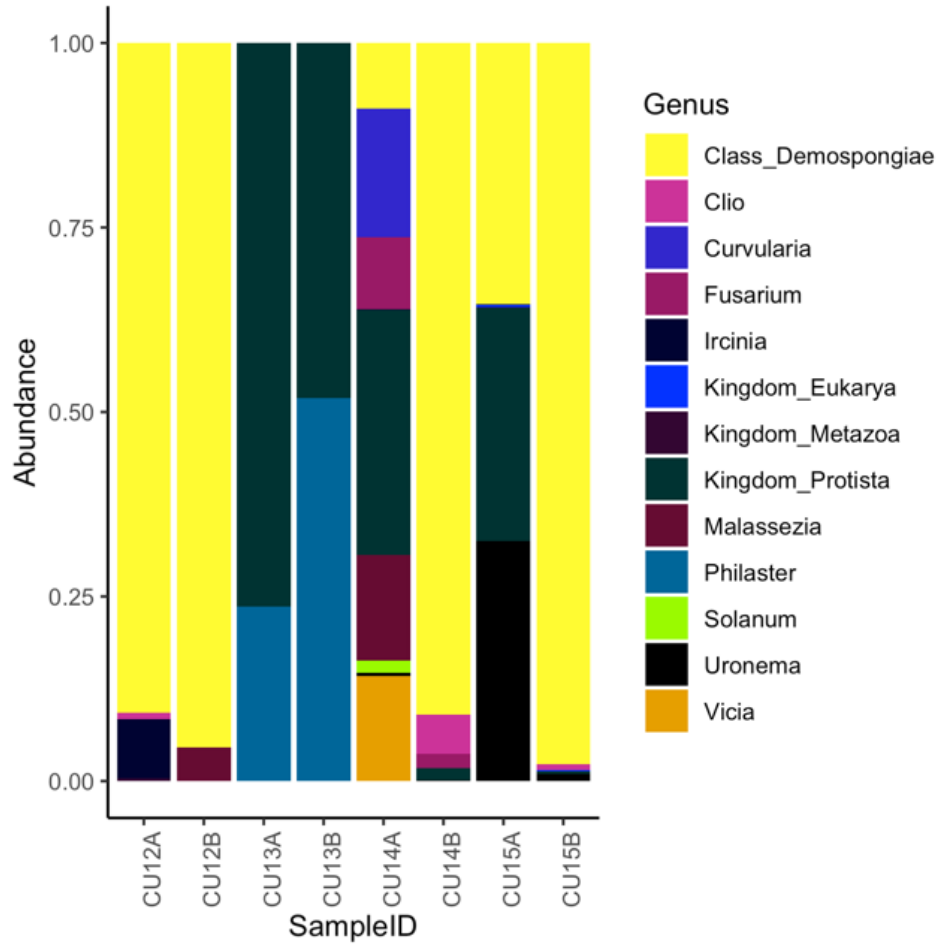


Figure 4.3a-Genera of eukaryotic reads found in *Lendenfeldia chondrodes* comparing gDNA (labelled as CU*A) and cDNA (labelled as CU*B) based sequencing of communities. Top 20 genera hits associated with *Lendenfeldia chondrodes* prefiltering for Porifera sequences. The number of taxa identified pre-filter was 81.

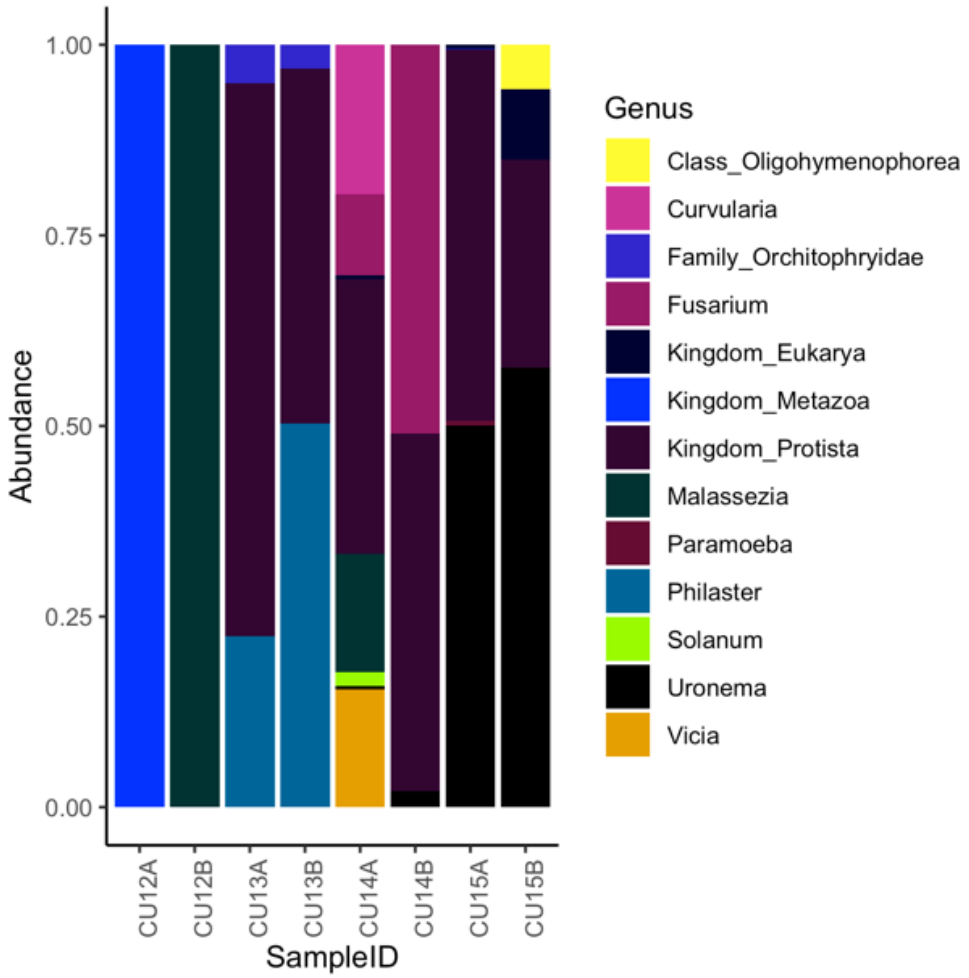


Figure 4.3b-Genera of eukaryotic reads found in *Lendenfeldia chondrodes* comparing gDNA and cDNA based sequencing of communities post-filtering for poriferan reads. Top 20 genera hits associated with *L. chondrodes* post filtering for Porifera sequences. The number of taxa identified post filtering was 65. Unidentified reads were run in BLAST and identified as *Lendenfeldia chondrodes*.

Figure 4.3a&b show how the abundance and diversity of the microeukaryote communities differ pre and post filtering of the host. Pre-filtering, most samples contained sequences matching to Demospongiae, which is expected. Post-filtering of host reads yielded fungal sequences (Curvularia, Malasezzia, and Fusarium) and other

eukaryotes consisting of ciliates (Oligohymenophorea, Orchitophryidae, Paramoeba, Philaster, and Uronema) and two plant groups (Solanum and Vicia).

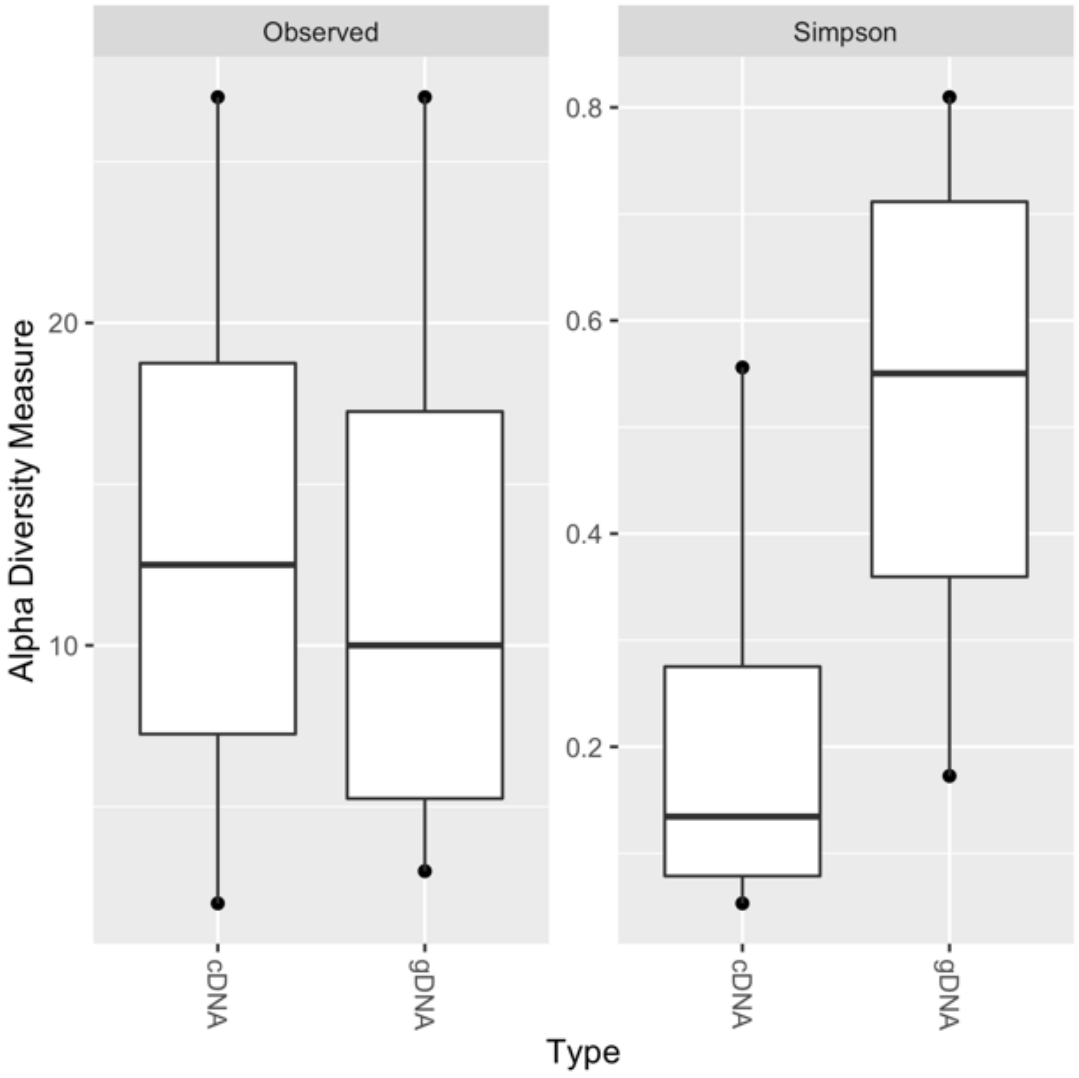


Figure 4.4a-Richness boxplot of observed ASVs and the Simpson Index for the *L. chondrodes* samples split by cDNA (the active community) and gDNA (the overall community). Observed ASVs show the counts of ASVs from the samples and the Simpson index shows the diversity of each sample. Porifera sequences were included for the boxplot.

In Figures 4.4a&b, a comparison is drawn between the observed ASVs and the Simpson diversity based on the presence of poriferan reads. Figure 4.4a contains poriferan reads and the contrast between the observed OTUs (R^2=0.0029, *p*-value=0.8991) and Simpson diversity (R^2=0.3125, *p*-value=0.1497) is low and no significant differences were found. When coupled with Figure 4.3a, the cDNA samples contain high levels of Demospongiae sequences. The intent of using cDNA was to identify active organisms. With the high levels of poriferan sequences present in those samples, other community members were overshadowed by the host sequences resulting in a seemingly lower diversity.

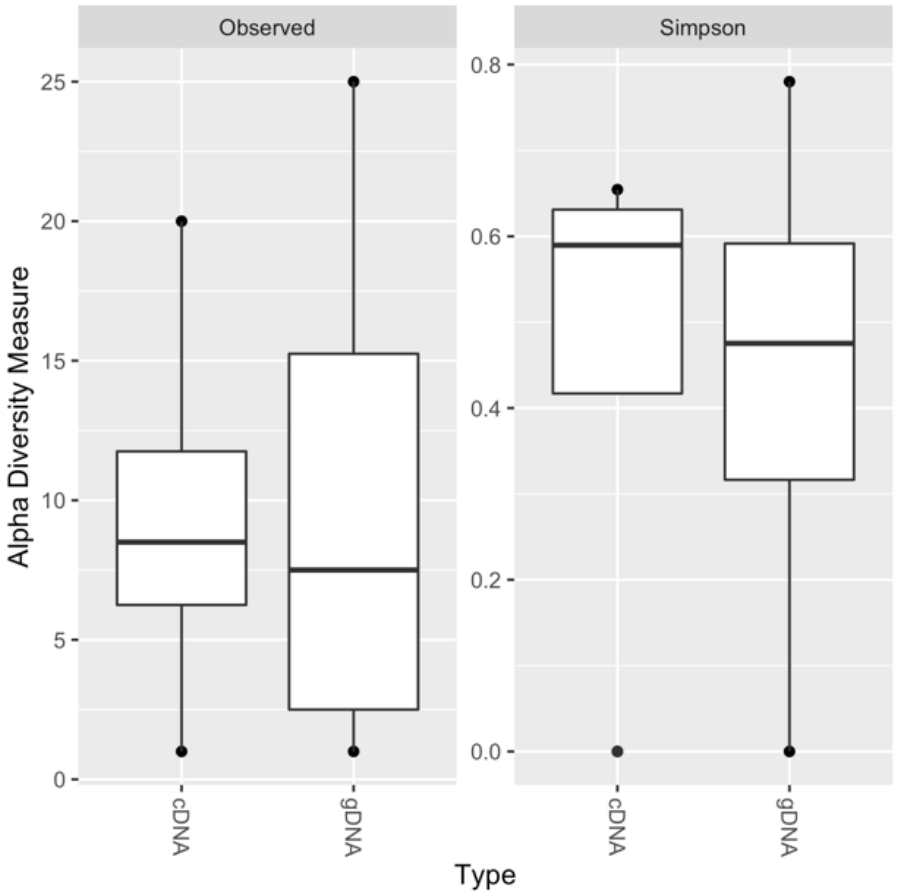


Figure 4.4b-Richness boxplot of observed ASVs and the Simpson Index for the *L. chondrodes* samples split by cDNA (the active community) and gDNA (the overall community) and labelled by collection timepoints. Observed ASVs show the counts of ASVs from the samples and the Simpson index shows the diversity of each sample. Porifera sequences were filtered from the resulting boxplots.

In Figure 4.4.b, the Simpson diversity of the cDNA group increases from 0.1 to 0.6 indicating a higher level of diversity and the number of observed ASVs decreases slightly due to the loss of poriferan sequences, but overall, there is no statistical difference between the nucleic acid types (R^2=0.002193, *p*-value=0.9123). The gDNA samples show a minor change in terms of observed ASVs and Simpson diversity where the median of the observed ASVs decreases slightly and the Simpson diversity also decreases. The decrease in observed ASVs can be attributed to a loss of host reads, but the differences in the observed ASVs show no significance (R^2=0.002065, *p*-

value=0.9149). In the initial taxonomic breakdown of the gDNA samples, only a few species of microeukaryotes are found and they are largely poriferan sequences. Overall, the differences between filtered and non-filtered reads do not show enough significance.

4.3.3 Top taxa in *Haliclona sp.*

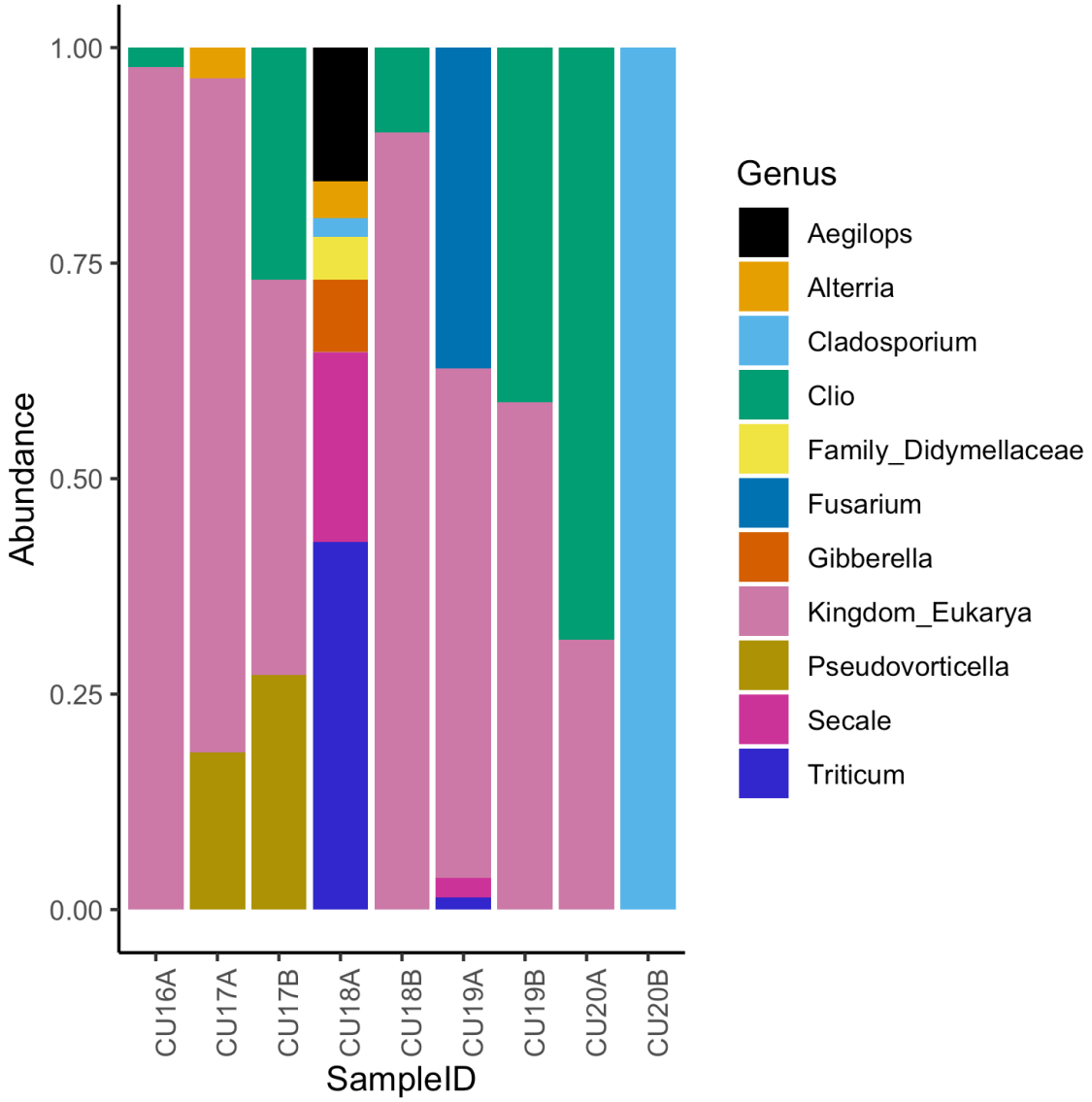


Figure 4.5a-Top 20 taxa hits associated with *Haliclona sp.* prefiltering for Porifera sequences. The number of taxa identified pre-filter was 240.

In Figure 4.5a, most reads identified as fungus (*Alterris*, *Cladosporium*, family Didymellaceae, and *Gibberella*). One ciliate group was identified (*Pseudovorticella*) and a gastropod (Clio) was also identified. The remaining groups identify to different plants. Interestingly, there are no poriferan reads identified in any of the samples. Kingdom Eukarya is present, and it is possible that the poriferan reads match within that group, but do not match well with other porifera groups in the UNITE database. The number of reads per sample pre- and postfiltering are listed in Supplementary Table 2 ranging from 160 in CU16B, which ended up being filtered out downstream to 85050 in CU18A prefiltered. The sequence count for these samples remained the same post filtering.

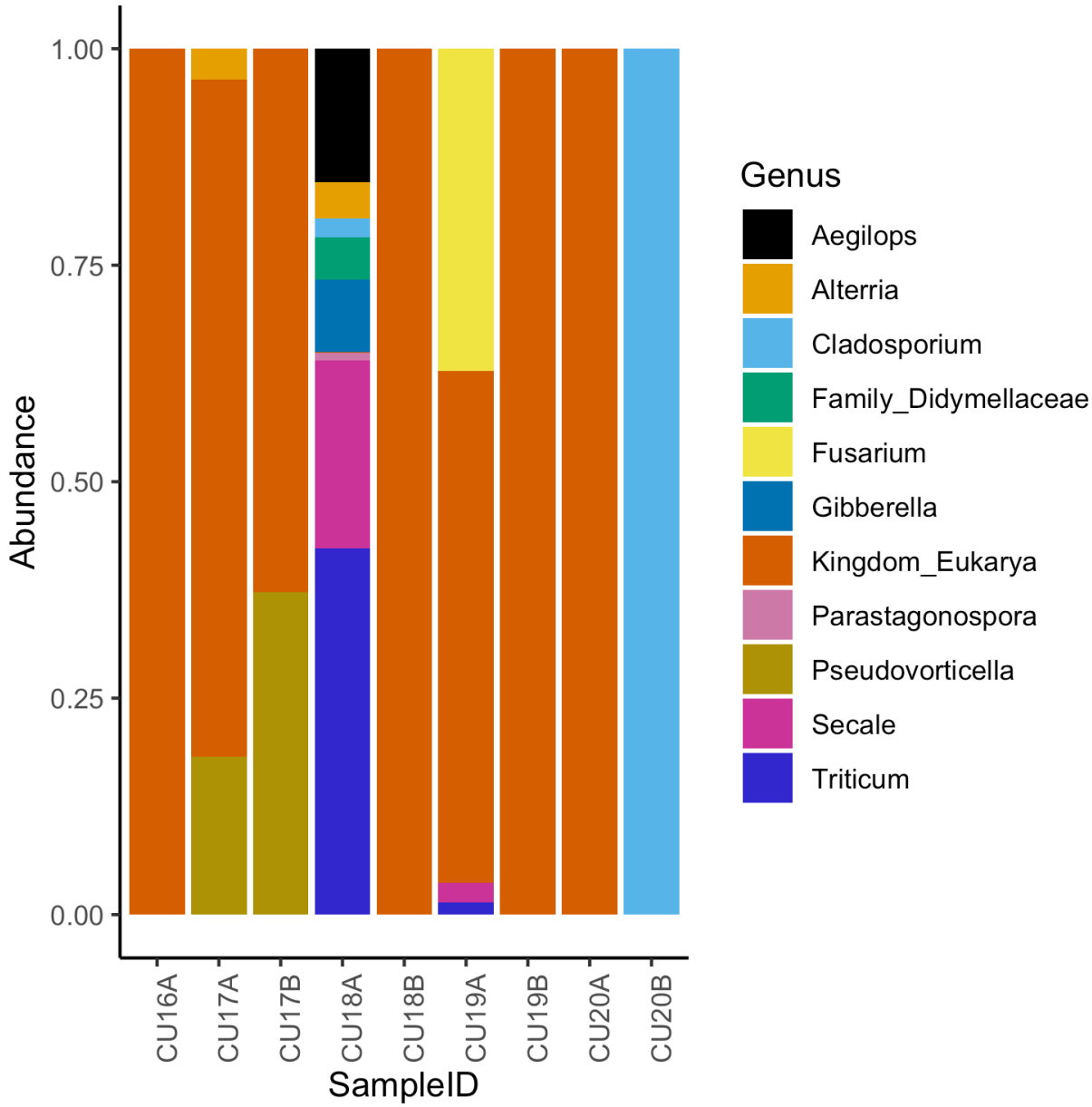


Figure 4.5b-Top 20 taxa hits associated with *Haliclona sp.* post filtering for Porifera sequences. The number of taxa identified post filtering was 233. Reads identified as kingdom Eukarya are not well resolved in the UNITE database.

Post filtering, most of the identified reads match to fungal sequences and a new group of fungal sequences (*Parastagonospora*).

There is no statistical difference in the observed ASVs (R^2=0.00763, p-value=0.8232) nor a significant difference in diversity (R^2=0.3379, *p*-value=0.1007) for *Haliclona* sp. when including the Porifera reads (Figure 4.6a). Post-filtering, there is no observable, significant difference was detected for the observed ASVs (R^2=0.00763, *p*-value=0.832), but there was a noted difference in the Simpson diversity (R^2=0.5321, *p*-value =0.02573).

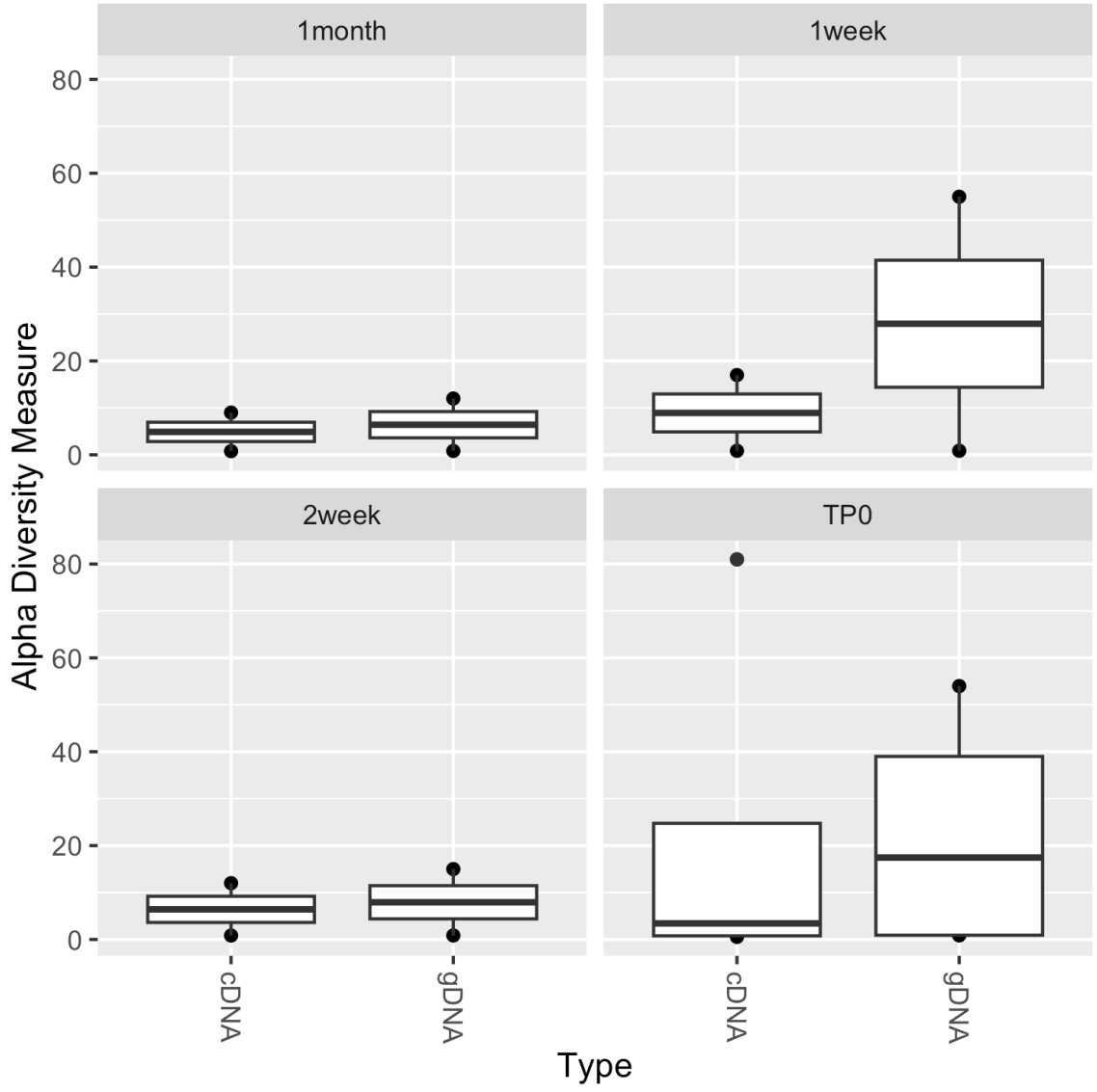


Figure 4.6a-Richness boxplot of observed ASVs and the Simpson Index for the *Haliclona sp*. samples split by cDNA (the active community) and gDNA (the overall community) and labelled by collection timepoints. Observed ASVs show the counts of ASVs from the samples and the Simpson index shows the diversity of each sample.

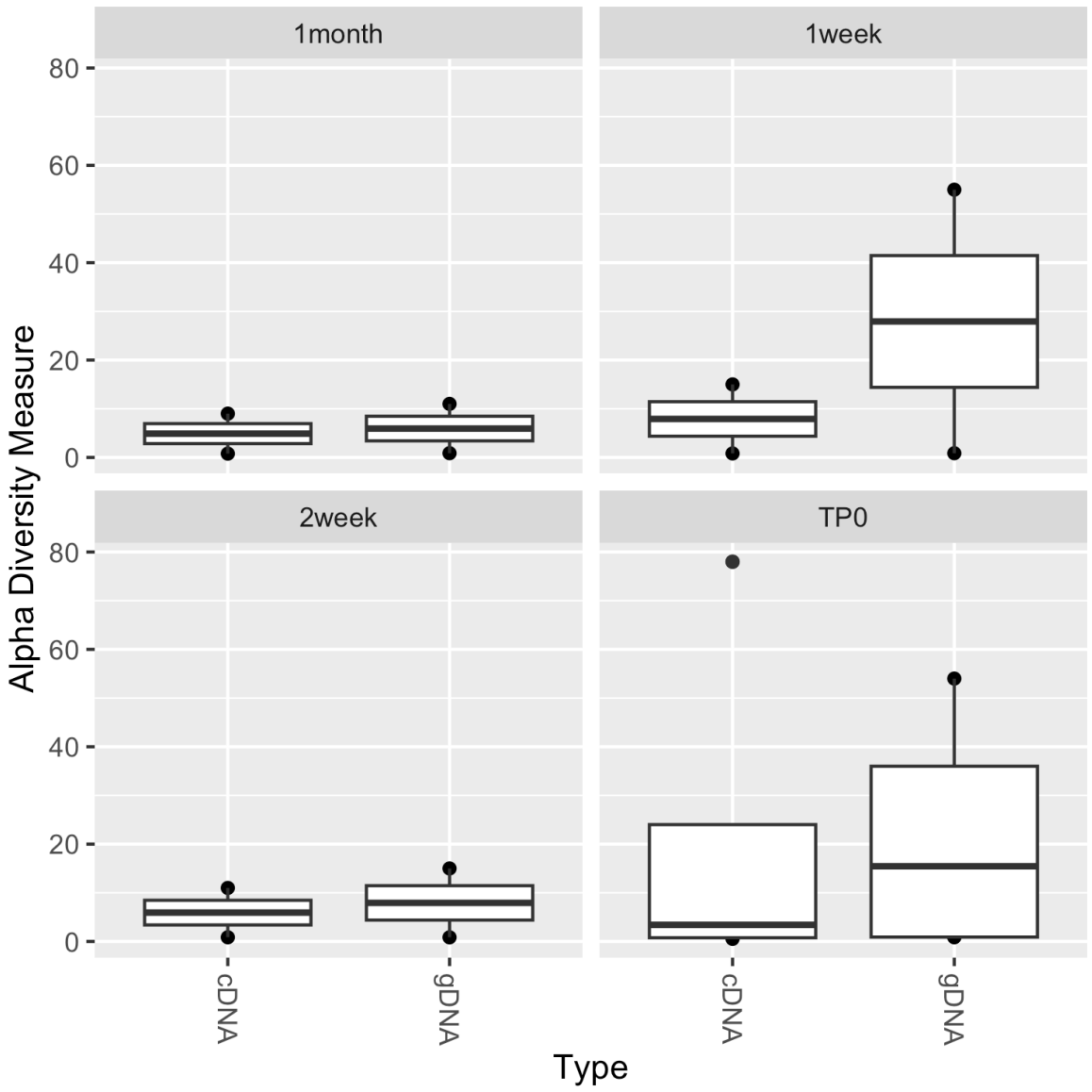


Figure 4.6b-Richness boxplot of observed ASVs and the Simpson Index for the *Haliclona sp*. samples split by cDNA (the active community) and gDNA (the overall community) and labelled by collection timepoints. Observed ASVs show the counts of ASVs from the samples and the Simpson index shows the diversity of each sample. Porifera sequences were filtered from the results.

4.3.4 Top Taxa in *Tedania ignis*

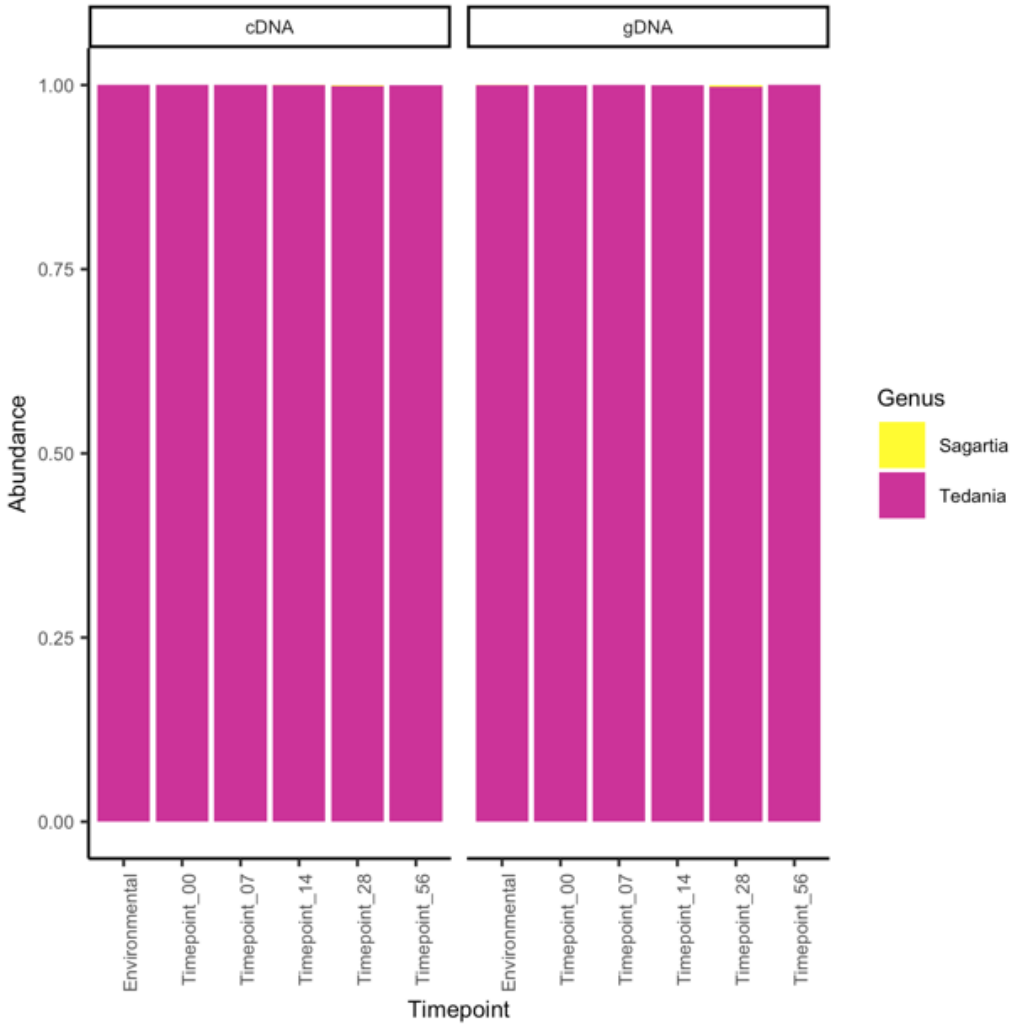


Figure 4.7a-Top 20 taxa hits associated with *Tedania ignis* prefiltering for Porifera sequences. The number of taxa identified pre-filter was 184.

In Figure 4.7a, all samples are dominated by *Tedania ignis* reads. Reads for *Sagartia*, an anemone, are present in low abundance at Timepoint_28 for both the gDNA and the cDNA samples. The number of reads per sample can be found in Supplementary Table 3 and the range prefiltering is 0 to 385677. In terms of the gDNA versus the cDNA communities, more communities are present. It

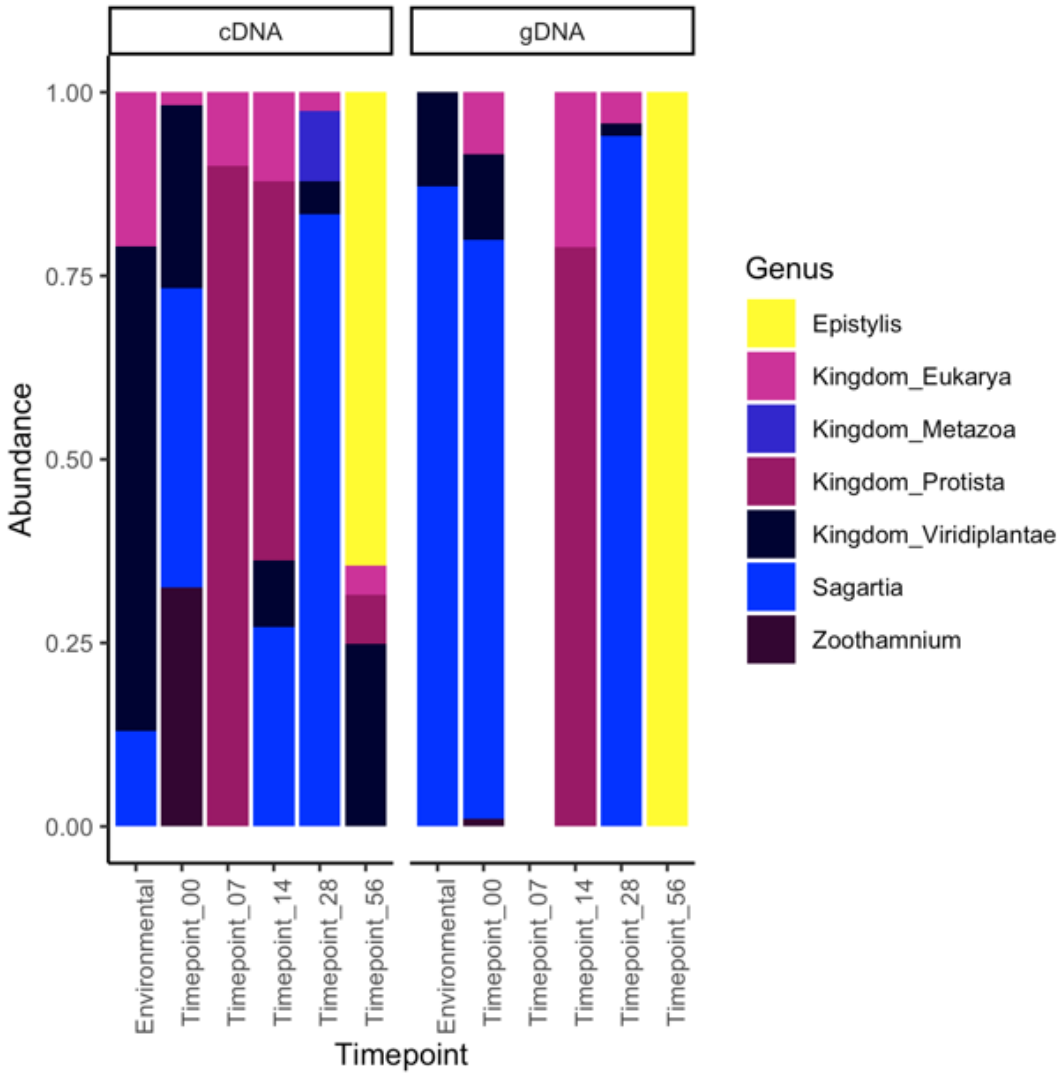


Figure 4.7b-Top 20 taxa hits associated with *Tedania ignis* post filtering for Porifera sequences. The number of taxa identified post filtering was 102.

Post-filtering of the poriferan reads reveals hidden diversity in the *Tedania ignis* samples. In addition to the *Sagartia* previously noted, ciliates like *Epistylis* and *Zoothamnium*, and eukaryotic green algae belonging to the Viridiplantae. The number of reads per sample can be found in Supplementary Table 3. The numbers range from 0 to 1343.

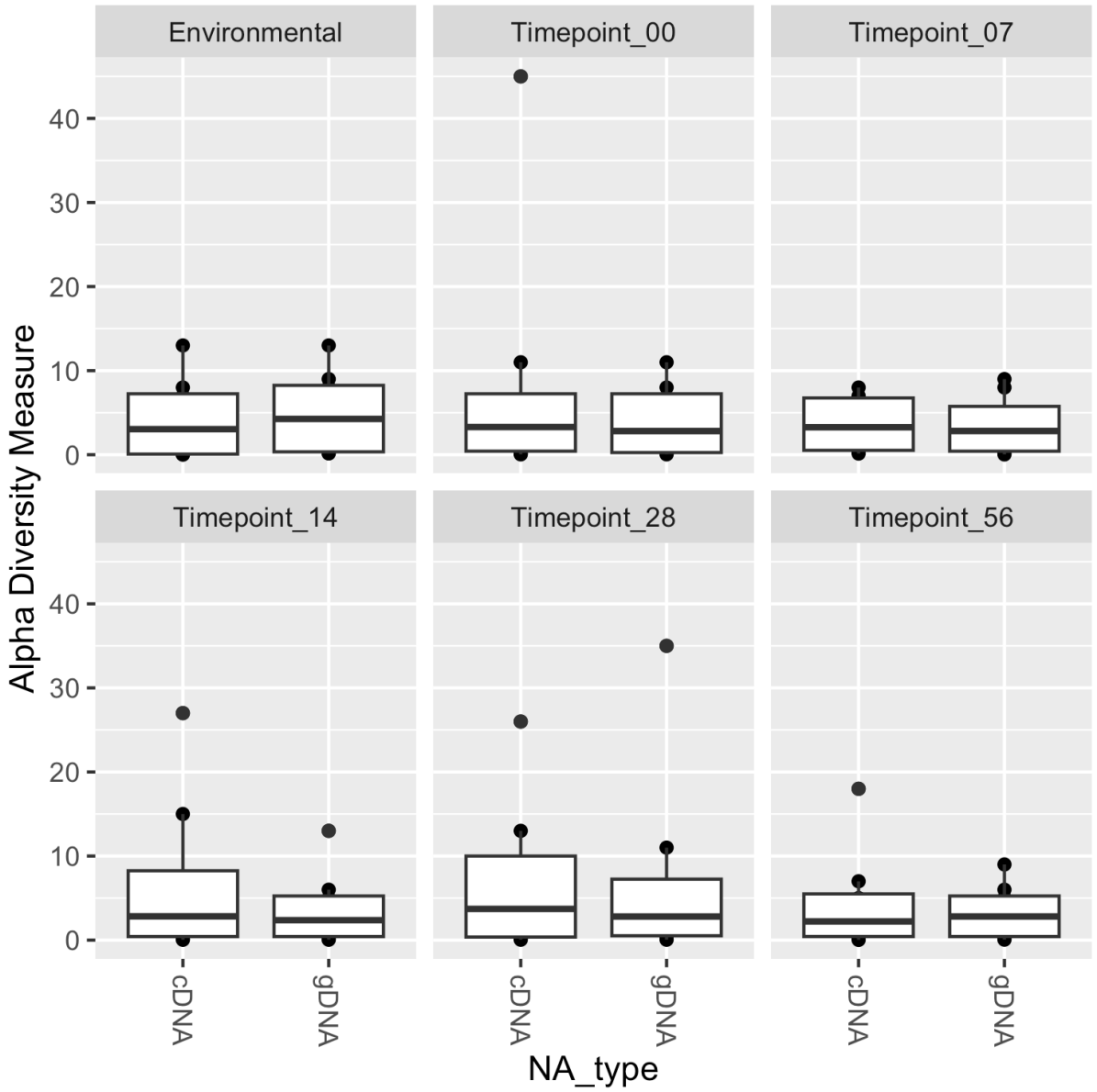


Figure 4.8a-Richness boxplot of observed ASVs and the Simpson Index for the *Tedania ignis* samples split by cDNA (the active community) and gDNA (the overall community). Observed ASVs show the counts of ASVs from the samples and the Simpson index shows the diversity of each sample. Porifera sequences were included.

In Figure 4.8a, the number of observed ASVs have a similar median between the two sample types, which can be accounted for by the inclusion of the host reads (ANOVA

R^2=0.0283 *p*=0.2531). As shown in Figure 4.7a, the samples were overwhelmed with host reads and no other organism was identified besides the *Sagartia*. A similar effect can be seen in the alpha diversity.

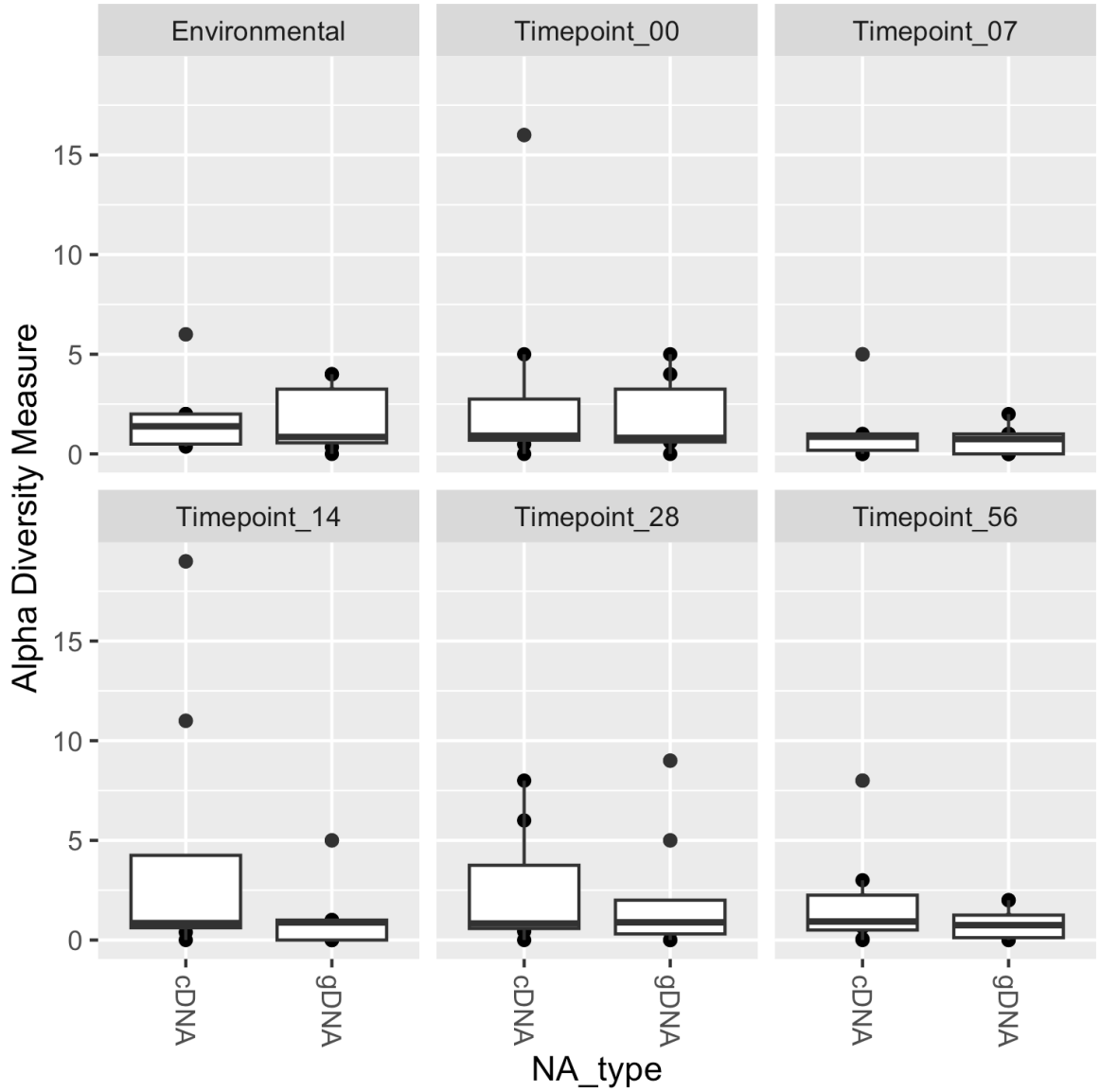


Figure 4.8b-Richness boxplot of observed ASVs and the Simpson Index for the *Tedania ignis* samples split by cDNA (the active community) and gDNA (the overall community) and labelled by collection timepoints. Observed ASVs show the counts of ASVs from the samples and the Simpson index shows the diversity of each sample. Porifera sequences were filtered out.

Postfiltered samples show a much lower amount of observed ASVs. Despite the lower observed ASVs, there is more diversity as seen in Figure 4.8b and previously observed in Figure 4.7b.

4.4 DISCUSSION

4.4.1 Challenges with microbial eukaryotic sequencing in mixed samples

The microbial eukaryote communities of marine sponges are still poorly characterized. Co-amplification of the host reads seems to reduce the amount of other eukaryote sequences generated from the sequencing run. Further, there could be indications of tissue necrosis or death of the sponge if higher numbers of fungal and other microeukaryotes are detected feeding on the dying tissue (Hyde et al. 1998; Thatoi et al. 2013).To better address this alternative methods have been proposed. One method utilized locked nucleic acid (LNA) based primers and PCR clamping by these primers to better amplify the ITS region (Ikenaga et al. 2016). This is achieved by having a small overlap of bases between the LNA primer with the ITS primer. This has also been performed with bacterial SSU to mitigate the amplification of organelle DNA (Ikenaga and Sakai 2014; Ikenaga et al. 2015). Another recent microeukaryote study utilized an anti-metazoan reverse primer and a universal 18S rRNA gene V4 hypervariable region forward primer (Minardi et al. 2022) which reduced the host reads and provided a more detailed view of the microeukaryome.

4.4.1 Taxonomic Profile

Fungi

Fusarium sp. was detected in both *L. chondrodes* and *Haliclona sp*. This mold species is commonly found in terrestrial soil environments and has been implicated in some diseases in humans (Nucci and Anaissie 2002; Dignani and Anaissie 2004; Anaissie and Nucci 2023) and plants (Aoki et al. 2014; Arie 2019). While most *Fusarium* species are considered harmless and some species can produce antimicrobial natural products (Xu et al. 2023), there are potential implications for human and animal health if it contaminates the food supply due to the production of mycotoxins (Ekwomadu et al.

2021; Johns et al. 2022). Pathogenic *Fusarium* species have been linked to diseases in Amazonian turtles and sea turtles, where the mycelium infects the nests resulting in hatch failures (Sarmiento-Ramírez et al. 2014; Gleason et al. 2020; Carranco et al. 2022; Kuschke et al. 2023).

In addition to Fusarium, other groups of fungi were identified in *L. chondrodes*, including *Malassezia*. *Malassezia* is a common fungus found in various environments (Steinbach et al. 2023) . As a pathogen, *Malassezia* can cause hypo- and hyperpigmentation of the skin, as well as seborrheic dermatitis (Saunte et al. 2020; Karray and McKinney 2023). In the marine environment, the role of *Malassezia* is not well established but has been determined to be diverse(Steinbach et al. 2023), but has previously been shown to be dominant species in mairne sponges using 18S rRNA amplicon analysis (Naim et al. 2017).

In *Haliclona* s*p*., a related genus, *Giberella*, was detected. *Giberella* is associated with various plant diseases such as ear rot in maize (Lana et al. 2022) and produces the hormone gibberellin, stimulating plant growth (Castro-Camba et al. 2022). Giberella has previously been detected in other sponge species and was found in the freshwater sponge, *Lubomirskia baicalensis* as part of a metagenomic survey of polyketide synthase genes (Kaluzhnaya et al. 2012). Other fungi found in *Haliclona* sp. include the family Didymellaceae and genus *Alternaria*, misnamed as *Alterria* in the taxonomic results, while in *L. chondrodes*, *Curvularia* was detected. These fungi, belonging to the same order of Pleosporales, are typically found in diverse environments, including the marine environment. Both *Alternaria (Thomma 2003; Tsuge et al. 2013)* and *Curvularia* (Chen et al. 2013; Shi et al. 2017; Pang et al. 2018; Santos et al. 2018; Cui et al. 2020)are plant pathogens in the terrestrial environment. In the marine environment, *Alternaria* and *Curvularia* have been associated with algal (Greve et al. 2008; Dai et al. 2010) and sponge hosts, and are a source of naturlal products . A sponge associated *Alternaria* sp. and an algal associated species, *Alternaria alternata* k21-1 (Shi et al. 2017) were found to produce anticancer metabolites in the form of sesterpenes and derivatives of molecules called perylenequinones. In addition to anti-cancer treatment,

A. alternatia is being explored for bioremediation. *A. alternata* contains roughly 153 genes related to the degradation of polyethylene (Gao et al. 2022), an inert plastic. When allowed to decompose, it generates microparticles which can get into soil and waterways causing severe impact to the environment.

Tedania ignis had no abundant fungal reads detected in contrast to previous mycobiome analyses which identified various groups of Ascomycota and Basidiomycota (Hardoim et al. 2021). In a related species, *Tedania anhelans* from the Indo-Pacific Ocean, the species *Aspergillus, Acrostalagmus,* and *Cladosporium* were identified (Nguyen and Thomas 2018). Possible reasons behind the lack of fungal reads from *T. ignis* could be that the PCR temperature needed to be raised to create more specificity or that the number of host reads were so overwhelming that fungal DNA was not amplified in detectable amounts. Of note in the *T. ignis* sequences, the genus *Sagartia (Cylista)*, an anemone, was identified. It is generally found in the North Atlantic and Mediterranean Seas. *Tedania ignis* co-occurs with several anemone species in the mangroves and it is not unexpected to have co-amplified anemone DNA.

As a whole, fungi in other organisms and environments contribute to fitness and defense, but in sponges, the function and structure of fungi is still relatively unknown (Pita et al. 2018) except for a couple of studies (Gao et al. 2008; Passarini et al. 2013; Passarini et al. 2015; Nguyen and Thomas 2018; Hardoim et al. 2021). Initial evidence that a *Rhabdocline* sp. fungus, a pathogen to fir trees, an Ascomycota sp., and a Pleosporales sp. may play a role in a polymicrobial, necrotic disease in *Callyspongia biru.* Diseased specimens of this sponge have been found with the Rhabdocline fungus and a Rhodobacteraceae were shown to have similar pathology in aquaria sponges as in the wild (Sweet et al. 2015).

Ciliates

Each sponge had a unique set of identified ciliates. In *L. chondrodes*, the ciliate Oligohymenophorea, Orchitiphyridae, Paramoeba, Philater, and Uronema were detected. Oligohymenophorea are groups of ciliates that can occur as single cells, such

as *Paramecium*, or in colonies. Oligohymenophorea encompasses a wide range of ciliates, approximately 2,311 species (Corliss 1974; Lynn 2008) in various habitats, both freshwater and marine, and as symbionts. A study from 2008 found it to be associated with coral brown band disease on the Great Barrier Reef (Bourne et al. 2008).

In *T. ignis,* the ciliates *Epistylis* and *Zoothamnium* were identified. *Zoothamnium* is a colonial genus of ciliate that is generally found in warmer waters such as the Red Sea, the Gulf of Mexico, and tropical Atlantic (Ehrenberg and Ehrenberg 1838; Bright et al. 2014). It has a symbiotic relationship with a sulfur-oxidizing bacteria, *Candidatus Thiobios zoothamnicoli* and often occurs in sulfur-rich environments like mangroves (Bright et al. 2014). It forms white feather-like colonies from the sulfur-oxidizing bacteria on its surface (Pflugfelder et al. 2005; Himmel et al. 2009; Gruber-Vodicka et al. 2011). *Epistylis* is species of freshwater ciliate belonging to the class Oligohyemophorea and many species are parasites to fish (Esch et al. 1976; Pádua et al. 2016) and crustaceans (Turner et al. 1979; Romero and Jiménez 1997; Ma and Overstreet 2006) in marine estuarine, and freshwater environments (Utz and Eizirik 2007).

4.4.2 Primer design and efficacy

The primer sets chosen for these studies are remarkably well-conserved among eukaryotic organisms, so much so that getting high numbers of host reads is quite common. In traditional microbiome studies, 16S rRNA gene primers are designed to pick up on the prokaryotic sequences with only an occasional co-amplification of organelle DNA (Walker et al. 2020). Primers designed for the eukaryotic ITS and 18S rRNA are well conserved (Gray et al. 1984; Khot et al. 2009) between the host and microbial eukaryote groups causing more amplification of non-targeted sequences. A current strategy is to filter out the host and organelle sequences post sequencing. This results in a serious depletion of data depending on the sample and may not fully capture the microbial communities (Pereira-Marques et al. 2019).

4.4.2.1 Strategies to overcome host read contamination

Other strategies such as PCR clamping, which involves the use of a PCR blocking primer for non-target sequences, may be a way around this co-amplification to improve community characterization for microbiome (Lefèvre et al. 2020b) and mycobiome (Ikenaga et al. 2016) applications, with some success seen in plants. Primer efficacy depends on the region amplified and the length. For instance, with ITS, it is possible to amplify the whole ITS region (ITS1/ITS4) (Bazzicalupo et al. 2013; Bengtsson-Palme et al. 2013), the ITS1 region (ITS1/ITS2), the ITS2 region (ITS3/ITS4) (Manter and Vivanco 2007, p.1; Rivers et al. 2018, p.2) and the 5.8S rRNA in between. A pre-extraction strategy includes the use of a density gradient with Nycodenz (Hevia et al. 2015), cesium chloride (Mäkelä and Hildén 2018), Ficoll (Kim et al. 2016), etc. This is a common strategy in microbiome studies (Lindahl and Bakken 1995; Aakra et al. 2000), but may lend some bias, particularly with how a sample might be preprocessed. There might be cellular debris or particulate material in the layers of a density gradient that affects recovery of microorganisms and desired cells may be destroyed in the process(Barra Caracciolo et al. 2005).

4.4.3 Other Strategies for Sequencing for micro-eukaryotes

A drawback to this study is the lack of seawater samples, due mostly to a failure to amplify or loss of sample in transport. To begin to identify which groups might be a part of the sponge rather than a transient microbe, a seawater sample would have been needed. For the *L. chondrodes* and *Haliclona* sp., seawater from the source would have been welcome in addition to tank seawater. As it was, upon collection of the *L. chondrodes* sample, it had been moved from its original tank into a holding tank roughly 24 hours before collection. A negative control should also have been sequenced. Again, this was due to a lack of amplification and almost no detectable DNA on a nanodrop or Qubit. In 16S rRNA gene studies, kit contamination can skew results, particularly for those organisms or environments that have not been well studied. A kit mycobiome has yet to be performed and should be as environmental molds and yeasts are also common contaminants in more than just samples.

4.4.4 Concluding Statements

Microeukaryote communities in seawater are poorly studied and many of the microbial communities, particularly those of low microbial abundance sponges, tend to be transient microbes from the surrounding water. Both the *L. chondrodes* and *Haliclona* sp*.* samples came from aquariums, so there was no environmental water sample for comparison. The *L. chondrodes*, in particular, was taken from its original aquarium and held in a different tank before being collected for sampling. Another caveat it that there was no environmental sample of the sponge so determining if the detected communities are part of the overall sponge microbiome, or if they are a result of the sponge being kept in captivity is challenging.

In terms of diversity, *Tedania ignis* was the only one that showed any significant changes in diversity and observed ASVs. *Haliclona sp.* and *Lendenfeldia chondrodes* do not show any significant changes. Some of this can be explained by the small sample size. A larger sample size would show if this were more likely to be a true result. The material for these sponges was in limited supply, having come from two tourist aquariums in the United Kingdom.
Finally, there has been a call from the microbial ecology community to sequence the kits being used. Despite careful work by different companies to keep things sterile, some microorganisms still survive the process (Salter et al. 2014; Czurda et al. 2016; de Goffau et al. 2018), something that can be seen in prokaryotic microbiome studies. In addition, subsequent handling by the investigator that could lead to contamination of the kit(Weiss et al. 2014). While not conducted for this experiment, a kit blank sample should be used for further experiments to account for communities that may not actually be present in the sponge.

Supplementary Data

Supplementary Table 1-Collospongia auris ASVs pre and post filtering for Porifera reads

SampleID	Prefilter ASV count	Postfilter ASV count
CU12A	14224	58
CU12B	4092	187
CU13A	138	138
CU13B	2183	2183
CU14A	10810	9856
CU14B	46367	2020
CU15A	33700	21927
CU15B	70344	1197

Supplementary Table 2-Haliclona sp. ASVs pre and post filtering for Porifera reads

SampleID	Prefilter ASV count	Postfilter ASV count
CU16A	13064	12627
CU16B	160	160
CU17A	20976	20976
CU17B	50456	38309
CU18A	85050	85050
CU18B	9356	8292
CU19A	2964	2964
CU19B	2947	2021
CU20A	965	638
CU20B	838	838

Supplementary Table 3-Tedania ignis pre and post filtering for Porifera reads

SampleID	Prefilter ASV count	Postfilter ASV count
Ted2-0202cDNA	23017	29
Ted2-0202gDNA	30892	0
Ted2-0703cDNA	31114	36
Ted2-0703gDNA	79586	14
Ted2-1403cDNA	64795	106
Ted2-1403gDNA	40994	0
Ted2-2504cDNA	23760	99
Ted2-2504gDNA	37079	26
Ted2-2803cDNA	42661	424
Ted2-2803gDNA	23121	1343
Ted2-2902cDNA	57748	13
Ted2-2902gDNA	41599	7
Ted3-0202cDNA	33522	46
Ted3-0202gDNA	31683	21
Ted3-0703cDNA	43955	0
Ted3-0703gDNA	32306	2
Ted3-1403cDNA	10208	7
Ted3-1403gDNA	7870	0
Ted3-2504cDNA	3094	10
Ted3-2504gDNA	3100	9
Ted3-2803cDNA	88981	81
Ted3-2803gDNA	338010	94
Ted3-2902cDNA	7670	10
Ted3-2902gDNA	0	0
Ted5-0202cDNA	44297	35
Ted5-0202gDNA	100644	102
Ted5-0703cDNA	0	0
Ted5-0703gDNA	36516	0

Ted5-1403cDNA	21922	15
Ted5-1403gDNA	12761	12
Ted5-2504cDNA	8412	23
Ted5-2504gDNA	8593	47
Ted5-2803cDNA	35547	11
Ted5-2803gDNA	13318	2
Ted5-2902cDNA	21366	38
Ted5-2902gDNA	18235	30
Ted6-0202cDNA	16079	15
Ted6-0202gDNA	38750	22
Ted6-0703cDNA	1202	27
Ted6-0703gDNA	13264	3
Ted6-1403cDNA	16270	258
Ted6-1403gDNA	16221	55
Ted6-2504cDNA	34446	73
Ted6-2504gDNA	12545	23
Ted6-2803cDNA	14565	9
Ted6-2803gDNA	14584	0
Ted6-2902cDNA	385677	320
Ted6-2902gDNA	34764	182

5.0 METAGENOMIC ANALYSIS OF THE MARINE SPONGE, *CINACHYRELLA KEUKENTHALI*

5.1 INTRODUCTION

Three species of *Cinachyrella* are found throughout the tropical Caribbean in shallow coral reef waters >5 meters). It is a yellowish-orange globular sponge reaching a diameter of 10 cm. *C. apion* is found near mangrove areas and typically reaches about 4cm in diameter*. C. keukenthali* is a large sponge (10-20cm diameter) and often found on deeper reefs (2-18m) (Cárdenas et al. 2009). It is often difficult to distinguish *C. keukenthali* from *C. alloclada* except under careful observation of the microscleres but incorporating analysis of the microbial communities in the sponge is another viable way of distinguishing the two. *C. alloclada* tends to be less diverse than *C. keukenthali* and both only share about 95 OTUs (Cuvelier et al. 2014a).

The abundance of microbial communities in *Cinachyrella* varies with different species. Species like *Cinachyrella alloclada* are considered high microbial abundance (HMA) while others within the genus *Cinachyrella* are considered to be low microbially abundant (LMA) (Moitinho-Silva et al. 2017). Species of *Cinachyrella* are common to shallow, tropical waters and there may be upward of 4-6 different species (Schuster et al. 2017a).

There are currently few studies on the microbiota of *Cinachyrella* sp. from south Florida and the Caribbean where it is abundant (Cárdenas et al. 2009; Cuvelier *et al*. 2014b), with most studies focusing on overall gene expression in *Cinachyrella* sp*.* in response to perturbations in the environment (Smith 2013). More current research on *Cinachyrella* sp. is focused on its use as a non-model organism (Lopez et al. 2014; Voolstra et al. 2017). In Desplat et al. (Desplat et al. 2023), a related species, *Cinachyrella alloclada*,

was exposed to Macondo oil and dispersant from the Deepwater Horizon oil spill and assessed for changes in gene expression pre- and post-exposure as *Cinachyrella* is a globally distributed sponge that can be kept well in aquaculture and is a marker of reef health in Florida where the sponge was collected. Another study on *Cinachyrella* sp. has focused on the “holo-transcriptome” as removal of non-sponge DNA is challenging (Desplat et al. 2021). Currently, the available kits are designed for model organisms such as human and mouse and are based host DNA methylation (Feehery et al. 2013). The methylation of sponge DNA is currently unknown.

Based on preliminary 16S rRNA gene sequencing data, it appears that *Cinachyrella* sp. splits into two distinct groups based on their microbiome (Cuvelier et al. 2014b). Group1 tends to have a lower diversity of bacteria, dominated by alpha proteobacteria. Group2 is of higher diversity and contains more of the rare microbiota. Between the two groups, there are 631 shared OTUs (12 archaeal and 627 bacterial). The Archaea belong to two phyla and the bacteria belong to 12. Both groups had 95 OTUs in common with 30.5% belonging to Group 1 and 22.4% to Group 2.

Cinachyrella keukenthali is one of the sponges actively being sequenced as part of the Global Invertebrate Genomics Alliance (GIGA) (Lopez et al. 2014), whose aims are to collaborate with groups working on invertebrate genomics and overcome challenges such as specimen selection and sequencing. It is currently on a list of priority sponge specimens (Voolstra et al. 2017) for this project given its ability to be maintained in long term aquaculture experiments (Smith 2013). Scientists within the GIGA consortium have found that the sponge subclass Spirophorina, of which *Cinachyrella* belongs, are hotspots for group I introns, many of which are thought to have originated in fungi (Schuster et al. 2017b).

Cinachyrella keukenthali has been poorly studied with regards to its chemotaxonomy. However, the similar species, *C. apion* and *C. alloclada*, have been found to contain a

type of steroid, 6-hydroximino-4-en-3-one steroids, that contain an oxime group, which has not been previously reported in marine sponges and show cytotoxicity against human cancer cell lines (Rodríguez et al. 1997; Cui et al. 2008). In a related species on the Great Barrier Reef, *Cinachyrella enigmata*, a metabolite called enigmazole A was first isolated in 2010 (Oku et al. 2010). This molecule is of particular interest as it interferes with c-Kit signalling pathways in cells. Briefly, the c-Kit aids in cell survival, proliferation and differentiation of healthy cells (Liang et al. 2013; Stankov et al. 2014). This c-Kit can be overexpressed in cancer and enigmazole A acts as a barrier to the cell signalling. Other compounds from *Cinachyrella* sp. include mitogenic lectins (Atta et al. 1989), a group of molecules that bind to T-cell receptors and stimulate the production of interleukin 1 and interleukin 2 (Kilpatrick 1999) which play important roles in inflammation (Kaneko et al. 2019). A PCR-independent taxonomic and functional profile will be generated for *Cinachyrella keukenthali* to identify a baseline observation of the complete microbial makeup (bacteria, archaea, and eukaryotes) and functional genes without the bias attributed to PCR-based microbiome.

5.2 METHODS

5.2.1 Sponge Collection

Samples of *Cinachyrella keukenthali* were collected by the Gilliam laboratory at NSU Halmos College of Natural Sciences and Oceanography on 20/11/2015 and kept in a mariculture setup for 3 days before storage. Briefly, a piece of tissue was cut and stored in RNALater at 22°C (referred to as CRL) and the rest was snap frozen. Before shipping to Cardiff, the snap-frozen piece was transferred into RNALater (referred to as CFRL) to account for accidental sample thawing. Upon arrival, samples were immediately stored at -80°C until DNA extraction was ready to be performed.

5.2.2 DNA Extraction and Purification

DNA was extracted from these sponges using two methods, 1) QIAgen DNEasy kit (Qiagen, Inc., Hilden, DE), 2) CTAB extraction. Up to 30mg and 50mg of tissue was cut from the sponge and soaked 1X TE buffer while it equilibrated to room temperature and

rinse off the storage buffer for the DNEasy and CTAB extraction respectively. The samples were spun at 14000 x g for 5 minutes to pellet the tissue. The TE/buffer supernatant was discarded before beginning the DNEasy extraction. 100ug/µL of RNAse A was added to the sample and vortexed. The tissue was extracted according to the manufacturer's Instructions. DNA was extracted using a CTAB/PVP buffer. Briefly, the CTAB recipe consisted of 5mL 1M Tris, pH 8.0, 2 ml 0.5M EDTA, pH 8.0, 4.15g NaCl, 1g CTAB, 1g PVP. The mixture was heated to 65°C. For the CTAB extraction, the sample was added to 500µL of 65°C CTAB/PVP buffer and macerated using a pellet pestle. Another 500µL of CTAB/PVP was added and the sample was vortexed briefly to mix. 10% beta-mercaptoethanol and 40 uL of QIAGEN proteinase K were added and the samples and placed 65°C heat block until the sample was completely lysed. Samples were centrifuged and pelleted at 14,000 x g for 5 minutes and the supernatant was transferred to a fresh microcentrifuge tube. One volume of chloroform was added to the sample and vortexed until an emulsion formed. Samples were spun at 14,000 x g for 10 minutes. The top layer was transferred to a new tube. 250µL of 1X TE was added to the original samples and back extracted again with chloroform and combined in the previous tube. One volume of room temperature isopropanol was added to the sample and kept overnight at -20°C. Samples were isopropanol precipitated with room temperature molecular grade isopropanol followed by washes with 70% molecular grade ethanol. The samples were resuspended in QIAGEN EB and treated with 100ug/ul of Rnase A. Treatment with Rnase A in both DNA extraction protocols is utilized to remove the stable RNA that gets co-extracted with genomic DNA.

The samples were loaded onto a 2% w/v LMP TAE gel and run out for one hour at 40V. High molecular weight bands (over 10,000bp) were cut out with sterile scalpel blade and put through a GELase High Activity protocol. Post-digestion, the samples were ethanol precipitated using 0.1M sodium acetate, 0.7 volumes of room temperature isopropanol, rinsed twice with 70% v/v ethanol, and resuspended in Qiagen EB buffer (pH 8.5). The resulting DNA was quantified on the Qubit 2.0 fluorometer.

5.2.4 Sequencing Library Preparation

DNA, 500ng, was prepared using the NextFlex Rapid DNASeq kit with a PCR-free step. The libraries were quantified using the QIAGEN QiaSeq NGS Library Quant assay kit. Before loading, the libraries were checked using the Agilent TapeStation. Samples were prepared using the NextSeq500 preparation protocol from Illumina on a High-Output 350 cycle cartridge.

5.2.5 Sequence Analysis

Raw sequences were first run through Trim Galore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to remove sequencing adaptors using the default auto-detect feature. A Bowtie2 (Langmead and Salzberg 2012) index was made using a phiX sequence downloaded from NCBI (NCBI Reference Number NC_001422.1). Sequences from both the MiSeq and NextSeq500 were filtered together with Bowtie2 against the index and combined for each sample. Only the reads that did not align with the phiX index were written out. Filtered forward and reverse reads were uploaded along with sample metadata to MG-RAST (Meyer et al. 2008). Sequences are available in MG-RAST under project IDs MGM4775061.3 and MGM4775062.3. Raw fastq files are available from the ENA under accession number PRJEB28367.

5.3 RESULTS

Sample CFRL contained 42,579,879 total sequences prior to quality checking with an average sequence length of 175 base pairs. 3,955,201 sequences from this data did not meet the default quality checking parameters of MG-RAST and were excluded from the analysis. Of these, excluded reads, 2,985,466 were identified as artificial duplicates. 126,293 of the passed reads contained ribosomal RNA (rRNA) genes and 16,184,694 contained proteins with known functions. The remaining 18,502,572 reads contained proteins of unknown function. Further details on sequence analysis are in Table 5.1.

Table 5.1- Sequence statistics from MG-RAST for *Cinachyrella keukenthali* in Frozen RNALater (CFRL) Statistics from the results of MG-RAST analysis showing the number of reads pre- and post-quality checking for the *Cinachyrella keukenthali* sample that was previously snap frozen and then stored in RNALater for shipment. Additionally, the numbers of sequences that matched to protein and ribosomal RNA features are also noted.

Cinachyrella Frozen RNALater Only Sequence Statistics	
Upload: bp Count	7,449,331,221 bp
Upload: Sequences Count	42,579,879
Upload: Mean Sequence Length	175 ± 49 bp
Upload: Mean GC percent	56 ± 13 %
Artificial Duplicate Reads: Sequence Count	2,985,466
Post QC: bp Count	6,821,610,718 bp
Post QC: Sequences Count	38,624,678
Post QC: Mean Sequence Length	177 ± 49 bp
Post QC: Mean GC percent	55 ± 13 %
Processed: Predicted Protein Features	22,788,423
Processed: Predicted rRNA Features	194,739
Alignment: Identified Protein Features	4,314,889
Alignment: Identified rRNA Features	4,203
Annotation: Identified Functional Categories	3,317,179

Sample CRL contained 34,393,495 sequences prior to quality check with an average length of 166 base pairs. 3,355,154 sequences failed quality check and of these, 2,261,507 were artificial duplicates. For the sequences that passed, 416,501 contained ribosomal RNA sequences, 12,760,523 sequences are proteins of known function, and 14,135,310 sequences are proteins that identify to no known function. Further details are found in Table 5.2.

Table 5.2-Sequence statistics from MG-RAST for *Cinachyrella keukenthali* RNALater only (CRL) Statistics of the *Cinachyrella keukenthali* sample stored in RNALater containing the subsequent QC results and in addition, the number of alignments to protein and ribosomal RNA features

Cinachyrella RNALater Only Sequence Statistics	
Upload: bp Count	5,714,554,724 bp
Upload: Sequences Count	34,393,495
Upload: Mean Sequence Length	166 ± 48 bp
Upload: Mean GC percent	57 ± 14 %
Artificial Duplicate Reads: Sequence Count	2,261,507
Post QC: bp Count	5,216,107,759 bp
Post QC: Sequences Count	31,038,341
Post QC: Mean Sequence Length	168 ± 48 bp
Post QC: Mean GC percent	57 ± 13 %
Processed: Predicted Protein Features	17,079,924
Processed: Predicted rRNA Features	413,393
Alignment: Identified Protein Features	2,967,197
Alignment: Identified rRNA Features	3,071
Annotation: Identified Functional Categories	2,297,048

These samples contain similar taxonomic profiles, representative of the same individual. Briefly, the make-up of both samples at the domain level is approximately 50-60% Bacteria, 20-30% Eukarya, 4-6% Archaea, and 0.2% unclassified sequences (Figure 5.1).

Alpha diversity profiles for both samples are also similar with 794 species present in the sample stored solely in RNALater and 795 species in the sample that had first been frozen then moved to RNALater.

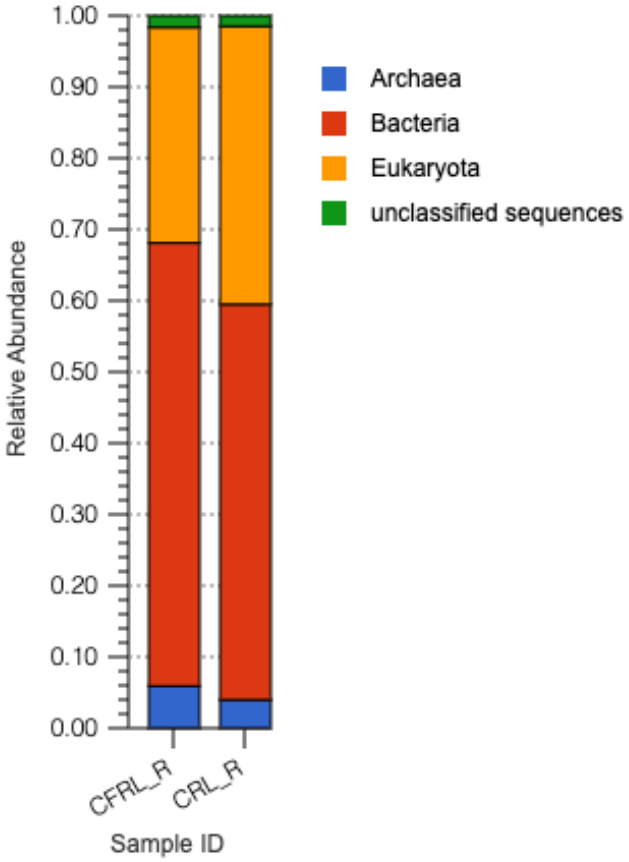


Figure 5.1-Domain makeup of samples CFRL and CRL. As noted previously, the majority of the taxa present are bacteria at 63% and 55%, followed by eukaryotes at 30% and 40%, and archaea at 6% and 4%, respectively. The remaining 1% are unclassified. The taxonomy data was generated using the SILVA SSU (rRNA) database available in MG-RAST.

Cinachyrella keukenthalli contains a large number of prokaryotic sequences. Using the SILVA small subunit database, it was determined that the top taxa, excluding unclassified sequences, in both samples of *Cinachyrella keukenthali* were Proteobacteria (26-33%), class Actinobacteria (14-18%), and Candidatus *Poribacteria* (17-25%) (Figure 5.2). The presence of Ca. *Poribacteria* likely indicates that this is a high microbial abundance (HMA) sponge, as HMA sponges tend to have Ca. *Poribacteria* present (Bayer et al. 2014). What is unusual is the lack of Chloroflexi, as it is an indicator of an HMA sponge in addition to Ca. *Poribacter* (Schmitt et al. 2011; Schmitt et al. 2012; Bayer et al. 2014).

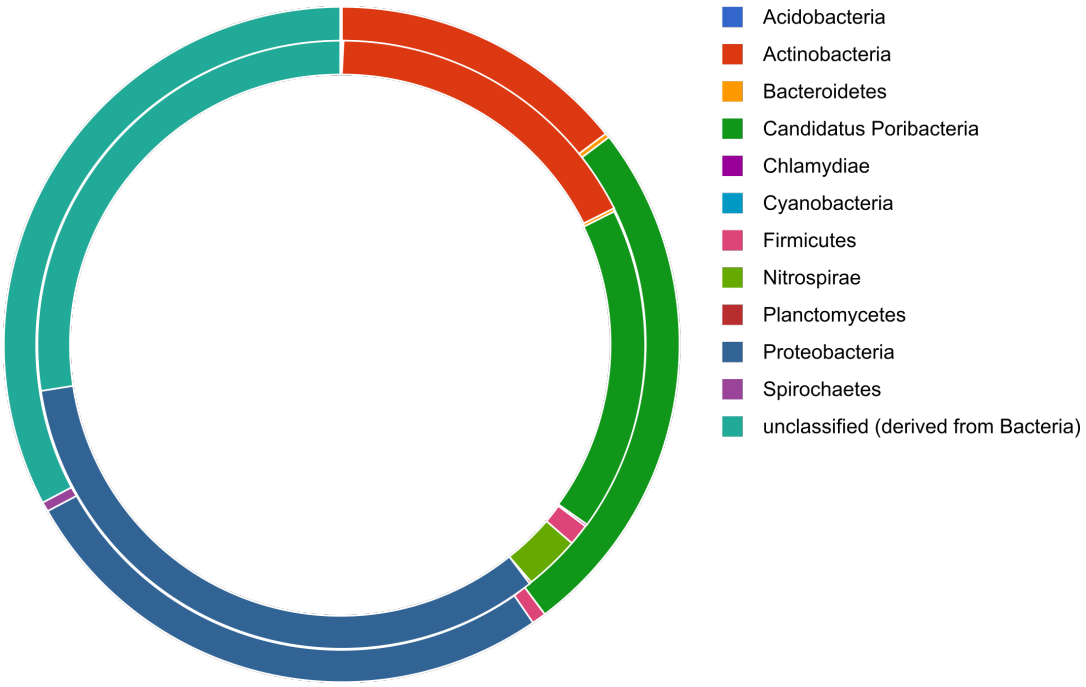


Figure 5.2-Circle graph of dominant bacterial phyla present in both samples of *Cinachyrella kuekenthali*. Both samples contain a similar taxonomic makeup of bacteria at the phylum level with the dominant phyla being Proteobacteria, Cyanobacteria, Candidatus Poribacteria, and Actinobacteria. With the presence of high levels Candidatus Poribacteria in both species of *Cinachyrella*, it is possible to speculate that this is likely *Cinachyrella kuekenthali*, a high microbial abundance sponge.

Among the Proteobacteria, most sequences were made up of Gammaproteobacteria (80-81% of Proteobacteria) with the remainder consisting of Deltaproteobacteria (8-10%),

Alphaproteobacteria (6-7%), and Betaproteobacteria at a much lower percentage (0.6-0.7%) based on rRNA genes detected with the Silva SSU database in MG-RAST.

The archaea present in *C. keukenthali* were of the phylum Thaumarchaeota mostly belonging to the orders of Cenarchaeales and Nitrosopumilales (72-77%). Sequences belonging to the genus *Crenarchaeum* (23-28%) match up most to *Crenarchaeum symbiosum* (23-28%). Nitrosopumilales sequences belong to *Nitrosopumilus maritimus* (17-21%) and *Candidatus Nitrosopumilus sp*. NM25 (55-57%) (Figure 5.3).

Crenarchaeum symbiosum is a unique archaeal species to sponges (Preston et al. 1996; Margot et al. 2002). It belongs to the phylum Thaumarchaeota, however, it grows at temperatures 60°C below the normal cultivation of other Thamarchaeota. The presence of *C. symbiosum* and the *Nitrosopumilus* species are expected and fits previous findings of other Thaumarchaeota in sponges (Simister et al. 2012; Fiore et al. 2013; Hardoim and Costa 2014).

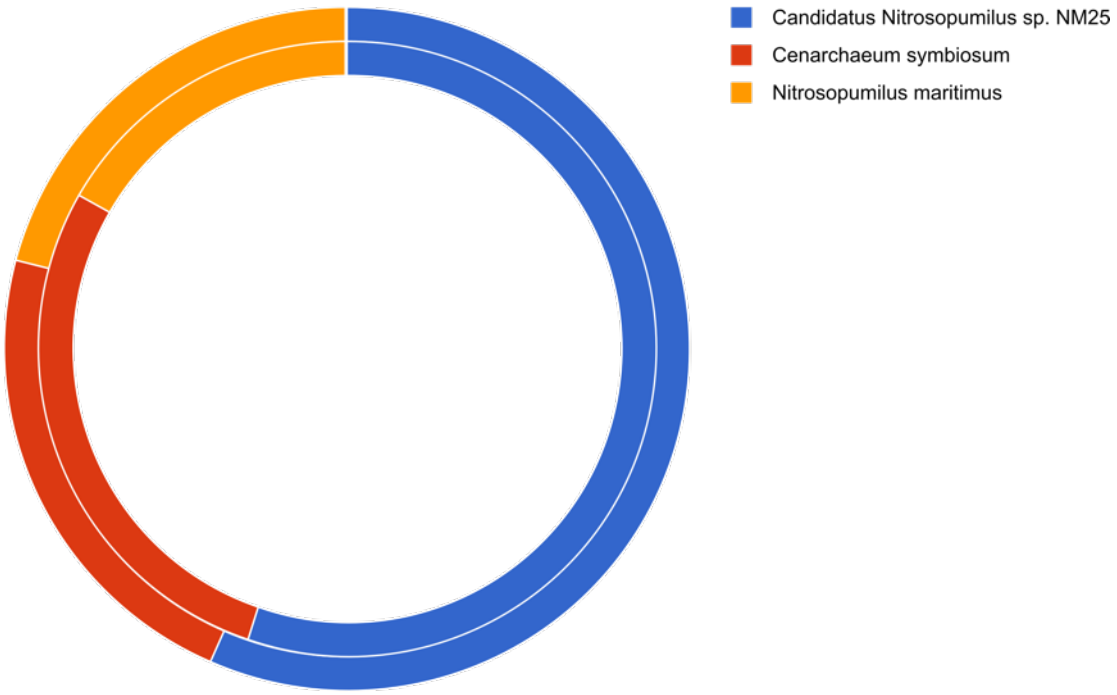


Figure 5.3-Archaeal taxa of *Cinachyrella keukenthali*. Three archaeal species dominate *C. kuekenthali*. *Crenarchaeum symbiosum* is a symbiont of sponges that belongs to the Thaumarchaeota, first found in marine sponges.

Upon further analysis of the eukaryotic sequences to the species level in MG-RAST, it was found that the sequences match up closest to *Cinachyrella keukenthali* and *C. apion* and share similarity with other sponges (97-100%). Also of note, 2% of the sequences in CFRL matched up to the phylum Chytridimycota, a group of mostly aquatic, parasitic fungi. Other unclassified eukaryotic organisms present, but in extremely low numbers.

Using KEGG Orthology (KO), both samples have similar hierarchal profiles. Sample CRL generated 3,707,798 functional genes, and CFRL generated 4, 798,741 functional genes. The most genes in each sample correlate to metabolism consisting of about 60% of the total reads for each sample, followed by genetic information processing at 20%, environmental information processing at 15%, and low levels of cellular processes and organismal systems at under 5% each. Cellular processes encompass genes responsible for motility, cell to cell communication, transport/catabolism, and regulation of growth and

death. In environmental information processing, genes relate mostly for membrane transport and signal transduction. This allows the organism to take cues from the environment and respond. For bacteria something like a change in pH or the addition of an outside compound allow it to respond in ways like making a biofilm or reduce/increase their metabolic output. Further analysis of genetic information processing shows an abundance of translation genes, followed by replication and repair genes, folding, sorting, and degradation genes, and transcription pointing to active microbial growth and proliferation within the sponge. Among these, genes responsible for metabolism were highest in abundance. Metabolism includes a large variety of genes, particularly those for metabolising amino acids, carbohydrates, cofactors and vitamins, and nucleotide metabolism. Metabolic genes influence how energy flows and is recycled and adaptation and resilience in the sponge. Low levels of other genes were detected such as those for xenobiotic biodegradation, metabolism of polyketides and terpenes, and other secondary metabolites. (Figure 5.4).

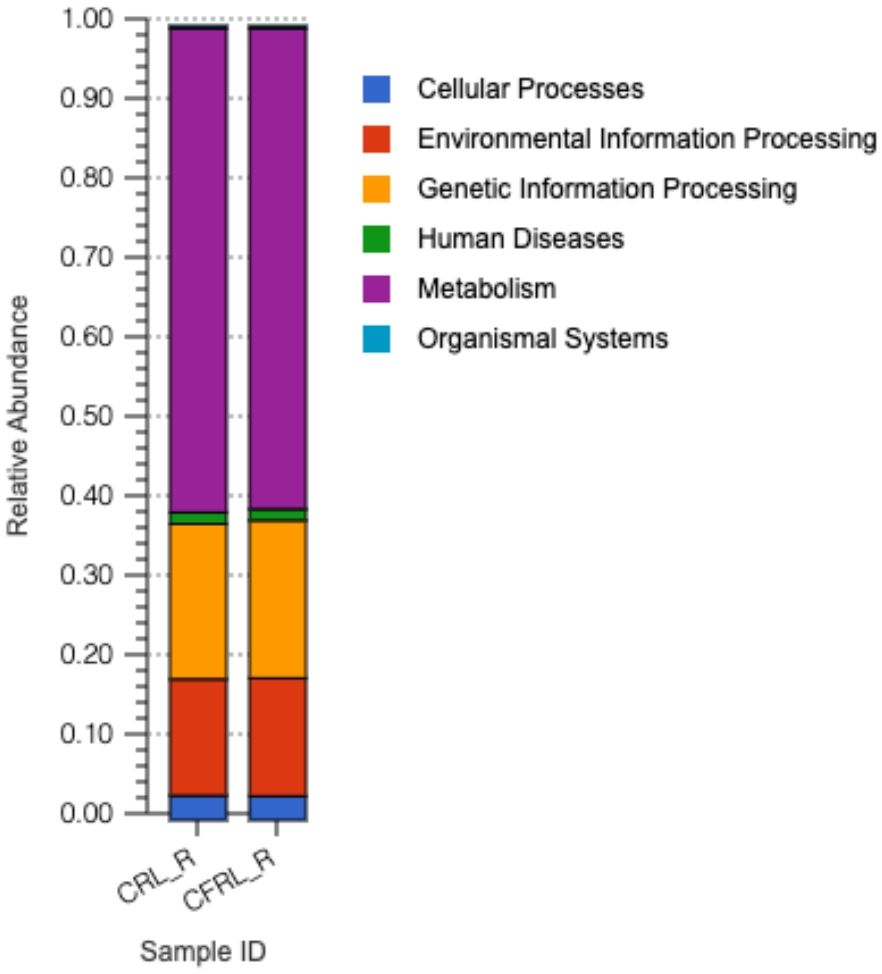


Figure 5.4-Relative abundance of KEGG Orthology (KO) genes at level 1 within Cinachyrella keukenthali Metabolism genes are the highest in abundance between both samples. Metabolic genes are important not only for the basic functions within the sponges, but also to produce secondary metabolites.

Further analysis shows diverse groups of metabolic genes within *Cinachyrella keukenthali*. The group of metabolic genes in highest abundance are for amino acid metabolism, followed by carbohydrates, energy, cofactors and vitamins and nucleotides. In lower numbers, lipid metabolism, metabolism of secondary metabolites, terpenoids and polyketides, and xenobiotics are also present (Figure 5.5).

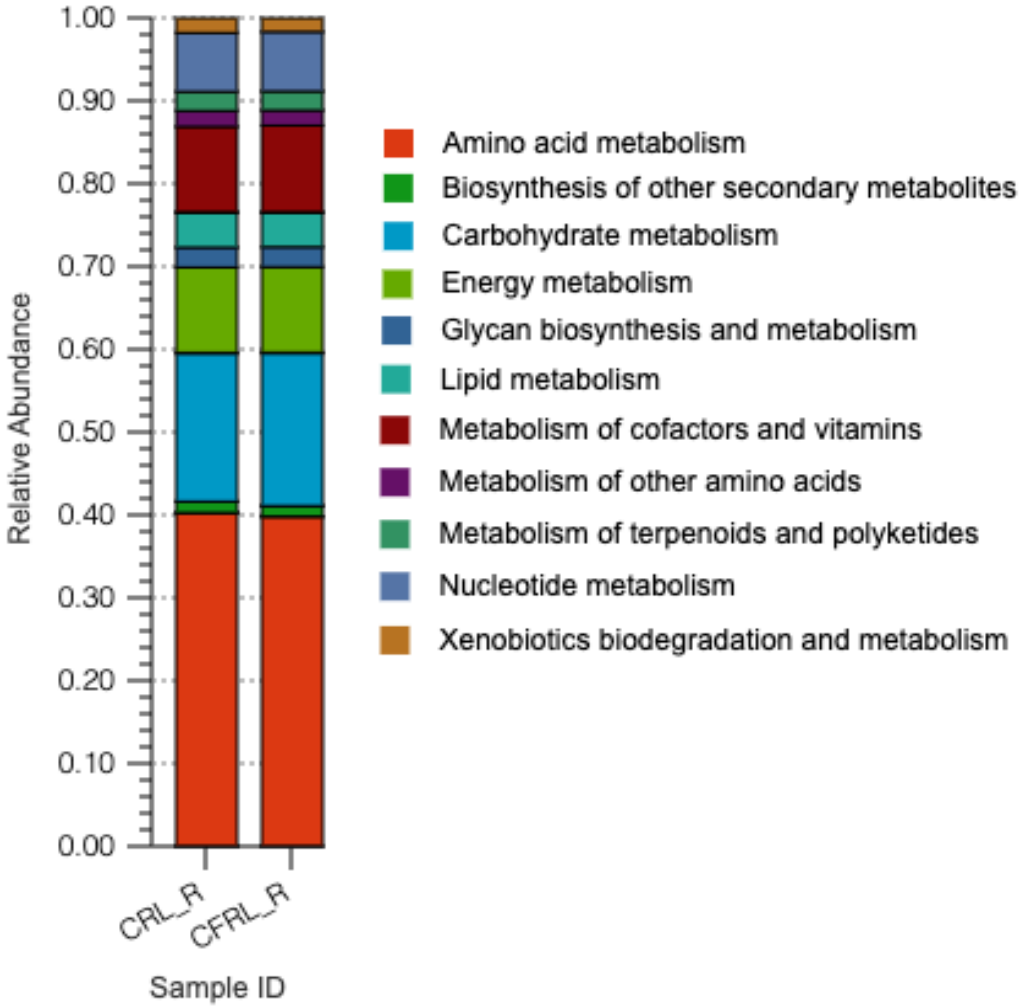


Figure 5.5-Level 2 KEGG Orthology of metabolism genes from *Cinachyrella keukenthali* High levels of amino acid, carbohydrate, energy, and cofactors and vitamins are found, serving as important components to life of the sponge. To a lesser extent, genes for lipid metabolism, biosynthetic genes like terpenoids and polyketides, xenobiotics, and others are shown to exist in low abundance (<5%).

Terpenoids and polyketides are important molecules produced by the sponge and its symbionts. They serve as protective chemicals to protect from disease and predators. Among the genes found for polyketides and terpenoids, those in highest abundance are the genes for geraniol degradation and terpenoid backbone biosynthesis.

5.4 DISCUSSION

Cinachyrella keukenthali is a common sponge in the shallow waters of the tropical Atlantic. Its small size makes it amenable to mariculture. Due to these features, *C. keukenthali* was selected to be one of many sponges to be sequenced as part of the Global Invertebrate Genome Alliance (GIGA). In addition to understanding it from a genomic perspective, *C. keukenthali* is an important sponge for understanding reef dynamics and has potential for use in blue biotechnology.

C. keukenthalli is dominated by bacterial taxa, *Candidatus Poribacteria*, Actinobacteria, and Proteobacteria, predominantly Gammaproteobacteria based on the Silva SSU results from MG-RAST. In Cuvelier et al. (Cuvelier et al. 2014) the microbial communities contained enough divergent classes to put the study sponges into two groups, SG1, containing Actinobacteria, Alphaproteobacteria, and Creanarchaeota and SG2, containing Ca. Poribacteria, archaeal phylum Thaumarchaeota, Gammaproteobacteria, and Chloroflexi. Overlap was found for both of these groups of sponges with the shotgun metagenome of this study, suggesting that this sponge could be part of its own group, or potentially, there may be some differences due to PCR bias with the 16S rRNA primers (Abellan-Schneyder et al. 2021). The biggest case is the small sample size as it is not reflective of the sponge as a species, but rather the sponge as an individual. More samples are required to truly draw these or other conclusions.

Most of the functional genes in the sponge belong to metabolic genes which play a role in how the sponge and its symbionts process different nutrients. Sponges are important nutrient recyclers and the abundance of metabolic genes, particularly that of amino acid and carbohydrate metabolism. Hanz et al. (Hanz et al. 2022) determined this via stable isotope analysis and comparing HMA sponges to LMA sponges. HMA sponges were determined to have a profile similar to that of basal food web organisms, suggesting the symbionts play a role in utilizing resources that higher trophic level organisms cannot. In contrast, LMA sponges show similarities to organisms of higher trophic levels. Similar

results were found in Caribbean sponges, wherein the HMA sponges had higher metabolic capacities compared to LMA sponges (Lesser et al. 2022)

In addition, smaller groups of important genes have also been identified such as those that produce terpenoids and polyketides, and for other bioactive molecules. *Cinachyrella* sp. have previously been identified as producing some biotechnologically relevant compounds such as galectins, which have potential for cancer treatment, steroidal compounds, and enigmazole A (Atta et al. 1989; Rodríguez et al. 1997; Cui et al. 2008; Oku et al. 2010).

While this chapter covers a general overview of the distribution of taxonomy and functional profiling of the reads from *C. keukenthali*, future studies should incorporate assembly and binning of the metagenomic data. Metagenomic binning is a way to pull individual bacterial genomes from a metagenomic library. Information obtained from these libraries include not only the taxonomic identifier, but also the groups of functions for that species of bacteria or archaea. Further, to determine the compounds and identify genes for those compounds, a metabolomic library would yield metabolic information about the sponge and to match biosynthetic gene pathways with a compound, a functional metagenomic library would be needed. Previous experiments in soil libraries have demonstrated this, but the caveat is finding the correct host as the host can affect how the gene is expressed, or it could be toxic to the heterologous host(Gillespie et al. 2002; Santana-Pereira et al. 2020).

6.0 AN ASSEMBLY-INDEPENDENT METAGENOMIC ANALYSIS OF THE WELSH SPONGE, *HYMENACIDON PERLEVIS*

6.1 INTRODUCTION

Sponges are filter feeding organisms that comprise a variety of marine and freshwater habitats. Each species contains a set of core and unique, rare microbiota (Thomas et al.

2016; Moitinho-Silva et al. 2017) with varying levels of richness, abundance, and diversity. These microbiota drive functions both within the sponge and the surrounding environment (Hentschel et al. 2006; Erwin et al. 2012; Hentschel et al. 2012; Thomas et al. 2016).

Sponge microbiota are known to have a variety of functions. One of the main functions is driving host adaptability (Rosenberg et al. 2007) and the cycling of nutrients within the sponge and even providing food to the sponge (Thacker 2005; Erwin and Thacker 2007; Erwin and Thacker 2008). In other instances, sponges and their symbionts produce bioactive molecules meant to deter (Armstrong et al. 1999; Kennedy et al. 2008; Esteves et al. 2013; Santos-Gandelman et al. 2014) predators (Bakus et al. 1986; Pawlik et al. 2002; Paul and Ritson-Williams 2008).
These same defensive molecules are also of importance to biomedicine (Radjasa et al. 2011; Wilson et al. 2014). Of interest are the metabolites produced by sponges and their symbionts (Schmitz et al. 1984; Gunasekera et al. 1990; Kenjiro Inaba et al. 1998) for the development of novel antibiotics (Bringmann et al. 2003; Muscholl-Silberhorn et al. 2008; Kennedy et al. 2009; Phelan et al. 2012), anti-cancer drugs(Schmitz et al. 1984; Gunasekera et al. 1990; Zheng et al. 2006; Costantino et al. 2009; Bhimba et al. 2013), and other biotechnological applications (Kennedy et al. 2007; Kennedy et al. 2008; Esteves et al. 2013; Santos-Gandelman et al. 2014).

Hymeniacidon perlevis, formerly *H. perleve*, is a common species found in the rockpools an intertidal and subtidal zones of the United Kingdom and Europe (Alex et al. 2012; Mahaut et al. 2013). It is characterized by its bright orange-red color on rocks and a yellow colour when submerged in the subtidal zone (Alex et al. 2012). *H. perlevis* has been shown to serve as an indicator of water quality in a study by Mahuat and colleagues in 2013 as it was shown to accumulate the highest concentration of contaminants from surrounding water. In addition to its ability to accumulate chemical contaminants in seawater, *H. perlevis* can function as an indicator of microbial pollution. In a 2010 study by Longo et al., *H. perlevis* was found growing in the wastewater pipes

for a land-based fish farm. Analysis of the sponge tissue showed an accumulation of the microbial pollution from the wastewater into the sponge tissue, indicating that some of the bacteria were not digested as a food source for the sponge. Overall, it shows the utility of this sponge for bioremediation purposes (Longo et al. 2010; Longo et al. 2022) and lays some groundwork for using sponges in general for bioremediation. Further studies of *H. perlevis* have also indicated that its microbial symbionts can accumulate and degrade other pollutants, such as the potent organophosphate lindane (Aresta et al. 2015), from the surrounding waters.

Hymeniacidon perlevis has been previously investigated for bioactive compounds such as hymenialdisine, an imidazole alkaloid (Meijer et al. 2000; Zheng et al. 2006; White et al. 2012). It also exhibits kinase inhibition activity (White et al. 2012). Further, oxime derivatives of this molecule were investigated as a potential cancer treatment. Downstream process showed that it did not inhibit cell growth but did exhibit an anti-proliferative effect. Hymenialdisine is isolated from other species of sponge such as *Axinella sp*. and S*tylissa carteri* (O'Rourke et al. 2016), and its origin is from the sponge cells, specifically the spherulous cells (Song et al. 2011). Other examples of natural products isolated from different sponge species include manzamine A, an alkaloid first described from an Okinawan *Haliclona* sp. (Sakai et al. 1986), has also been identified in *Acanthostrongylophora ingens* (Zhang et al. 2008), *Pachypellina sp.* (Ichiba et al. 1994), , and *Ircinia sp.* (Kondo et al. 1992). and halichondrin b , a macrolide, first described from a Palauan *Haliclona* sp. (Pettit et al. 1994), and also in *Phakellia carteri* (Pettit et al. 1993), and a Lissodendoryx sp.(Litaudon et al. 1994). Other compounds from *H. perlevis* include norharman (Zheng et al. 2006), a beta-carboline alkaloid derived from a symbiotic *Pseudoalteromonas piscicida* strain and spongiacidins A-D (Kenjiro Inaba et al. 1998) (Figure 6.1). The information on the sponges and metabolites came from the LOTUS natural products database which reference from PubChem (Kim et al. 2025) and compiles organisms associated with a compound (Rutz et al. 2022).

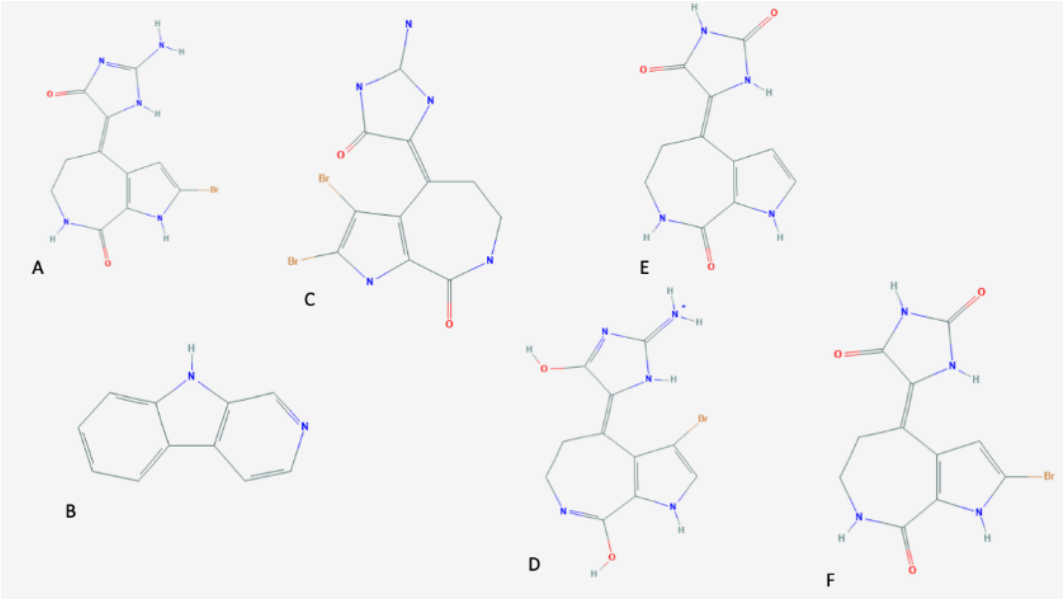


Figure 6.1-Compounds derived from *Hymeniacidon perlevis*;
A) hymenialdisine; (B) norharman; (C) Spongiacidin A; (D) Spongiacidin B; (E) Spongiacidin C; (F) Spongiacidin D.

H. perlevis contains groups of genetically diverse cyanobacteria consisting mostly of *Synechococcus*, but in recent research, *H. perlevis* was found to also contain groups of *Xenococcus* sp.-like cyanobacteria (Alex et al. 2012) and *Acaryochloris* sp., a group of cyanobacteria previously only found in ascidians (López-Legentil et al. 2011; Alex et al. 2012). In a related species, *H. heliophila*, it was found that there were differences in the microbial composition based on the location (subtidal vs. intertidal) of the sponge. Specifically, Weigel and Erwin observed changes in the relative abundance of dominant taxa and presence/absence of rare microbiota (Weigel and Erwin 2016).

Other groups of bacteria found in *H. perlevis* are the Alphaproteobacteria, Gammaproteobacteria which occur at the highest proportion in the sponge, while other groups, such as the Actinobacteria and the Bacteroidetes have also been shown to occur in the sponge, albeit at much lower proportions (Alex et al. 2013).

In this study, I will investigate the microbial composition and functional potential of *H. perlevis* and its symbionts. This study aims to 1) identify the microbial symbionts in

Hymeniacidon perlevis using MG-RAST (Meyer et al. 2008), 2) determine potential functions of the sponge symbionts, 3) show the proportions of other microbial groups (Fungi, Protists, Viruses) in the sponge, and 4) begin to unravel the complex interactions of sponges and their symbionts.

6.3 METHODS

6.3.1 Sponge Collection

Two specimens of *Hymenacidion perlevis*, were collected in December of 2016 from exposed tidal rocks in Porthcawl, Wales (Figure 2) and placed into DESS buffer (20% DMSO, 0.1M EDTA, sodium chloride saturated). Samples were transported at room temperature, and stored for 24 hours at 20°C before final storage at -80°C.

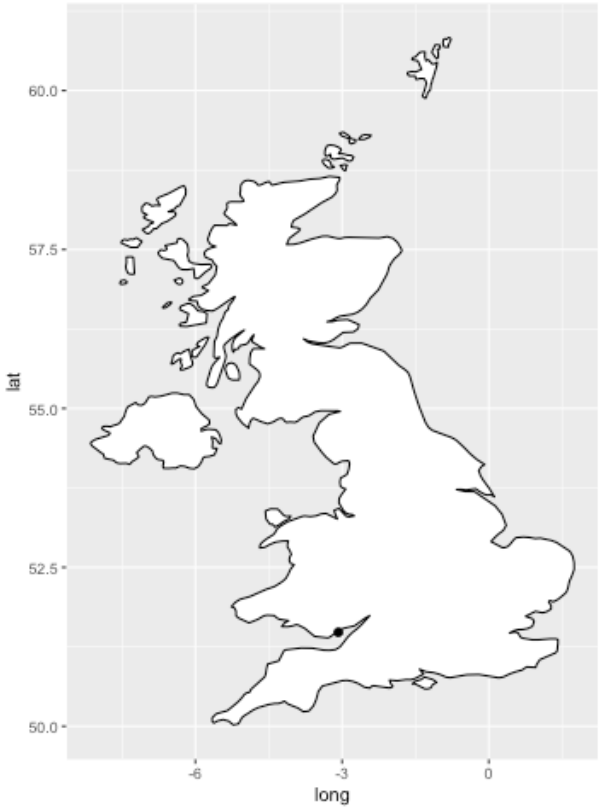


Figure 6.2-Sampling site location. Samples were collected at low tide from rockpools around Porthcawl Beach (noted with the black dot) and transported back to Cardiff, Wales at room temperature before being stored at -20°C.

6.3.2 DNA Extraction

DNA was extracted from these sponges using the Qiagen DNEasy kit, 2) CTAB extraction. For the DNAEasy extraction, two 30-50mg pieces of tissue for each extraction method, were cut from the sponge and soaked in 1X TE buffer while it equilibrated to room temperature. The samples were spun at 18000 x g for 5 minutes to pellet the tissue. The TE/RNALater supernatant was discarded before beginning the extractions. 100 µg/µl of RNAse A was added to the sample and vortexed. The tissue was extracted according to the manufacturer's instructions. The samples were loaded onto a 2% LMP TAE gel and run out for one hour at 40V. High molecular weight bands were cut out and put through a GELase High Activity protocol. Post-digestion, the samples were ethanol precipitated. The resulting DNA was quantified on the Qubit 2.0 fluorometer.

6.3.3 Preparation of libraries for shotgun metagenomics

DNA (500ng) was prepared for sequencing libraries using the NextFlex Rapid DNA_Seq kit with a PCR-free step. The libraries were quantified using the QIAGEN Qia-Seq NGS library Quant assay kit (Qiagen, Inc., Hilden, DE). Before loading, the library size was checked using the Agilent TapeStation (Agilent Technologies, Santa Clara, CA). Sequencing libraries were denatured and diluted using the NextSeq500 preparation protocol from Illumina on a High-Output 350 cycle cartridge.

6.3.4 Sequence Analysis

Raw and trimmed samples were uploaded to MG-RAST (Meyer et al. 2008) along with metadata. The raw sequences are available under sequence IDs mgm4821890.3, mgm4821893.3, mgm4821892.3, and mgm4821891.3. The trimmed samples are available under MG-RAST IDs mgm4821894.3 and mgm4821889.3. Profiling in MG-RAST constituted utilising the RefSeq database available through MG-RAST to compare whole genomes. For functional profiling, the eggnog (Huerta-Cepas et al. 2019) database was used.

6.3.5 Taxonomic Profiling in Kaiju

For taxonomic profiling in Kaiju (Menzel et al. 2016), the Illumina adapters were removed from the raw reads using Trim_Galore. Following, the PhiX (NCBI: NC_001422.1) genome was indexed using Bowtie2 and used to filter out any contaminating PhiX sequences. The filtered and trimmed reads were uploaded to the Kaiju webserver and analysed using the default parameters. Sequences were uploaded and analysed as paired ends Results were generated directly from the kaiju web server. The sequences were run against a kaiju database that contained the entire non-redundant protein database which contains proteins from bacteria, archaea, and viruses in RefSeq and microbial eukaryotic groups (nr +euk). Bubble plots at the phyla level were generated by the kaiju server. Each taxonomic group is represented by a bubble and larger bubbles indicate that more sequences identify with that particular taxon. While there are different bubbles representing the same phyla, each bubble represents further divisions within that phylum.

6.4 RESULTS

6.4.1 Kaiju Profiling

In addition to MG-RAST analysis, unassembled reads were run using kaiju as a preliminary view of the metagenome. Briefly, the adapter trimmed and PhiX filtered reads were uploaded to the kaiju webserver using the default parameters. In the first sample, 4,120,110 of 24,731,326, or 16.7% of the reads were classified. In the second sample, 3,859,409 of 41,637,762, or 9.27%.

6.4.1.2 Bacterial groups detected by kaiju

In the SP3 (Figure 6.3), there were 51,172 reads of unclassified Proteobacteria. Proteobacteria form a large part of the core microbiome in *H. perlevis* SP3. In addition to the unclassified Proteobacteria, there were also high numbers of Alphaproteobacteria (73,077 reads), *Achromobacter* (61,597), *Delftia* (44,562), and Rhodobacteriaceae (41,714). For unclassified Bacteria, there were 74,137 reads identified. From there, other species detected were *Candidatus Entotheonella* TSY-1 (206,326) and TSY-2 (12,030). Further, a group of bacteria only previously detected in metagenomic datasets from geothermal springs, *Candidatus Kryptonia* (19,789) were also detected.

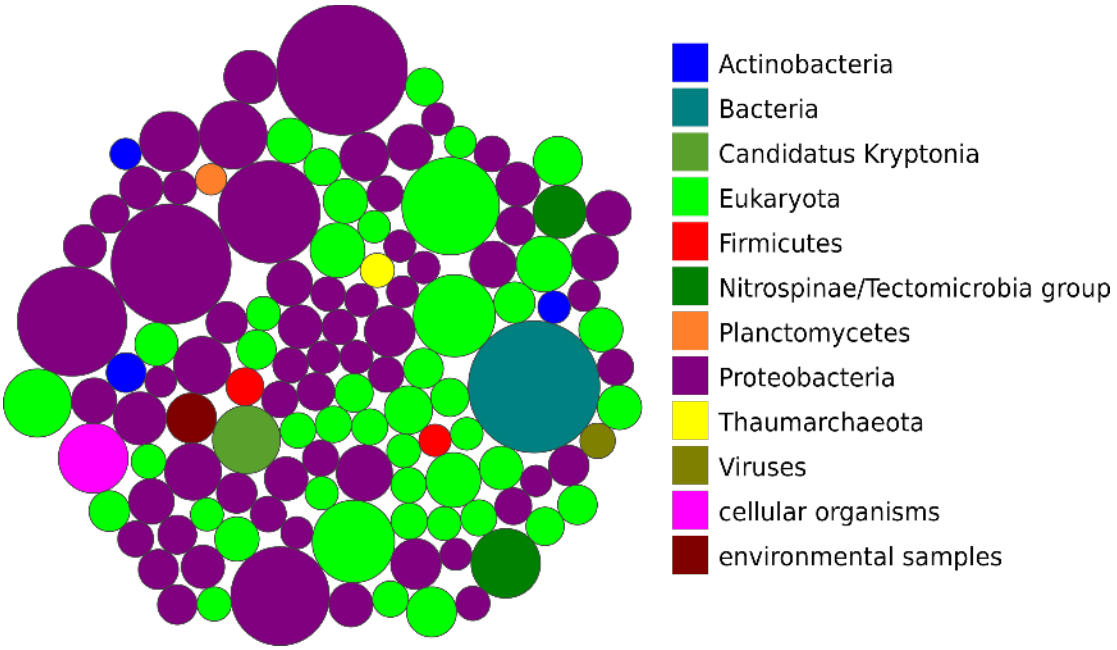


Figure 6.3- Kaiju NR + eukaryotes profile of unassembled (raw) reads in *Hymeniacidon perlevis* SP3. The bubbles represent binned reads. The size of the bubble is scaled to the number of reads detected for the different phyla. Cellular organisms and environmental reads are reads that did not resolve well when compared to the database.

In SP5 (Figure 4), a similar profile was detected. 35,763 reads were classified as being unclassified Proteobacteria. Reads identified as Alphaproteobacteria (55,722), Achromobacter (25,147), and Rhodobacteraceae (31,638) were also detected. For the unclassified bacteria, 64,629 reads were detected. Similar to SP3, Candidatus Entotheonella TSY-1-like sequences (26,940) and Tsy-2-like sequences (16,083) were also detected.

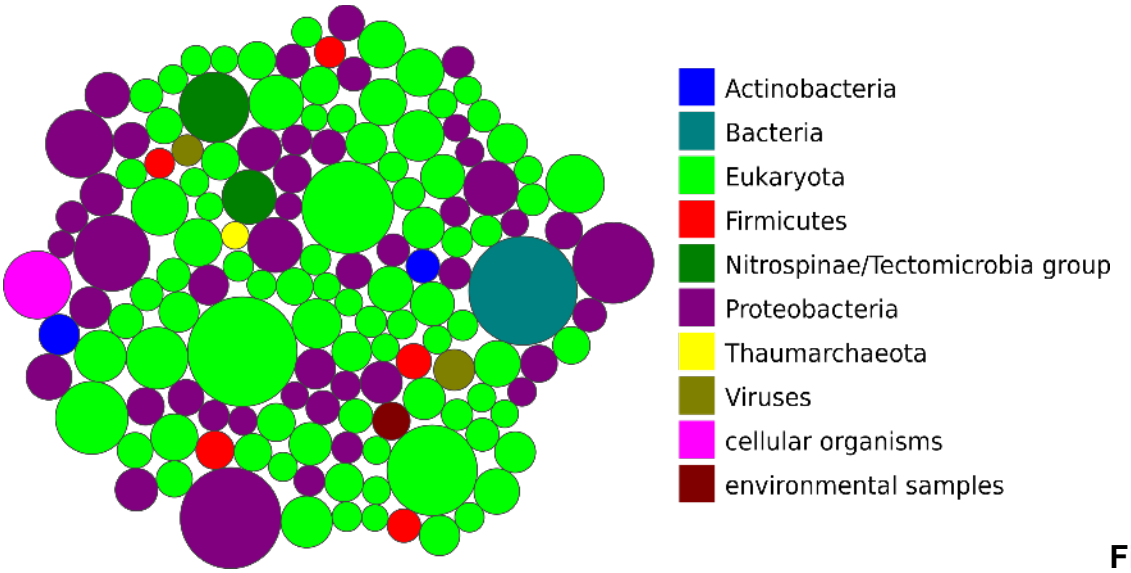


Figure 6.4- Kaiju NR + eukaryotes profile of unassembled (raw) reads in *Hymeniacidon perlevis* SP5. Between the two samples, two groups in SP3 are absent in SP5, Candidatus Kryptonia and Planctomycetes.

Kaiju uses protein level sequence classification and searches for exact matches to proteins in the reference genomes of each database. When matches are found, kaiju outputs the taxonomic identifier or determines the last common ancestor (Menzel et al. 2016) to that sequence, hence all of the different bubbles. Some can be different kingdoms or phyla or since kaiju uses protein level classification, it could be matches to different proteins in the reference genomes. Curiously, cyanobacterial reads were not detected in the kaiju dataset, despite this phylum normally being found in high numbers in this sponge species. Also of note, are the number of Eukaryota reads present in each sample, more so in SP5 than SP3.

6.4.1.3 Archaeal groups detected with kaiju

For archaeal sequences, only 5,024 reads were classified. These sequences matched to *Nitrosopumilus* sp. A similarly low number, 4,144 reads, were detected in the SP5. The viral component in each sample was low. In SP3, 5,388 reads were identified as uncultured Mediterranean phages. A similar profile was detected in the SP5.

6.4.1.4 Eukaryotic groups detected with kaiju

Eukaryotic sequences were among some of the highest detected sequences. In general, unclassified eukaryotes were detected for 28,843 reads in sponge sample 1 and 44,828 sequences in SP5. The next highest eukaryotic group were the unclassified Fungi with 19,878 reads identified in sponge sample 1 and 28,956 identified reads in SP5. In SP3, 10,783 reads matched to the fungus *Serendipitta vermifera* strain MAFF305830. This was not seen in SP5.

Other important eukaryotes detected in each sponge sample matched to a group called the choanoflagellates. In sponge SP3, 40,702 reads matched to *Salpingoeca rosetta* and 29127 to *Monosiga brevicollis* MX1. In SP5, 65,845 reads matched to *Salpingoeca rosetta* and 46,519 to *M. brevicollis* MX1.

There is an absence of poriferan reads in both datasets post-processing in kaiju. The sequences were analyzed using the nr+euk database generated and provided by kaiju. The nr database is a subset containing Archaea, Bacteria, and Viruses. Additionally, to address the eukaryotes, there is a nr + euk pre-generated database consisting of the subset nr database with microbial eukaryotes and fungi. Mentioned earlier, of the sequences analyzed, only 16.7% of SP3 and 9.27% of SP5 were classified. The remaining sequences are likely the host reads. When checked in MG-RAST, the number of reads identified as Porifera is low. The figure shows the eukaryotic annotation based on the MG-RAST default setting of 60% identity. This was important to show the sequence identity based on MG-RAST as it will allow connection of taxa to different functions. To verify the accuracy of this result, subsets of the reads identified as Porifera were downloaded MG-RAST. Non-porifera eukaryotic reads were also downloaded for comparison. The sequences were analyzed with BLAST using the megablast setting (Altschul et al. 1990; Morgulis et al. 2008). The reads identified as Porifera were came back as *Hymeniacidon perlevis.* A portion of the non-poriferan sequences also came back as *Hymeniacion perlevis.*

6.4.5 MG-RAST Results

Sequences were subsequently analysed using MG-RAST (Meyer et al. 2008) which is a public platform for metagenomic and metatranscriptomic studies. MG-RAST gives an assembly-independent overview of microorganisms in a sample. MG-RAST performs an internal quality check and measures basic sequencing statistics and generates profiles of metagenomic data.

6.4.5.1 Sequence Statistics

A total of 41,446,274 sequences were uploaded into MG-RAST for sample SP3 (Table 6.1). Post quality checking in MG-RAST, which consists of adapter trimming with Skewer (Jiang et al. 2014), denoising (Aronesty 2013), and the removal of sequencing artifacts/duplicates (Gomez-Alvarez et al. 2009). Sequence duplicates and artifacts are removed to prevent an overestimation of taxon diversity and functional genes. It is important to note that the sequences processed were pre-trimmed prior to being run on MG-RAST. This pre-processing resulted in the loss of 5,485,043 sequences, or a 13.23% loss of sequences post-QC. In SP5 (Table 6.2), a total of 60,619,728 sequences were initially uploaded and processed in the same manner. Quality checking resulted in a loss of 8,514,084 sequences or a 14.05% loss.

Table 6.1- Sequence statistics from MG-RAST for *Hymeniacidon perlevis* (SP3) Sequence statistics showing the change in the number of sequences in SP3 pre- and post-QC. No identified functional categories were identified in this sample.

Hymeniacdion perlevis specimen 1 Sequence Statistics	
Upload: bp Count	6,709,336,602bp
Upload: Sequences Count	41,446,274
Upload: Mean Sequence Length	162 ± 41 bp
Upload: Mean GC percent	43 ± 11 %
Artificial Duplicate Reads: Sequence Count	4,435,377
Post QC: bp Count	5,892,346,608 bp
Post QC: Sequences Count	35,961,231
Post QC: Mean Sequence Length	164 ± 40 bp
Post QC: Mean GC percent	43 ± 10 %
Processed: Predicted Protein Features	22,216,590
Processed: Predicted rRNA Features	572,735
Alignment: Identified Protein Features	2,495,955
Alignment: Identified rRNA Features	23,389
Annotation: Identified Functional Categories	Unknown

Table 6.2- Sequence statistics from MG-RAST for *Hymeniacidon perlevis* (SP5) Sequence statistics showing the number of reads pre- and post- quality filtering in MG-RAST.

***Hymeniacdion perlevis* specimen 2 Sequence Statistics**	
Upload: bp Count	10,217,516,413 bp
Upload: Sequences Count	60,619,728
Upload: Mean Sequence Length	169 ± 47 bp
Upload: Mean GC percent	39 ± 9 %
Artificial Duplicate Reads: Sequence Count	7,388,367
Post QC: bp Count	8,910,406,687bp
Post QC: Sequences Count	52,105,644
Post QC: Mean Sequence Length	171 ± 47 bp
Post QC: Mean GC percent	39 ± 9 %
Processed: Predicted Protein Features	27,627,603
Processed: Predicted rRNA Features	485,317
Alignment: Identified Protein Features	1,781,092
Alignment: Identified rRNA Features	22,823
Annotation: Identified Functional Categories	Unknown

6.4.5.2 Taxonomic Profile

MG-RAST provides a more in-depth view of metagenomic samples using the complete RefSeq database wherein the relationship between the number of host reads can be seen compared to the metagenome. A comparison of three different methods (kaiju, MG-RAST, and a subset of megablast searches) was performed to cross validate the MG-RAST findings, highlight any discrepancies and attempt to capture a broader

diversity in the reads and attempt to determine why some expected bacterial groups were in low numbers or non-existent.

In SP3, a total of 6,312,380 and 5,280,745 sequences were classified using RefSeq. Overall, most of the sequences in both samples were comprised of high levels of bacteria (80% and 66%) . Of these bacterial sequences, Proteobacteria, comprising 68% and 56% respectively, were identified as the highest occurring bacterial phylum in both samples. Further broken down, Alphaproteobacteria were found to comprise 64% and 71% of the total Proteobacteria, followed by Gammaproteobacteria 11% and 9% and Betaproteobacteria 14% and 11%, respectively. Other important phyla such as the Actinobacteria make up 3% and 2% of the total bacterial sequences, followed by Firmicutes at 3% for both samples and cyanobacteria at 2% for both samples. A comparison of RefSeq to kaiju and MEGABLAST results were to confirm taxonomy. It is likely that the taxonomic resolution may be inaccurate due to short reads, and at broader levels, like the domain, the reads would be more accurate in identifying the organismal group.

In addition to the bacterial sequences, archaea (Figure 6.5) were found to occur at extremely low abundance in each sample (0.8% and 0.6%). Despite these low numbers, a few interesting groups were still identified. The identified sequences belong to three major archaeal groups, Thaumarcheota (44% and 35%), Euryarchaeota (49% and 59%), and Crenarchaeota (6% both samples).

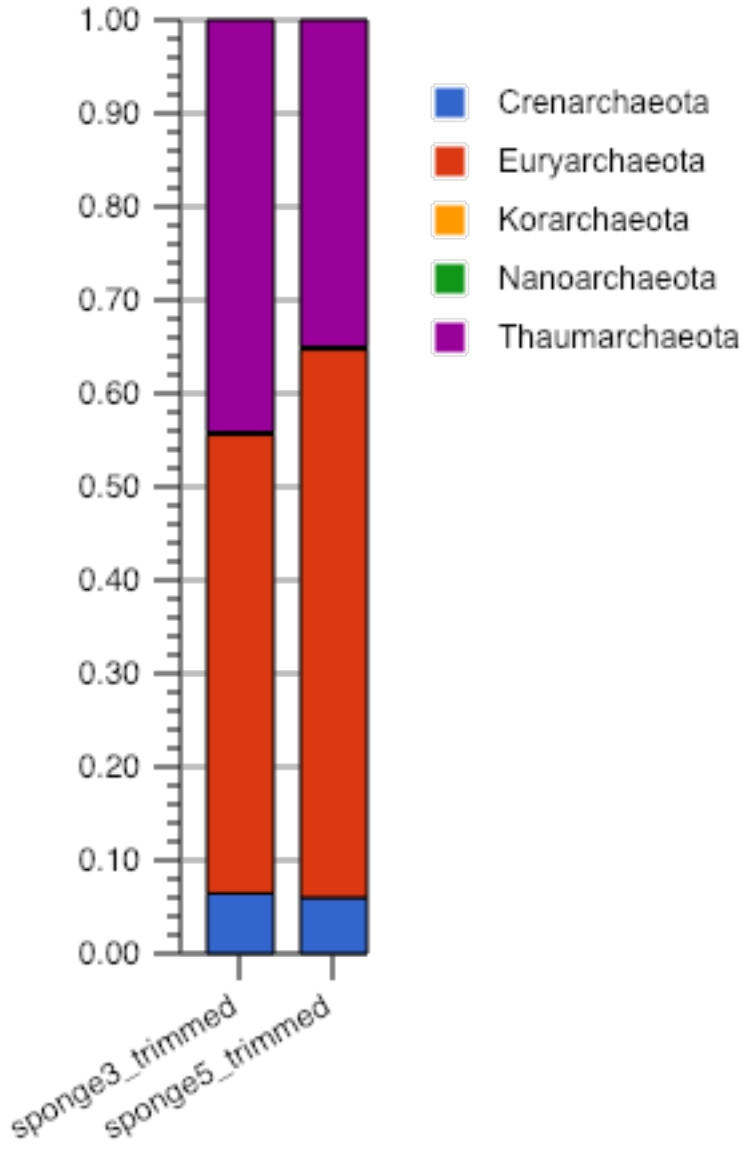


Figure 6.5-Archaeal phyla present in *Hymeniacidon perlevis*. Archaea are present in low numbers in *H. perlevis.*

Eukaryotic sequences have also been identified in each sample, making up 19% and 34% of the total sequences, respectively. Major groups identified in the eukaryotic sequences include chordates (39% and 41%), arthropods (15% and 16%), cnidarians (10% and 11%), and echinoderms (5% both samples) as the highest identified groups. As for the microbial eukaryotes such as fungi and plant-like organisms, these number are much lower with the fungal groups being split into two major groups, the

Ascomycota (Figure 6.6A) and the Basidiomycota (Figure 6.6B). Most other microbial eukaryotes are present at less than 1%. Porifera reads were identified as about 2% in both samples. Porifera sequences were run through MEGABLAST and identified as *Hymeniacidon* and similar species. An unusual number of Chordata were detected and checked with BLAST. Most sequences were determined to be unidentifiable, but some yielded results for various cnidaria and sponges. One reason for such discrepancy could be due to the nature of RefSeq used in MG-RAST versus something more sensitive like BLAST. RefSeq contains curated reference sequences with complete entries for genomes, chromosomes, contigs, and scaffolds (Haft et al. 2023; Goldfarb et al. 2024). This limits the size of the database, speeding up taxonomic identification. In contrast, BLAST uses the nucleotide (nt) database unless otherwise specified.

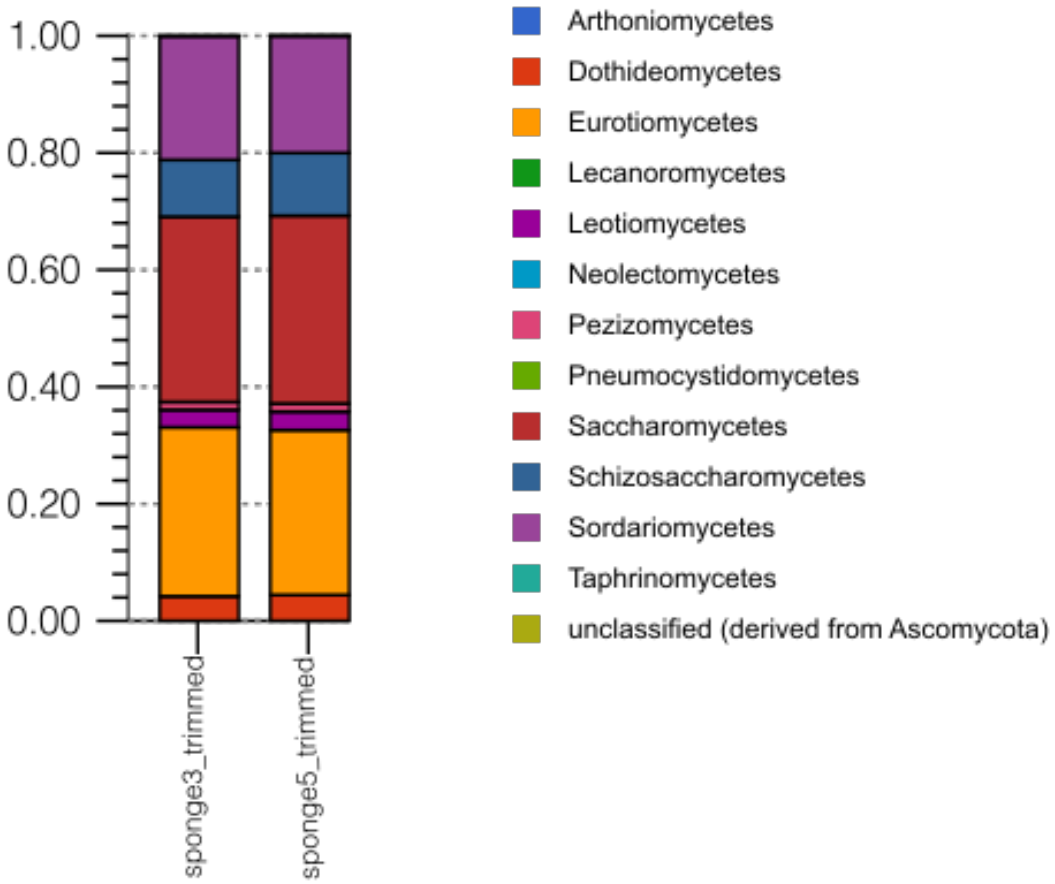


Figure 6.6A-Ascomycota fungi present in *Hymeniacidon perlevis*-Sequences were run against RefSeq and filtered within MG-RAST to get the Ascomycota groups. Total Ascomycota sequences make up 4% in SP3 and 3% in SP5. Euritomycetes, Saccharomycetes, Sordariomycetes, and Schizosaccharomycetes are present in higher numbers while, others such as Leotiomycetes and Pezizomycetes are present at much lower levels.

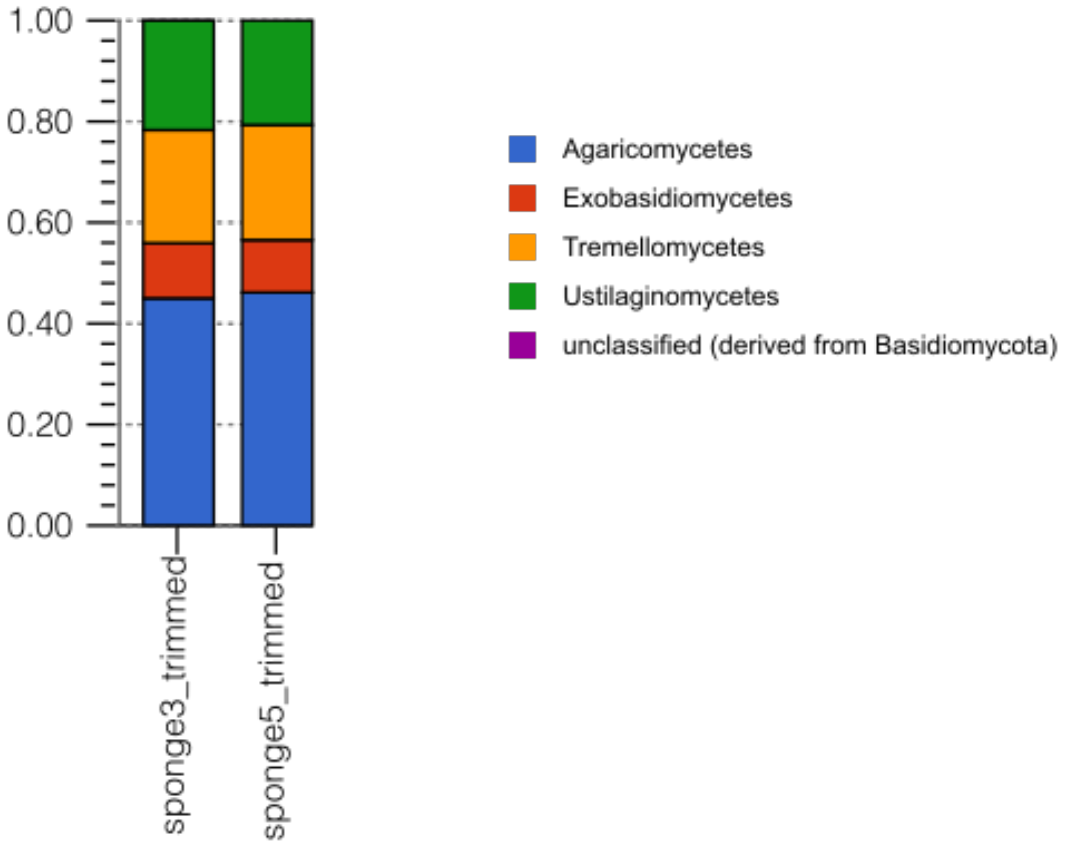


Figure 6.6B-Basidiomycetes groups within *Hymenicacidon perlevis* using RefSeq. Basidiomycota represents 0.1% of the fungal groups identified in both samples. Of the basidomycete groups, Agaricomycetes and Tremellomycetes are among the most dominant followed by Ustilaginomycetes and Exobasidiomycetes.

Also of importance to note are the low levels of viral sequences identified in each sample (Figure 6.7). Of the viral sequences, the majority identified with RefSeq belong to the unclassified viruses Phycodnaviridae (36% and 43%), Iridoviridae (7% and 6%), Baculoviridae (3% and 4%), Myoviridae (20% and 21%), Siphoviridae (14% and 10%), and Podoviridae (10% and 8%).

Figure 6.7-Viral sequences at the family level in *Hymeniacidon perlevis*. Viral sequences were matched from RefSeq. While viruses were found to be present in extremely small amounts (<0.01%), some sequences were able to be mined out and still show incredible diversity despite such low occurrence. Total viral sequences make up 0.1% in SP3 and 0.2% in SP5.

6.4.5 Functional Profile

Non-supervised Orthologous Groups (NOGs)

Non-supervised orthologous groups (NOGs) present in *H. perlevis* show similar profiles between each sample. Important to note are the overall distribution of NOGs between samples and the percentage of NOGs within each sample. On a general level, most NOGs remain poorly characterized with 62% of these NOGs identified in SP3 and 68% in SP5. Following this, metabolism NOGs make up the highest group of identified NOGs at 20% in SP3 and 18% in SP5. In SP3 the second highest identified NOGs were for information storage and processing at 9.2% followed by 8.5% in cellular processes and signaling. In SP5, cellular processes and signaling NOGs were identified at 8.6% followed by information storage and processing at 5.9%.

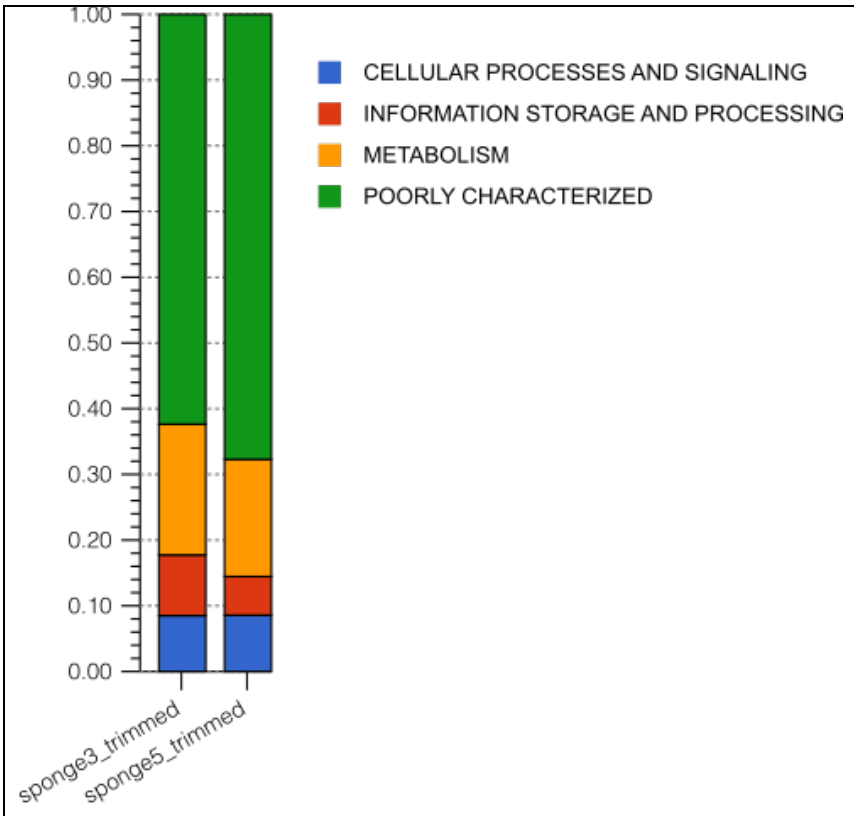


Figure 6.8 -Illustration of the level 1 NOGs present in each sample of *H. perlevis*. Most of the reads were not characterized well. Metabolism is the highest group of functions in the sponge.

Each sample showed high levels of genes coding for carbohydrate transport and metabolism, energy production and conversion, co-enzyme transport and metabolism and lipid transport and metabolism (Figure 6.9). Overall, the three highest metabolism NOGs identified were for energy production and conversion, carbohydrate transport and metabolism, and coenzyme transport and metabolism. Within SP3, energy production and conversion and carbohydrate transport and metabolism were among the highest occurring NOGs at 38% and 24% respectively. Lipid transport and metabolism was found to be the third highest occurring NOGs at 8.7% followed by amino acid transport and metabolism at 7.7%, inorganic ion transport and metabolism at 6.7% and secondary metabolite biosynthesis, transport and metabolism at 5.5%. In SP5, energy production and conversion and carbohydrate transport and metabolism were also among the highest occurring NOGs at 41% AND 36% respectively. Following,

coenzyme transport and metabolism occurred at 7.1% followed by lipid transport and metabolism at 5.2%, amino acid transport and metabolism 4.0% inorganic ion transport and metabolism at 3.6% and secondary metabolite biosynthesis transport and metabolism at 3.1%. Further filtering in MG-RAST found that Alphaproteobacteria in both samples had the highest amount of hits for metabolism genes at 1024 for sponge3_trimmed and 797 for sponge5_trimmed. Betaproteobacteria came up behind at 557 and 235 respectively and Gammaproteobacteria at 256 and 94 hits for both samples. Cyanobacteria had 286 hits in SP3 and 318 hits in SP5. In each group the dominant metabolism gene groups were for carbohydrate transport and energy production and conversion. The fungal group Ascomycota had hits for only carbohydrate metabolism at 62 and 74 hits in each sample. No viral functions were detected.

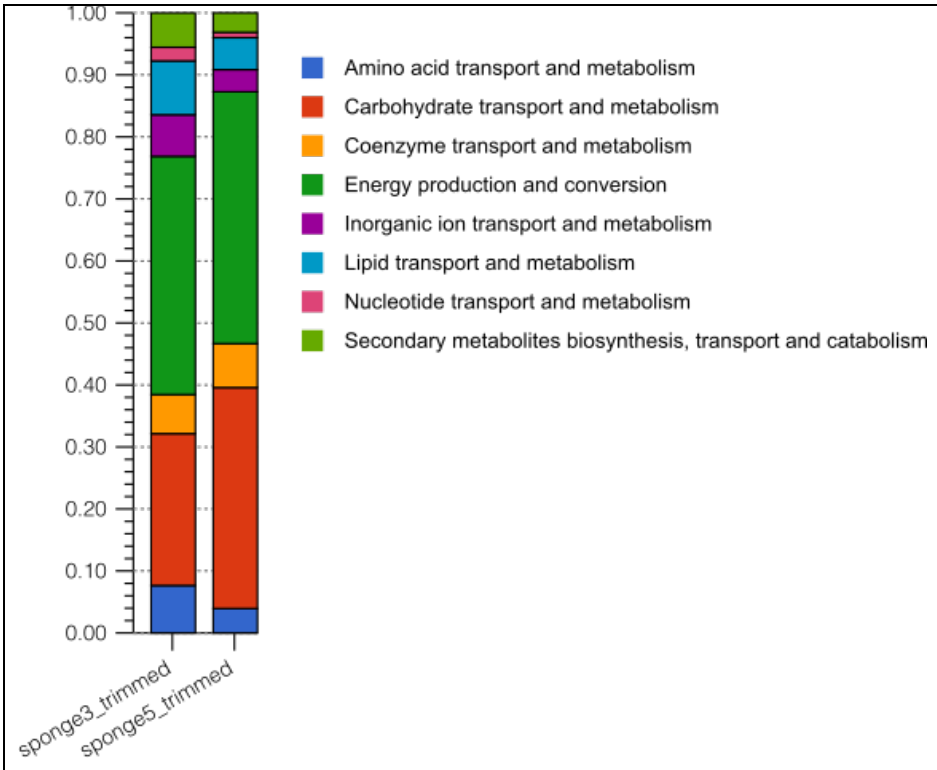


Figure 6.9-Metabolism NOGs present in each sponge sample. The top two between both encompass energy production and carbohydrate transport and metabolism. Each sample begins to deviate at this step with higher levels of lipid metabolism genes in SP3 than in SP5

The two highest groups found in information systems processing and storage were transcription and replication, recombination, and repair. In SP3, transcription NOGs occurred at 67% followed by replication, recombination and repair NOGs at 32%. For SP5, transcription NOGs occur at 59% and replication, recombination and repair NOGs occurred at 40%. Further refinement in MG-RAST showed that genes for information storage and processing were highest in betaproteobacteria at 103 hits in SP3 and 41 in SP5 and in gammaproteobacterial at 127 in SP3 and 49 in SP5. Further, 87 hits were identified in the alphaproteobacterial in SP3 while only 28 were identified in SP5. Other groups such as cyanobacteria and Ascomycota were found in much lower abundance.

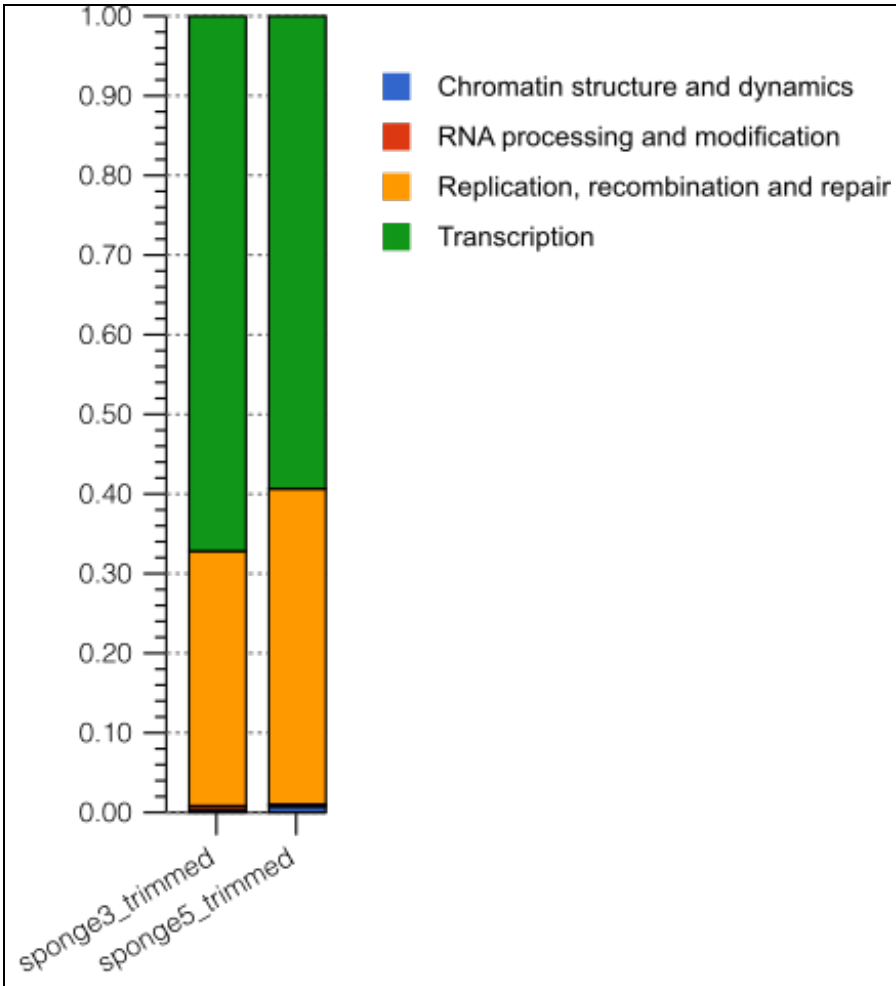


Figure 6.10-Information Storage and Processing NOGs.

The highest number of functional groups found in cellular processes and signaling were posttranslational modification, protein turnover, and chaperones, cell

wall/membrane/envelope biogenesis, and intracellular trafficking, secretion and vesicular transport. In SP3, posttranslational modification, protein turnover, and chaperones, cell wall/membrane/envelope biogenesis and intracellular trafficking, secretion and vesicular transport were the highest occurring NOGs at 53%, 19%, and 8.2% respectively. Following were signal transduction mechanisms at 6.5%, cytoskeleton at 5.8% and defense at 6.5%. In SP5 the highest occurring NOGs were posttranslational modification, protein turnover, and chaperones, cytoskeleton, and cell wall/membrane/envelope biogenesis at 51%, 13%, and 11% respectively. Following, defense NOGs were present at 11%, intracellular trafficking, secretion and vesicular transport occurred at 7.6% and signal transduction mechanisms occur at 4.9%. Further filtering in MG-RAST showed high levels of cell processes and signaling genes in the three proteobacterial groups. In the Alphaproteobacteria, 234 hits were identified to SP3 and 249 hits to SP5. The Betaproteobacteria also had high numbers with 243 hits in SP3 and 98 hits in SP5. Finally, the Gammaproteobacteria had 280 hits in SP3 and 115 hits in SP5.

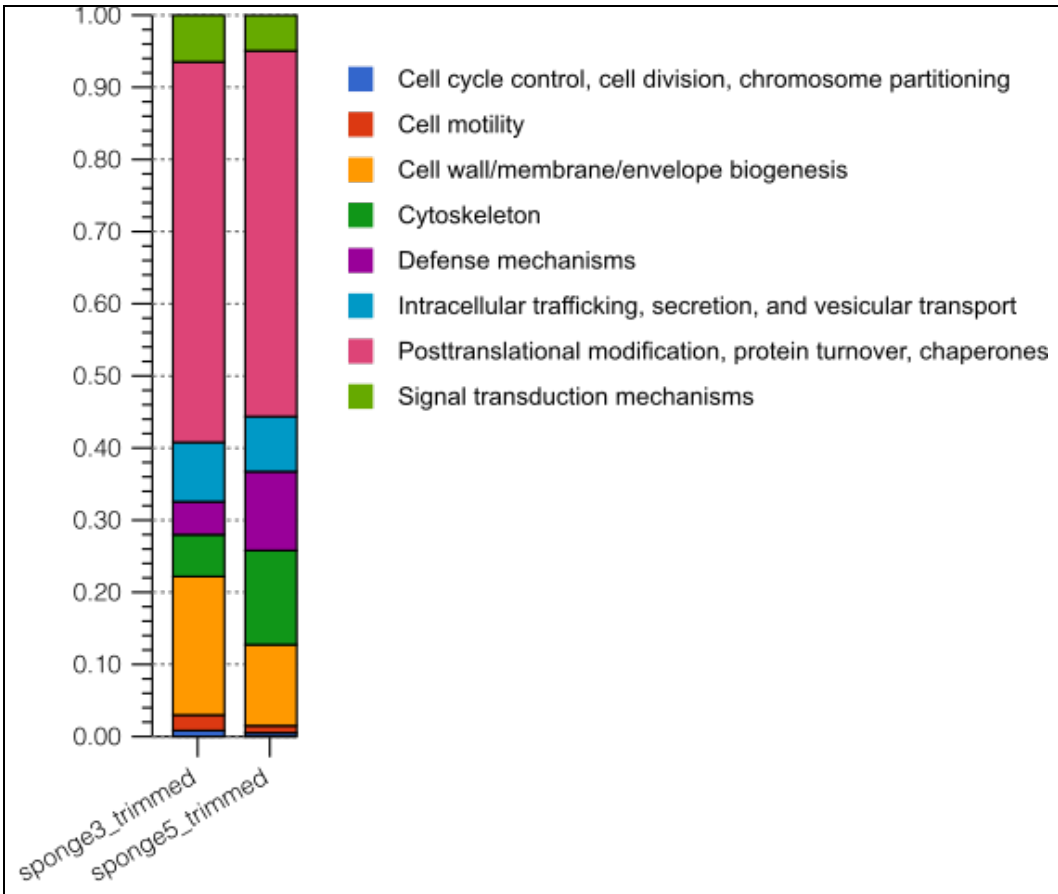


Figure 6.11-Cellular Processing and Signaling

6.5 DISCUSSION

Candidatus Kryptonia was identified in sponge SP3 from the kaiju dataset. This particular phylum has only recently been discovered in hot springs metagenomic datasets. From the same study, metabolic reconstruction showed that this candidate phylum lacked genes for various pathways and likely relies on the microbial community for appropriate nourishment (Eloe-Fadrosh et al. 2016). This group was not detected in the MG-RAST RefSeq data. Candidatus Kryptonia is a member of the Fibrobacterota-Chlorobiota-Bacteroidota (FCB) group of bacteria. This group had previously remained undetected due to primer mismatch. It was first detected and seems to be exclusive to pH-neutral geothermal springs. Members of the FCB group were detected in the MG-RAST data as well, but Candidatus Kryptonia was not specifically detected and it is not listed within the results.

Another important sponge bacterium detected in the kaiju data was the candidate phylum, Candidatus Entotheonella. Ca.Entotheonella is a candidate phylum of interest in the production of natural products. Originally isolated from the sponge *Theonella swinhoei* (Piel et al. 2004; Mori et al. 2018), this phylum has been identified in the microbiome of sponges that produce various natural products such as *Discodermia dissoluta* (Schirmer et al. 2005; Brück et al. 2008). The bacteria itself produces a large repertoire of secondary metabolites (Wilson et al. 2014; Lackner et al. 2017; Mori et al. 2018). This candidate phylum has only been identified through metagenomic datasets and clone libraries (Lackner et al. 2017). *Ca.* Entotheonella and *Ca.* Kryptonia do not seem to be present anywhere in MG-RAST. Kaiju detects taxa by translating the reads into amino acid sequences and comparing them to a protein database rather than nucleotides, with the rationale being that a homology based comparison is more sensitive than nucleotide comparisons (Menzel et al. 2016).

Proteobacteria are components of the core sponge microbiome in many animals. and are seen throughout various species of sponges, specifically the Alpha and Gammaproteobacteria groups (Kennedy et al. 2014; Thomas et al. 2016) and were detected in great abundance in the kaiju data. Alpha- , beta-, and gammaproteobacterial were the most abundant proteobacterial groups found in *H.perlevis* from the MG-RAST RefSeq data. Alpha- and gammaproteobacterial groups tend to be dominant in marine sponges as part of the core microbiota, and the enrichment of Gammaproteobacteria and betaproteobacteria might suggest that *H.perlevis* is a low microbial abundance (LMA) SP3. Further bacterial groups found in *H.perlevis* were the Actinobacteria, Firmicutes, and low levels of Cyanobacteria. Actinobacterial groups in *H.perlevis* tend to be quite diverse and are a source of bioactive compounds (Xin et al. 2008; Izumi et al. 2010; Manivasagan and Kim 2016).

Cyanobacteria also tend to be highly abundant in sponges (Alex et al. 2012). Cyanobacteria comprised about 2% for this sponge according to the MG-RAST results, though a higher amount of cyanobacteria was expected from previous evidence and the

life history of the sponge (Alex et al. 2012). A relative sponge, *Hymeniacidon heliophila* has also shown cyanobacteria counts at about 8.9%. While higher, it was not at dominant as alpha- and gammaproteobacteria, identified at 37% and 18%, respectively(Weigel and Erwin 2016). In the case of *Hymeniacidon perlevis*, it is possible that sequences matching to cyanobacteria were classified as general bacteria or potentially there was low sequence coverage, but overall, we do not have an explanation as to why cyanobacterial reads were not well detected.

Other groups detected from the kaiju data, though not found in abundance in the MG-RAST data were *Delftia* and *Achromobacter*. *Delftia* and *Achromobacter* are ubiquitous to the environment and are common contaminants to contact lenses (Vijay and Willcox 2018) and have previously been detected in the reagents of common DNA purification kits done by sequencing the negative control in amplicon sequencing studies (Salter et al. 2014). As a negative control was not sequenced, primarily due to no DNA being detected prior to sequencing, it is difficult to determine if *Delftia* and *Achromobacter* are present due to a contamination of reagents, being a low abundance symbiont of the sponge, or simply a misidentification by the kaiju data as these were not detected initially in the MG-RAST dataset.

The archaeal sequences detected on MG-RAST included groups from three phyla Thaumarchaeota, of which the genus *Nitrosopumilus* sp. was detected from the kaiju data, Euryarchaeota, and Crenarchaeota. In sponges, the archaeal groups tend to be dominated by Crenarchaeota. These groups are associated with ammonia oxidation, particularly the Crenarchaeota and the genus *Nitrosopumilus* (Walker et al. 2010). Ammonia oxidation is the first step in the nitrogen cycle, which is important for the generation of metabolic energy (Christman et al. 2011). In the sponge, these groups are important for nitrogen metabolism which make sponges an important nitrogen sink (Hoffmann et al. 2009) and regulating the amounts of nitrogen species in the environment[63–65].

Within the sponge, groups of eukaryotes were identified. Many of the groups came from chordate animals, arthropods, cnidarians, and echinoderms. Possibilities for the identification of these groups could be sample contamination, misidentification, or possibly the detection of environmental DNA (eDNA). Environmental DNA is becoming an important component in conservation science in that an organism does not need to be seen or captured but can still be detected in its environment (Willoughby et al. 2016; Hunter et al. 2017). Other important groups of eukaryotes detected from the sponge include the fungal groups Ascomycota, the sac fungi, and Basidiomycota, the cup fungi. Information on the fungal groups of marine sponges is limited (Webster and Thomas 2016) with only a few pure culture studies performed (Baker et al. 2009; Baker et al. 2012; Passarini et al. 2013; Naim et al. 2017) and limited community sequencing studies (Naim et al. 2017; Nguyen and Thomas 2018). While still limited in scope, sponge-associated fungi have the potential to be new sources of bioactive compounds (Höller et al. 2000; Baker et al. 2009; Baker et al. 2012).

One other important eukaryotic group detected in the kaiju data and at low levels in the MG-RAST data were choanoflagellates, the sister group to Metazoa (Carr et al. 2008; King et al. 2008). What makes this particular group so fascinating is the similarity of these cells to the choanocytes within sponges, suggesting an evolutionary link (Mah et al. 2014). Choanoflagellates are thought to be a common ancestor to metazoans. Certain species, like *Salpingoeca rosetta*, expresses developmental genes and can alternate between a unicellular and multicellular state (Cavalier-Smith 2017; Laundon et al. 2019). As far as the detection of porifera reads in both datasets, porifera reads were not included in the kaiju database. For MG-RAST, other groups of invertebrates and even Chordata were identified but analysis with BLAST showed that reads identified as Porifera and other phyla identify as *Hymeniacidon perlevis*.

Much like the eukaryotic groups, viruses are poorly understood in sponges (Webster and Thomas 2016). No viral sequences were detected in the kaiju data. With the NR database, protein sequences were used and if any viral sequences were in the dataset, they likely did not match to what was in the NR dataset. This is not to say that viruses

are not present in sponges, however, viral metagenomics is still an emerging niche in metagenomic analysis.

Viral groups made up a small proportion of the sequences identified from RefSeq. These groups included the Phycodnaviridae, Iridoviridae, Baculoviridae, Myoviridae, Siphoviridae, and Podoviridae. To properly identify the viral structure of marine sponges, a targeted study of the viral metagenome will need to be completed. It has been suggested that sponges may predate upon viruses , much like they do with bacteria and particulate matter and in previous studies, viral-like particles have been detected (Vacelet and Gallissian 1978; Pascelli et al. 2018).

Expectedly, high levels of metabolism genes were found in each sponge. The abundance of high levels of metabolism genes, particularly those for carbohydrate metabolism and energy production, suggest that the bacterial groups present in the sponge are processing food such as particulate matter and other bacteria and converting it to energy to be used for growth and defense. Of metabolism genes 156 were identified from SP3 and 65 from SP5. Further analysis in MG-RAST matched the genes to the KEGG Orthology (Mao et al. 2005) database. The KO numbers identified were ko00627 for aminobenzoate degradation, ko00362 for benzoate degradation, and ko00360 for phenylalanine metabolism. Aminobenzoates are xenobiotic compounds that do occur naturally, but they can also be introduced in high levels into the environment from anthropogenic activities (Spain 1995), industrial sources such as from azo dyes (Liu et al. 2022) and via prophylactic treatment for sea lice, which has been found to be toxic (Lennox et al. 2020). Aminobenzoates have negative impact on the environment, like reducing the dissolved oxygen content in water (Hassan and Carr 2018) and mutagenicity in fish (Abe et al. 2018). It is suggested that organisms, particularly bacteria, that contain these genes and express them can be useful for bioremediation purposes (Peres et al. 2001). Mentioned earlier, *H. perlevis* has been presented as a candidate for the mitigation of microbial pollution (Longo et al. 2010; Mahaut et al. 2013; Longo et al. 2022). *H. perlevis* and its associated microorganisms could be considered

a source of xenobiotic degrading compounds, but further study is needed to determine if this is plausible on a large scale.

Further study of *H. perlevis* should include a functional metagenomic library for the degradation of aminobenzoates to verify the data from MG-RAST. In addition, expected secondary metabolite genes, such as those for polyketide synthases, were not detected. It could potentially be missed in the database so further analysis should include other functional databases such as KEGG (Kanehisa et al. 2004; Kanehisa et al. 2012; Kanehisa et al. 2016).

7.0 METAGENOMIC ANALYSIS OF THE INTERTIDAL WELSH SPONGE, *HALICHONDRIA PANICEA*

7.1 INTRODUCTION

Sponges represent some of the most ancient of animal life dating back to the Precambrian period (Peterson and Butterfield 2005). In the aquatic environment, sponges play an important role in filtering seawater (Bell 2008; Leys et al. 2011) and providing a food source to animals in tropical and temperate seas. Sponges are present in a variety of habitats from marine systems (Becking et al. 2013; Chambers et al. 2013; Turk et al. 2013) to freshwater lakes and streams (Karlep et al. 2013; Jung et al. 2014). Typically, sponges must be submerged in order to pump water (Leys et al. 2011) to collect particulate matter (Thurber 2007) and excrete waste products. Exposure to air can introduce air pockets and stop the pumping mechanism (Leys et al. 2011). Interestingly, some species can be found growing on rocks intertidal zone (Alex et al. 2012; Weigel and Erwin 2016) where they are exposed to air for extended periods of time. What allows these sponges to survive this exposure is the constant sea spray and wave activity that occurs in these intertidal areas (Weigel and Erwin 2016).

Sponges are also host to a variety of symbionts of the domains Archaea, Bacteria, and Eukarya (Hentschel et al. 2006; Jackson et al. 2013; Bayer et al. 2014). Each of these groups plays an important role in the sponge such as digesting particulate matter the sponge picks up while filter feeding and in the conversion of nutrients such as nitrogen (Hoffmann et al. 2009). Sponges are also important in the discovery of bioactive molecules for industry and medicine (Gunasekera et al. 1990; ter Haar et al. 1996; Zheng et al. 2006; Graça et al. 2013).

Halichondria panicea is a commonly dispersed European sponge found in tidal zones attached to rocks and in deeper areas, attached to the thallus of kelps and rocks (Figure 7.1). *H. panicea* is distributed throughout Europe from the Black Sea to the Adriatic and

Mediterranean Seas into the Artic regions of the northeastern Atlantic (de Voogd et al. 2023). Similar sponges identified as *H. panicea* have been documented in the southern Ocean and the northern Pacific Ocean. Based on work by Erpenbeck et al. in 2004, the sponges identified as *H.panicea* in Alaskan waters are closely related to Atlantic populations based on COI mitochondrial markers but are still distinct siter groups. (Erpenbeck 2004). This work suggests that the sponges for a species complex between the Atlantic and Pacific populations in addition to a related species, *H. bowerbanki* suggesting the possibility of a previous speciation event but more data is needed to confirm. As of a review in 2022 (Goldstein and Funch 2022), this has not been further solved, though an article from 2018 does suggest non-conspecificity between *H.panicea* and a new species found in the southwest Atlantic near Argentina (Gastaldi et al. 2018).

H. panicea is considered a low microbial abundance (LMA) sponge due to the dominance of only a few species of bacteria being present within its tissues (Wichels et al. 2006). Typical of LMA sponges, *H. panicea* shows a low density of microbial cells in its mesohyl and low diversity with this being backed up by large 16S rRNA sequencing studies (Giles et al. 2013). LMA sponges are also characterized as being dominated by one or two clades of bacteria, usually a group of Proteobacteria or Cyanobacteria and show little diversity with other symbionts (Althoff et al. 1998; Giles et al. 2013; Bayer et al. 2014; Thomas et al. 2016). With this low diversity in symbionts, there are also differences in physiology in the symbionts such as lower abundances of certain genes (Gloeckner et al. 2014).

Figure 7.1 *Halichondria panicea* growing on rocks from an exposed tide. *H. panicea* is easily found on exposed rocks at low tide. Photo by A. Campbell

In the study by Althoff et al. 1998, specimens of *Halichondria panicea* were collected from three areas of northern Europe-Rovinj, Croatia, Helgoland, and Kiel in Germany (Althoff et al. 1998). These sponges all had an identical 740 bp band found using DGGE. All three of these sponges contained high levels of Alphaproteobacteria and it was further found that all three sponges contained a species of Rhodobacter, dominant in the sponge. In a further study by Wichels et al 2006, it was found that *H. panicea* harbors not a Rhodobacter, but a Roseobacter group belonging to marine Alphaproteobacteria (Wichels et al. 2006). It is low in diversity but stayed consistent through all samples and further experiments showed that this group of bacteria shows some seasonal variability and that it tends to be the dominant bacteria in *H. panicea.*

In 2019, a group of researchers from Iceland discovered a candidate species in *Halichondria panicea* (Knobloch et al. 2019a) while trying to co-cultivate *H. panicea* and its symbionts in an *ex situ* setup. In the experiment, specimens of *H. panicea* were kept in a mariculture system for six months utilizing a new cultivation method where the sponge is separated by a membrane in a 24-well plate in a high flow through system to preserve the microbial symbionts in *H. panicea*. This led to an overall decrease in the bacterial abundance of the sponge, however, as earlier seen in Wichels et al (Wichels et al. 2006), the alphaproteobacterium, herein dubbed *Candidatus Halchondribacter symbioticus*, was still found in dominance within the sponge.

The aim of this study is to determine the microbial makeup and functional potential of *Halichondria panicea* using read-based taxonomic and functional annotation. Following this approach, biases normally introduced during PCR and constraints with metagenomic assembly are avoided.

7.2 METHODS

7.2.1 Sponge Collection

Three specimens of *Halichondria panicea* were collected in December of 2016 from tidal rocks Porthcawl, Wales and placed into DESS buffer (20% DMSO, 0.1M EDTA, sodium chloride saturated). Samples were transported at room temperature, and stored for 24 hours at 20°C before final storage at -80°C.

7.2.2 DNA Extraction and Purification

DNA was extracted from these sponges using the Qiagen DNEasy kit

The sample was loaded onto a 2% LMP TAE gel and run out for one hour at 40V. High molecular weight bands were cut out and put through a GELase High Activity protocol. Post-digestion, the samples were ethanol precipitated. The resulting DNA was quantified on the Qubit 2.0 fluorometer. Samples from the DNEasy extraction were used due to higher yields and sample purity.

7.2.3 Sequencing Library Preparation and Quantification

500ng of DNA was prepared for sequencing libraries using the NextFlex Rapid DNA_Seq kit with a PCR-free step. The libraries were quantified using the QIAGEN Qia-Seq NGS library Quant assay kit (Qiagen, Inc., Hilden, DE). Before loading, the library size was checked using the Agilent TapeStation (Agilent Technologies, Santa Clara, CA). Sequencing libraries were denatured and diluted using the NextSeq500 preparation protocol from Illumina on a High-Output 350 cycle cartridge. (Supplementary)

7.2.4 MG-RAST analysis

Raw sequence data was uploaded and analysed on the MG-RAST platform. Briefly, uploaded sequences had adaptors removed using skewer and denoised with fastq-mcf. Host DNA (human) was removed using a bowtie2 index through MG-RAST. This is a standard step in the MG-RAST pipeline. Gene calling was performed for ribosomal RNA using sortmerna and clustered using cd-hit. The similarity search was performed with blast. FragGeneScan was run to identify protein coding regions and the same processing protocol for the rRNA was followed for the protein features. Sequence data is available from MG-RAST under project ID mgp89090. Raw reads are also available from the European Nucleotide Archive under project ID PRJEB54767, available September 30, 2022.

The RefSeq and KEGG Orthology data were extracted imported into R for analysis. To view the data neatly, only the top 50 eukaryotes and top 150 entries are shown. Further, groups were subset. The RefSeq data was broken down by domain and further by phylum for the fungal sequences. Alphaproteobacterial reads were also checked for the presence of *Ca. Halichondribacter symbioticus.* The KO data was broken down into each level 1 group and further subset to show the distribution of genes for each KO group. To keep the graphs easy to read, only the top 100 function hits for each group are shown.

7.3 Results

7.3.1 RESULTS

Three sponge specimens were used for this study to determine the microbial consortia and functional potential of these organisms. The effects of sequencing quality checks are seen in each sponge through the MG-RAST process and showing the number of predicted and identified features for all three samples (Tables 7.1,7.2,7.3).

Table 7.1-Pre and post quality checking in MG-RAST of *H. panicea* sample number 1. A 1% drop in the GC content is noted through quality checking and a 16.5% drop in sequence numbers was also found. Mean sequence length increased between quality checking. Within this sample, 95,272,702 predicted protein features were found of which 5,857,789 (6.15%) were identified. For rRNA, there were 1,320,406 features found with only 30,550 (2.31%) of those features being identified.

Sponge 1 Table Data	
Upload bp Count	30,693,614,062
Upload Sequence Count	182,603,597
Upload Mean Sequence Length	168 ± 46
Upload Mean GC %	39 ± 9 %
Artificial Duplicate Reads	28,432,659
Post QC bp Count	26,091,411,688
Post QC Sequence Count	152,343,888
Post QC Mean Sequence Length	171 ± 46
Post QC Mean GC %	38 ± 9 %
Predicted Protein Features	95,272,702
Predicted rRNA Features	1,320,406
Identified Protein Features	5,857,789
Identified rRNA Features	30,550
Identified Functional Categories	Undefined

Table 7.2-Pre and post quality checking in MG-RAST of *H. panicea* sample number 2. QC content remained the same for this sample through quality checking. An 11.9% drop in sequence count was also found. A small increase in mean sequence length was also observed. Within this sample, 18,372,100 predicted protein features were found of which 1,750,269 (9.53%) were identified. For rRNA, there were 254,977 features found with only 10,833 (4.25%) of those features being identified.

Sponge 2 Table Data	
Upload bp Count	5,961,839,577
Upload Sequence Count	35,279,611
Upload Mean Sequence Length	169 ± 48
Upload Mean GC %	44 ± 10 %
Artificial Duplicate Reads	3,619,216
Post QC bp Count	5,328,597,931
Post QC Sequence Count	31,086,386
Post QC Mean Sequence Length	171 ± 48
Post QC Mean GC %	44 ± 9 %
Predicted Protein Features	18,372,100
Predicted rRNA Features	254,977
Identified Protein Features	1,750,269
Identified rRNA Features	10,833
Identified Functional Categories	Undefined

Table 7.3-Pre and post quality checking in MG-RAST of *H. panicea* sample number 4. QC content remained the same for this sample through quality checking. A 23.1% drop in sequence count was also found. A small increase in mean sequence length was also observed. Within this sample, 8,915,542 predicted protein features were found of which 1,485,552 (16.67%) were identified. For rRNA, there were 8,915,542 features found with only 4,285 (0.048%) of those features being identified.

Sponge 4 Table Data	
Upload bp Count	3,115,126,912
Upload Sequence Count	19,416,656
Upload Mean Sequence Length	160 ± 44
Upload Mean GC %	52 ± 20 %
Artificial Duplicate Reads	2,365,561
Post QC bp Count	2,475,079,665
Post QC Sequence Count	14,932,171
Post QC Mean Sequence Length	166 ± 45
Post QC Mean GC %	47 ± 15 %
Predicted Protein Features	8,915,542
Predicted rRNA Features	908,157
Identified Protein Features	1,485,552
Identified rRNA Features	4,285
Identified Functional Categories	Undefined

7.3.2 Taxonomic Profile

Halichondria panicea sample 1 was found to contain the most rRNA and protein features. From the rRNA features, roughly 2.31% were identified. Of these, most sequences matched to Bacteria and Eukarya. The sponge appears to be dominated by one group of bacteria and the remaining symbionts are present in much lower numbers, a trait seen in other low microbial abundance sponges. For the eukaryotic sequences, most of it is dominated by Chordata, which could be potential environmental DNA or misidentified reads and by Demospongiae, the host sequences. This is seen in all three sponge samples. In a follow up analysis with BLAST, a random subset of the reads identified as

Porifera and Chordata were run using the megablast (Zhang et al. 2000; Morgulis et al. 2008) algorithm against the nt/nr database. In the Porifera reads, the results were *Halichondria panicea* or related species. Interestingly, reads from the Chordata set, mostly consisting of *Branchiostoma floridae*, a lancet, and *Sus scrofa*, a wild hog species, also came back as *H. panicea*. There were some reads that showed no match in NCBI. In *H. panicea* specimen 2, a similar profile is found. There is a high abundance of bacteria seen once again followed by an abundance of eukaryotic sequences (Figure 7.2a). Further, at the class level, Alphaproteobacteria is seen again as the dominant group of bacteria and lower levels of other symbionts is also seen (Figure 7.2b). Other domains, such as the Archaea and Eukarya are present at much lower numbers. In *Halichondria panicea* specimen 4, the same high abundance of bacteria is seen at the domain level. Further, this specimen was dominated by Gammaproteobacteria and Alphaproteobacteria and Betaproteobacteria in contrast to the other two samples. Again, low numbers of Archaea and Eukarya were detected in this sample.

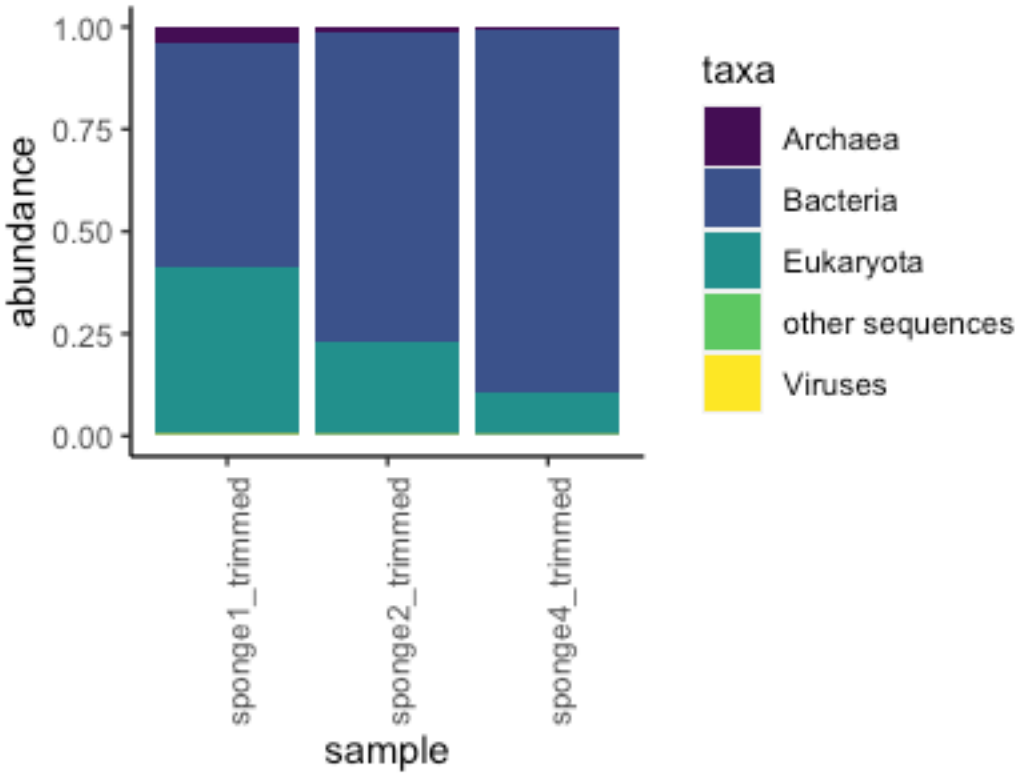


Figure 7.2a--Overall taxonomic makeup of *H.panicea*. Most taxa found in *H. panicea* belong to the domains Bacteria and Eukarya. Archaeal groups are present in small amounts. Viruses, while not show on the bar chart do comprise a small amount of the overall microbiome of the sponge. It is unclear why there are many bacterial reads for what is considered a low microbial abundance (LMA) sponge, but it maybe that the original definition does not hold true for all instances of this sponge and the LMA status may be influenced by other factors such as extraction efficiency.

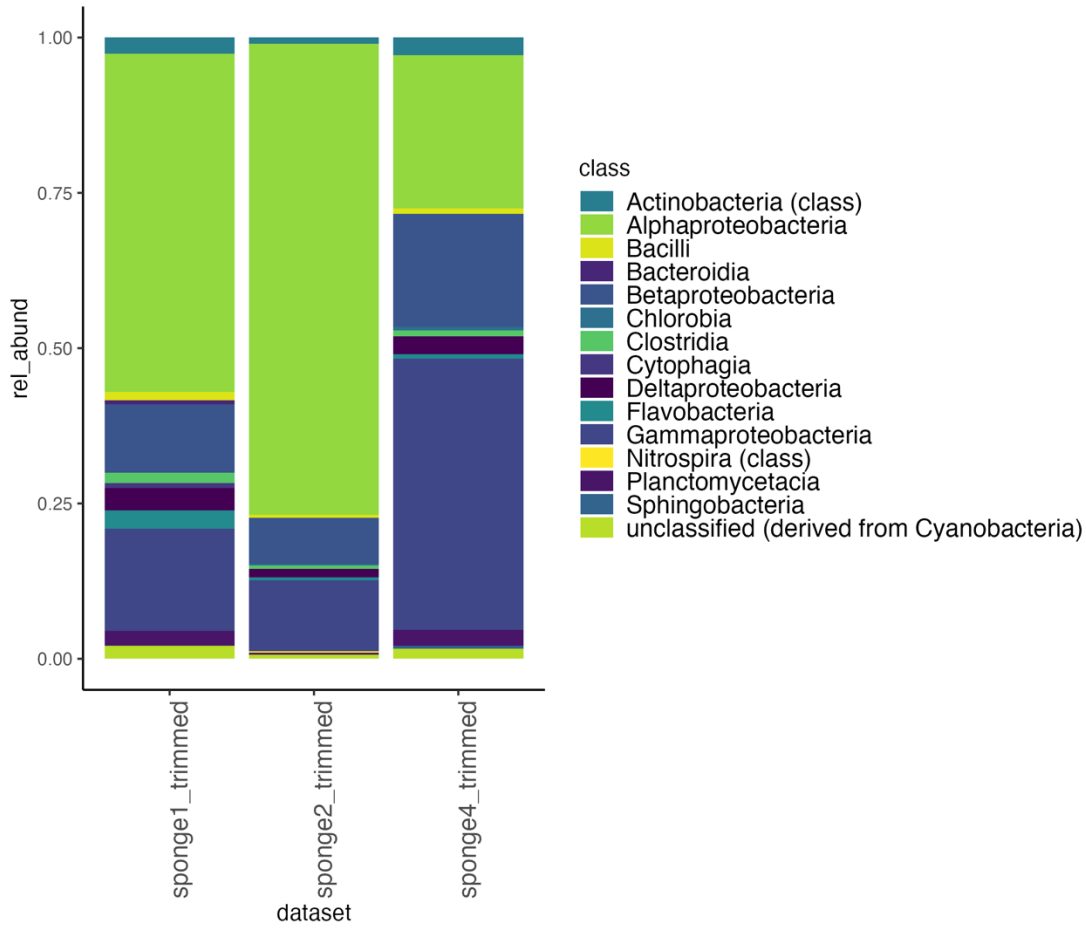


Figure 7.2b -Bacterial classes present in *H. panicea*. Three classes dominate the sponge-the Alphaproteobacteria, Betaproteobacteria, and the Gammaproteobacteria. While showing fluctuation in the relative abundance of each class, each sponge still shows a similar makeup of their dominant bacteria.

Each sponge showed a high abundance of Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria. Having an overwhelming dominance of proteobacteria is commonly seen in a low microbial abundance sponges, such as *Halichondria panicea*. In addition, have a high abundance of Alphaproteobacteria is a common feature to *H.panicea* and has been seen in many previous studies (Althoff et al. 1998; Wichels et al. 2006; Knobloch et al. 2019a). Interestingly, the alphaproteobacterial reads did not match to *Ca. Halichondribacter symbioticus* in a follow-up BLAST search, though there were matches to other bacteria within Roseobacter. It is still possible that some of the alphaproteobacterial reads are *Ca. H. symbioticus*, but factors such as the short

sequence length of the reads may prevent strong taxonomic resolution as seen with 16S rRNA gene studies (Nagy et al. 2008; Winand et al. 2019). In addition, it was previously noted that not all sponges identified as *H. panicea* contained *Ca. Halichondribacter symbtiocus* (Knobloch et al. 2020).

Eukaryotes made up a variable number of sequences from each sponge and quite a large amount from sponge1. Most sequences, surprisingly, did not match to sponge but to other organisms (Supplementary Figure 7.1). This can perhaps be contributed to the data available in the databases or perhaps environmental DNA. This could also be due to the default cutoff in MG-RAST, which is for a 60% identity. Upon raising the percent identity to 90%, more sponge reads were present, but still low comparatively. With the default values, *Porifera* reads were present at 2%, 5%, and 7% in Sponge 1, 2, and 4 respectively. At the higher percent identity, the percentage of Porifera reads increased to 11%, 28%, and 30%, though there is still a high amount of reads that were identified as other eukaryotes. Mentioned earlier, subsets of these reads were put into BLAST and determined to be *H. panicea* and other poriferan reads. Fungi, while not present in high volumes, were still detected in the sponge.

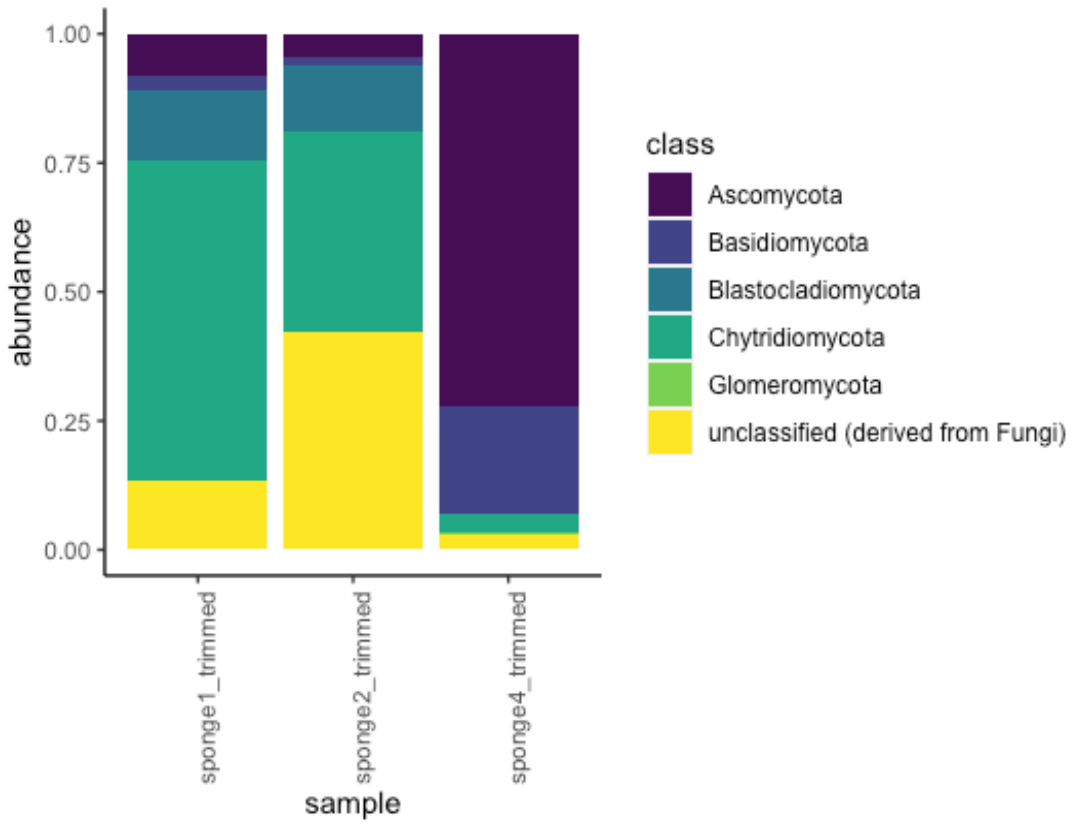


Figure 7.3-Fungal classes detected in the sponge *Halichondria panicea*. Fungal groups are present at low amounts in the sponge samples, but upon further analysis, a few groups were identified. The dominant groups were the Eurotiomycetes, Saccharomycetes and Sordariomycetes.

In all sponge specimens, there were three main classes detected at high levels-the Eurotiomycetes, Saccharomycetes, and Sordariomycetes. The three classes show a similar makeup to that of previous studies on *Haliclona simulans* wherein, sponges were cultured for fungi (Baker et al. 2009). Blastiocladomycetes were also a high abundance group but were not detected in sponge 4. Most groups of fungi found in the *H. panicea* are marine yeasts.

Each sponge's functional potential was determined using KEGG Orthology (KO) in MG-RAST. Each sample contributed several hits. *H. panicea* specimen one had 4,582,498 hits, *H. panicea* specimen 2 had 2, 195,817 hits, and *H.panicea* specimen 4 had 1,627,067 hits.

In Figure 7.4a, the first level of KO is shown and for each sample, the highest abundance of genes is utilized for metabolism. Second most are the genes for genetic information processing which include tRNA biosynthesis and ribosome formation. The next group is the environmental information processing genes which include transporters, secretion, and signaling pathways. Figure 7.4b shows the breakdown of metabolism genes within *H. panicea*.

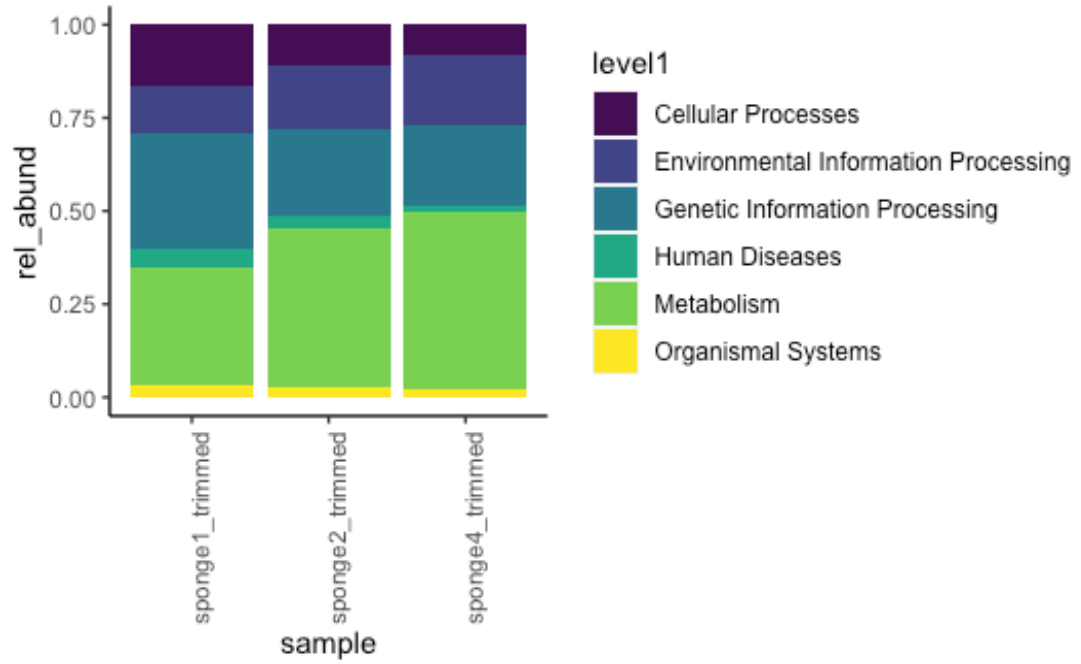


Figure 7.4a-Breakdown of the KEGG Orthology functional groups found in each sample. A similar structure of functional genes is found across all samples. The highest among these are the metabolism genes at 31% in specimen 1, 42% in specimen 2 and 48% in specimen 4. For genetic information processing 31% of the reads belong to specimen 1, 24% to specimen 2, and 21% to specimen 4. Environmental information processing 13%, 17%, and 19%.

The highest metabolic genes are those for amino acid metabolism, which support the growth of bacteria by regulating energy and proteins. Protein production is important in bacteria for the breakdown of proteins from food and the synthesis of new proteins to sustain the bacteria and the sponge. Of the metabolism genes, amino acid metabolism genes account for about 13% in sponge1 and 11% in both sponge2 and sponge4. Energy metabolism is the second highest and is an important part of utilizing other compounds as an energy source. Energy accounts for 7% in sponge1 and sponge2, and 5% in sponge4. Following energy metabolism is carbon metabolism. Genes in this group are essential for allowing organisms to utilize carbon as an energy source. In addition, carbohydrate metabolism is important in the synthesis of the cellular wall of bacteria. Carbohydrate metabolism genes are present at 6% in sponge1 and 5% in sponge2 and sponge4.

Other genes are present in smaller numbers but still have important functions in the sponge and its microbial symbionts. Bacteria and other cells utilize their nucleotide metabolism, present at 2% across each sample, for the breakdown and production of DNA as well as to initiate RNA transcription. Genes of special interest include the hits for the metabolism of terpenoids and polyketides, present at 0.006% in sponge1 and 0.004% in sponge 2 and sponge 4, and the biosynthesis of secondary metabolites, present at 0.2% in sponge1 and sponge4 and at 0.1% in sponge2.

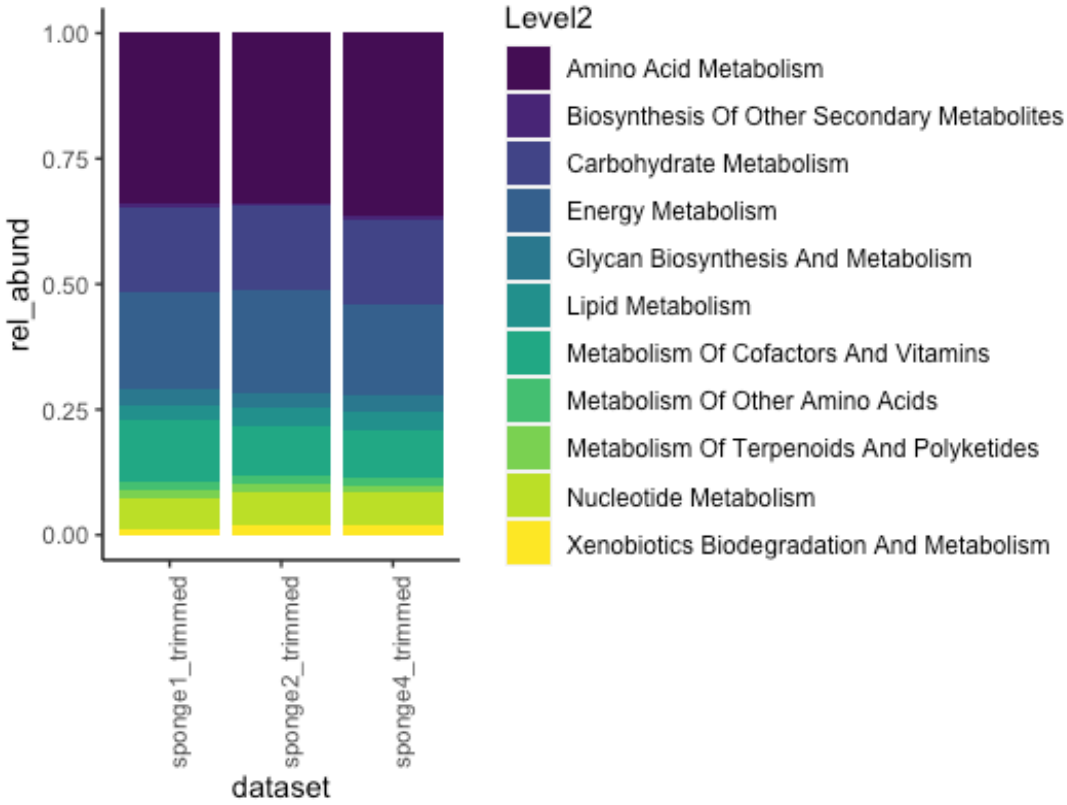


Figure 7.4b -KO Metabolism genes described at level 2. Four groups dominate the functional genes in each sponge. These are the genes for nucleotide metabolism, energy metabolism, carbohydrate metabolism, and amino acid metabolism.

7.4 DISCUSSION

Halichondria panicea shows a suite of microbial reads and a variety of metabolic functions. The overall taxonomic profile shows an overwhelming number of bacterial reads compared to eukaryotic reads, even though *H. panicea* is considered an LMA sponge. It is unclear why this occurred. A reason for this could be extraction efficiency. Certain microbes and euakaryotic cells can be difficult to lyse due to cell wall thickness in eukaryotic organisms and Gram positive bacteria, the presence of inhibitors, and endospores (Feinstein et al. 2009; Roopnarain et al. 2017; *The Lysis Bias Crisis* 2019; Demkina et al. 2023), which in turn can bias the results. While this is unusual, the make-up of the bacterial communities is more standard for LMA sponges. Analysis of the

prokaryotic communities reveals a dominance of Proteobacteria, mainly Alphaproteobacteria, then Gammaproteobacteria, and Betaproteobacteria. Previous studies utilizing denaturing gradient gel electrophoresis (DGGE) found that *H. panicea* contains high levels of Alphaproteobacteria (Althoff et al. 1998; Wichels et al. 2006; Knobloch et al. 2019a). Investigations have shown previously that *H. panicea* is dominated by one group of Alphaproteobacteria. Similar results have been found with 16S rRNA gene sequencing and further analysis has revealed that this Alpahproteobacteria is specific to a *Roseobacter* clade (Wichels et al. 2006) and further work has identified an important symbiont, *Candidatus Halichondribacter symbioticus*. Surprisingly, this bacterium was not detected in this study. As noted by Knobloch et al., not all *H. panicea* contain *Ca. H. symbioticus* (Knobloch et al. 2019; Knobloch et al. 2020).

Betaproteobacteria are another interesting group within the sponge. In the 1998 study by Althoff (Althoff et al. 1998) which utilized PCR and cloning, Betaproteobacteria were detected, though this was not seen in a follow up (Wichels et al. 2006) which used denaturing gradient gel electrophoresis. Betaproteobacteria were detected in a 2019 study which utilized 16S rRNA gene sequencing of the *H. panicea* community following stays in different mariculture conditions (Knobloch et al. 2019b; Knobloch et al. 2019a). Another species of sponge, *Crambe crambe*, is dominated by Betaproteobacteria (Croué et al. 2013) and a clade identified in *C. crambe* was associated with LMA sponges and other marine invertebrates. An attempt was made to compare betaproteobacterial sequences with BLAST to determine if this clade was present in *H. panicea*, but the BLAST results were inconclusive due to the MG-RAST reads not being 16S rRNA gene reads. Gammaproteobacteria are common symbionts to sponges and perform important functions like host protection and the fixation of nitrogen (Li et al. 2006; Webster et al. 2012; Li et al. 2016; Bibi et al. 2020). While only one sample, sponge 4, showed higher levels of Gammproteobacteria (about 48%), numbers of detected Gammaproteobacteria were similar to that of a prior 16S rRNA gene study of *H. panicea* (Knobloch et al. 2019a; Knobloch et al. 2019b).

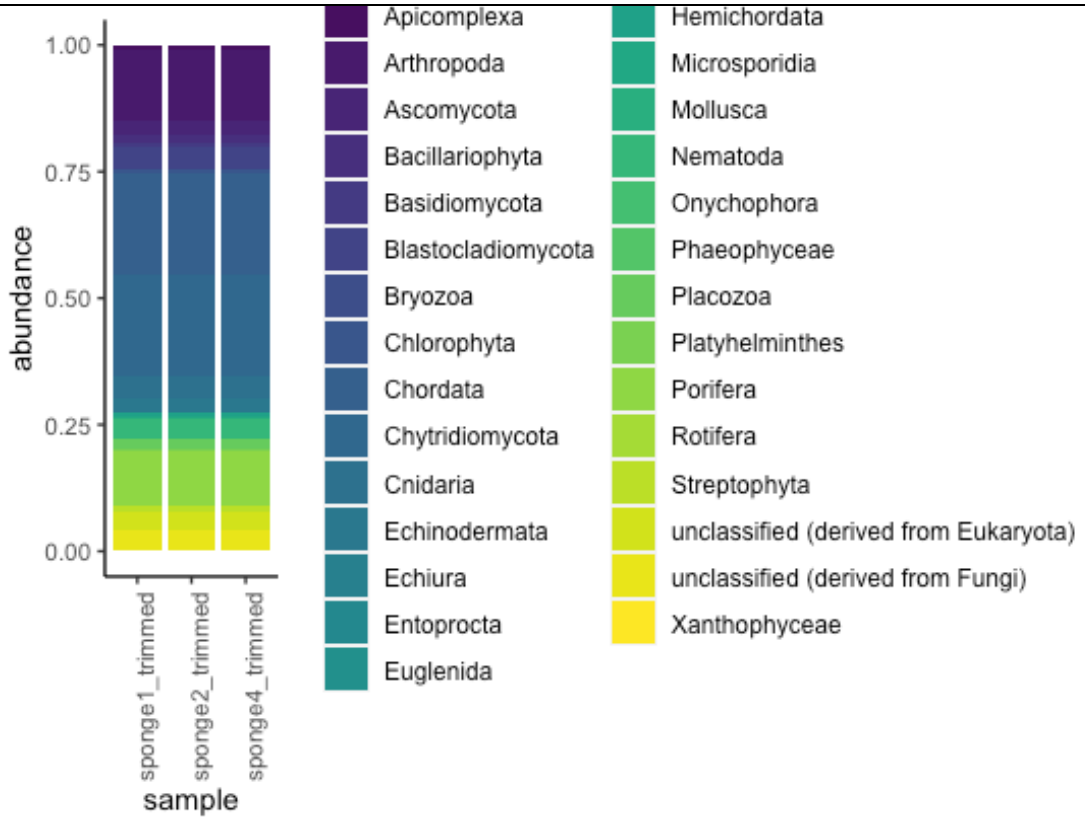
In addition to the bacterial groups in *H. panicea*, a small number of fungi were detected in the sponge. Fungi are an often understudied group in sponges with only a small handful of known studies that have mainly focused on culturable fungi (Wiese et al. 2011; Baker et al. 2012; Passarini et al. 2013). Only a few studies exist that utilized NGS to distinguish fungal groups (Naim et al. 2014; Naim et al. 2017; Nguyen and Thomas 2018). Often, with PCR-based NGS studies of fungi, in a mixed sample like a sponge, co-amplification of host DNA can be an issue as the primers for fungi target genes like the internal transcribed spacer (ITS) (Bazzicalupo et al. 2013; Bengtsson-Palme et al. 2013) and 18S ribosomal RNA genes (Naim et al. 2017). Post-filtering of host reads is essential in many NGS studies. Filtering is recommended as it reduces computational load and for assembly based approaches, improve the assembly quality (Rumbavicius et al. 2023; Gao et al. 2025). The approach of shallow shotgun sequencing can alleviate some of this as there is no PCR bias (Hillmann et al. 2018) and in the case of this study, see the amount of host reads in context with the symbiont reads. Taxonomic assignment, however, is dependent on the database used. In this work, taxonomic assignments generated by MG-RAST and the RefSeq database did not always match those from a megablast search. Megablast offers use of the core_nt (Staff 2024) databases which contain complete, partial, and draft genomes and RefSeq whieh contains well defined reference genomes and transcripts (Haft et al. 2023; Goldfarb et al. 2024).

An advantage to shallow shotgun sequencing is not only discerning the microbial constituents, but in also obtaining the functional potential. In Figure 7.4a, a breakdown of broad KEGG Orthology (Kanehisa et al. 2004; Mao et al. 2005; Kanehisa et al. 2012; Kanehisa et al. 2016) shows the structure of various functions. Metabolism is the highest occurring function. It encompasses basic needs for the sponge such as carbohydrate, lipid, energy, and nucleotide metabolism which are important for maintaining sponge and microbial cells.

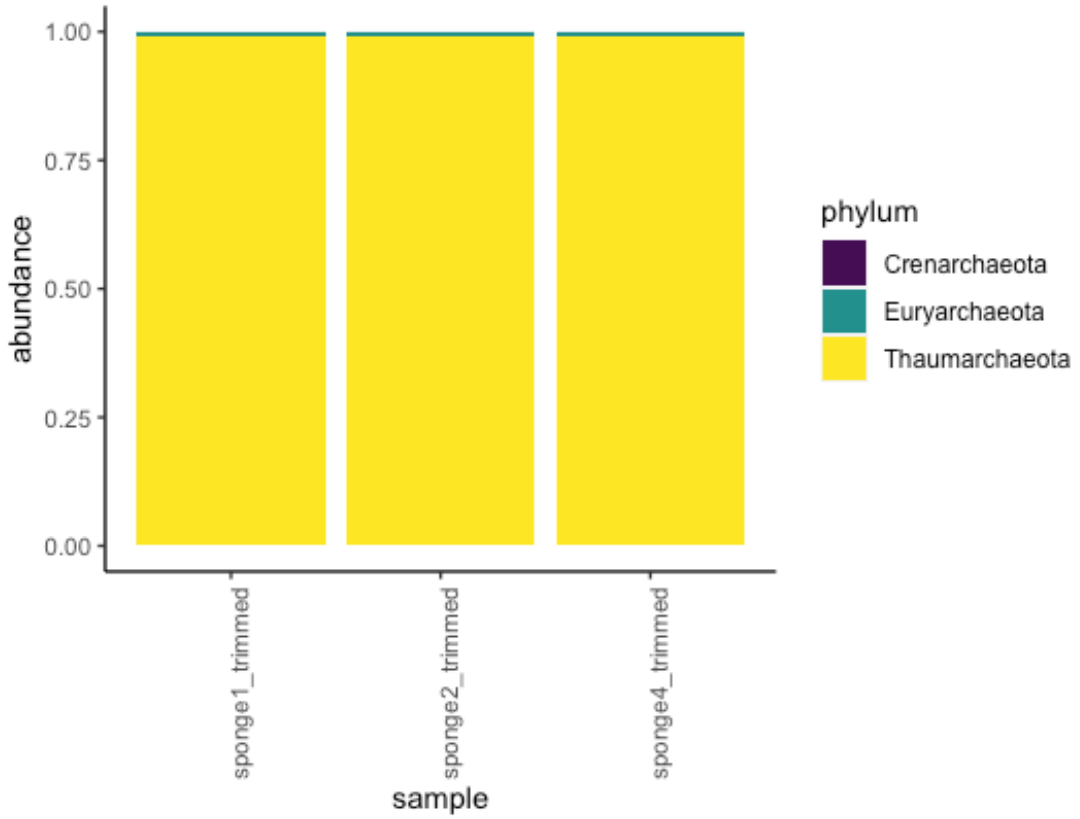
To a smaller extent, within *H. panicea* are subgroups of metabolism are the functions for producing terpenoids and polyketides, which are key to the production of certain secondary metabolites. (Schirmer et al. 2005; Hochmuth and Piel 2009; Della Sala et al.

2013). Terpenoids and polyketide synthase metabolism is important as these types of molecules are important in the pharmaceutical industry in the production of potential antibiotics and anti-cancer treatments (Schirmer et al. 2005; Hochmuth and Piel 2009; Della Sala et al. 2013; Mrudulakumari Vasudevan and Lee 2020). In the sponge, whether produced by the sponge or the symbionts, polyketides and terpenes function as a defense against predation and invading microbes (El-Bondkly 2014; Ueoka et al. 2015; Tholl et al. 2023). Previous works have found an associated with polyketide synthases (PKS) with Actinobacteria associated with *H. panicea* (Schneemann et al. 2010). This phylum was present at around 2% for sponge1 and sponge4, and at 1% in sponge2. In the 2010 study by Schneemann et al., 122 compounds with 88 yet to be known compounds were detected isolated from an actinobacterial strains associated with *H. panicea*. Further, *Streptomyces sp.* HB202 from a concurrent study (Schneemann et al. 2010) was found to produce a PKS based antimicrobial compound, mayamycin, which exhibits broad antibiotic activity and cytotoxicity. While only detected in low amounts, these genes are important for maintaining symbiont relationships and reducing predation on the sponge (Hochmuth and Piel 2009; Jenke-Kodama and Dittmann 2009).

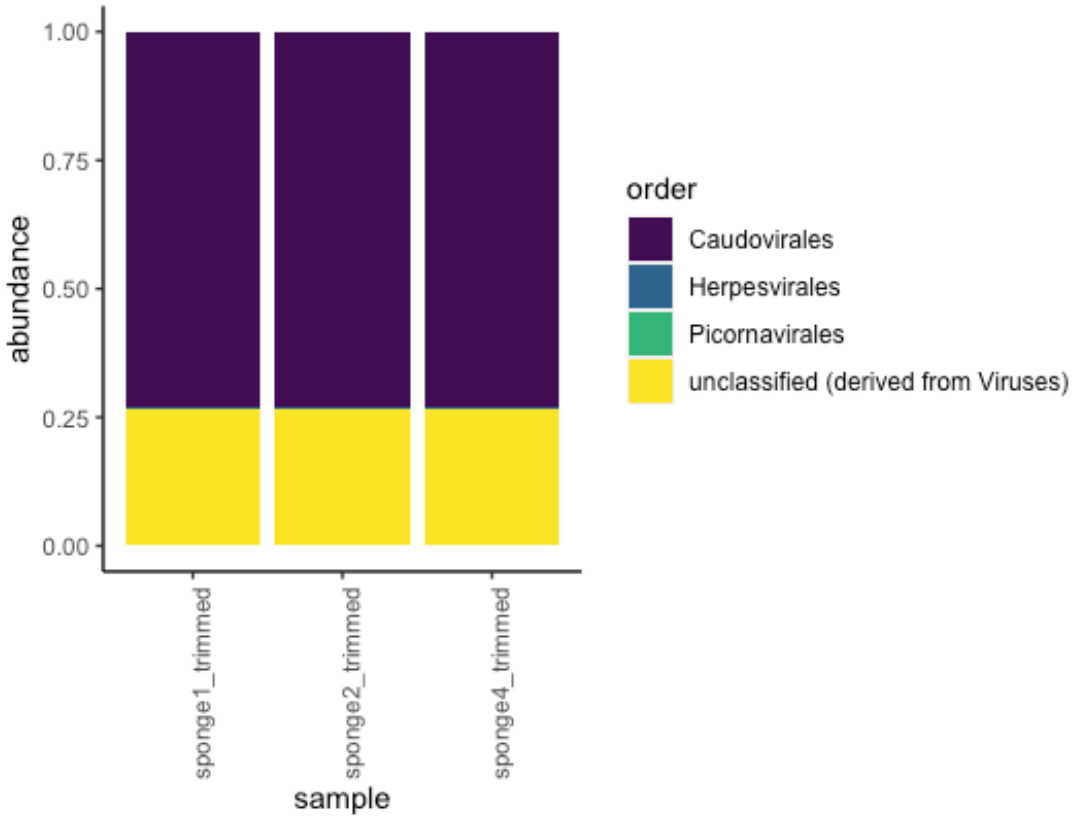
SUPPLEMENTARY FIGURES



Supplementary Figure 7.1 -Eukaryotic phyla detected in *H. panicea.* Despite the sample being directly extracted and sequenced from the sponge, there is an overwhelming amount of non-poriferan and non-microeukarytic reads present in each sample.



Supplementary Figure 2-Archaeal phylum of the sponge, *Halichondria panicea*. Further breakdown of the phyla revealed archaeal classes belong to unclassified Thaumarcheota. Other groups that dominate the sponge are the Methanomicorbia and the Halobacteria. Groups with lower relative abundance also include the Thermoprotei and Thermococci.



Supplementary Figure 3-Viral orders detected in the sponge, *Halichondria panicea*. Low levels of viral sequences were detected, but Caudovirales were able to be detected.

8.0 GENERAL CONCLUSIONS AND DISCUSSION

A comprehensive look at the metagenome of sponges and analysis of microbial community structure throughout a mariculture setup were undertaken. This research has highlighted areas of sponge microbial ecology that would be of relevance to biotechnology and explored under researched areas.

In this chapter, the general conclusions of each main work area will be recapped with respect ot the aims and objectives highlighted in Chapter 1.0. In addition, the inferences of this study will be discussed in a broader context about sponge microbial ecology and metagenomics along with future areas of work.

8.1 CONCLUSIONS

1) **Determine the total and active community structure of the tropical marine sponge, *Tedania ignis*, in a mariculture setup, using 16S rRNA amplicon sequencing (Chapter 3.0)**

Most microbiome studies focus on the total microbial community which may overtake the rarer communities of microbes due to high copy number of rRNA, but these higher copy number bacteria may not be fully active in the sponge. As such, alternative methods are needed to be able to view some of the rarer microbial communities that may be acting within the sponge, but on a smaller scale. While some communities may be seen as stable with total microbial community sequencing using genomic DNA (gDNA), utilization of complementary DNA (cDNA) shows that the microbial communities in the sponge are more dynamic than previously thought. It was hypothesized that bacterial communities in *Tedania ignis* would show a shift in the dominant community over time in a mariculture setup and that active community members would show a strong shift towards lesser abundant, but more active groups of bacteria and archaea. The basis behind it being that RNA is synthesized from DNA to eventually make proteins in living cells and has previously been used in a couple of sponge microbiome studies (Kamke et al. 2010; Moitinho-Silva et al. 2014). Indeed, microbial communities sequenced from gDNA showed more stability whereas those sequenced from cDNA show marked shifts, particularly in the rarer microbiome. For *T. ignis*, a large portion of bacteria were unclassified, but others, such as *Candidatus*

Branchimonas, a fish pathogen (Toenshoff et al. 2012; Mitchell et al. 2013) and the family Oceanospiralles, in which the marine invertebrate symbionts, *Endozoicomonas*, (Nishijima et al. 2013; Neave et al. 2017), were present. The presence of *C. Branchiomonas* is not well understood in the sponge, but in previous work has been detected at sponges found in more polluted areas (Turon et al. 2018; Turon et al. 2020). *Endozoicomonas* sp*.,* however, are of great interest as they are important nutrient cyclers (Nishijima et al. 2013; Schreiber et al. 2016) and may produce novel natural products for the sponge and other invertebrates (Nishijima et al. 2013) and likely has a different role depending on the invertebrate organism it inhabits (Neave et al. 2017). Broken down further, in the genomic DNA samples the abundant microbial symbionts have been identified as Mycobacteria (Moeseneder et al. 2001) and Sulfurovum. Mycobacteria are found in many environments and have been implicated in cases of diseases of humans (Tortoli et al. 1996; Ucko et al. 2002; Smith 2003; Ucko and Colorni 2005; Petrini 2006; Sette et al. 2015), birds (Biet et al. 2005), and livestock (Woodroffe et al. 2009). Sulfurovum was another group of identified bacteria and they serve an important purpose in the sulphur cycle as they uptake sulphur as an electron donor (Inagaki et al. 2004) rather than oxygen. The reasoning behind these communities being so high in an otherwise “low microbial abundance” sponge is still unclear. Most of the bacteria identified in the cDNA samples are unclassified. Lower levels of *Mycobacteria* and *Sulfurovum* were still detected in these samples. Overall, the incorporation of cDNA revealed more of the *Tedania ignis* microbiome. While it is a valid method of finding rarer biospehere organisms, other methods, such as live/dead staining should be used to confirm if there are high

numbers of dead bacteria within the samples. Further, to show if this had any effect on tedanolide and other metabolites, a follow up can include generating a metabolic profile concurrent with a microbiome study.

2) **Microeukaryote community analysis of three tropical sponges using high throughput sequencing methods (Chapter 4.0)**

Understanding of fungal communities within marine sponges is an often-understudied topic in microbiome research, with only a few studies and most focusing on the culturable fungi from sponges, with only a small portion dedicated to non-culture based studies utilizing techniques like denaturing gradient gel electrophoresis (DGGE) and one or two studies utilizing high throughput sequencing (HTS) methods. Particularly for HTS, many of the reads come back as the study organism with only a few that may be fungal or microeukaryotic in nature. To this end, fungal/eukaryotic databases are not as robust as those dedicated to prokaryotic organisms. It was hypothesized that the fungal structure of marine sponges could be determined using conventional ITS2 primers and high throughput sequencing. Most reads came back as the host organism with one set of host sequences identifying one sponge species, *Collospongia auris*, as a different one *Lendenfeldia chondrodes.* For the eukaryotic communities, after filtering of sponge sequences, samples became much more diverse. In *Lendenfeldia chondrodes*, the early sample showed little diversity with the first sample being dominated by metazoan reads. The cDNA sequenced sample showed a dominance of the common fungus *Malasezzia sp.* As the samples move forward in time, the communities start to become more diverse with further samples being dominated by protistan and ciliate reads. Overall, diversity in *L. chondrodes* was

fairly low. The *Haliclona* sp. reads in the first sampling were dominated by mostly ciliate and vague eukaryotic reads. Overall, diversity in this *Haliclona* sp. was low. In *Tedania ignis*, reads were dominated by ciliates and other protists and low levels of fungi. Findings from this experiment have led to the conclusion that the microeukaryotic diversity was fairly low. These results suggest either the sponges are all low abundance with fungi and other microeukaryotes, or it is possible that due to the presence of more dominant and active organisms in the sponge. Other reasons could include that the microeukaryote sequences did not amplify well, cell walls were not broken down enough to obtain DNA or RNA creating PCR bias, or possibly, the UNITE database may not be populated with enough fungi so reads match with the next best. A followup using other ITS regions, or other metabarcoding markers to determine fungi and other microeukaryotes in a culture-independent manner would be ideal. Fluorescent methods or TEM would also be ideal to determine the presence of yeasts or fungal hyphae in the sponge.

3) **A culture independent analysis, using shallow shotgun sequencing, to determine the microbial (bacteria, archaea, and fungi) components of the tropical sponge *Cinachyrella keukenthalli* (Chapter 5)**

In this research, one of the biggest objectives was to not only identify microbial components of *Cinachyrella keukenthalli*, but to also begin to understand the diversity of functional genes in the sponge. One of the first steps was to perform shallow shotgun sequencing on specimens of *Cinachyrella*. It was found to be dominated by

Proteobacteria, Candidatus Poribacteria, and lower levels of *Actinobacteria* based on culture- and assembly-independent analysis. Interestingly, this set of samples lacks *Chloroflexi* which in combination with *Candidatus Poribacteria* would identify this sponge as high microbial abundance (HMA) (Gloeckner et al. 2014; Chaib De Mares et al. 2017). This is also in contrast to previous *Cinachyrella* datasets from the same area wherein *Chloroflexi* was detected but in low amounts (Cuvelier et al. 2014) in one group of Cinachyrella, SG2, but it was not detected or below detection limits in SG1, despite being collected from the same area, showing that there are two groups of *Cinachyrella* based on their microbiome. With regards to eukaryotic sequences, most identified as chordates or as poriferan reads. Low levels of other eukaryotes like fungi and protists were detected. No viral reads were detected. In *Cinachyrella keukenthalli*, high levels of functional genes for amino acid, carbohydrate, lipid, and energy metabolism are detected. In lower, but still fairly detectable levels are genes for the metabolism of terpenoids and polyketides and for xenobiotic degradation. Xenobiotic degradation is important for sessile organisms to survive in certain environments. *Cinachyrella* is found in nearshore environments along the coast of the tropical and subtropical Atlantic. These locations are densely populated coastal areas increasing the possibility of xenobiotics entering the seawater. Genes for the biosynthesis of other secondary metabolites were detected in low numbers. *Cinachyrella keukenthalli* contained low levels of biosynthetic gene clusters, but terpenoid and polyketide genes were still detected which have some defense functions for the sponge and could be a future source of natural products. In order to highlight the microbial symbionts and metabolic profile more robustly, a larger sample size is needed to allow a full

comparison and make stronger conclusions about this sponge. The overall aim of this chapter was to provide a structural and metabolic profile and a further comparison to prior metabarcoding work. While there are interesting insights with the current data, the small sample size precludes making a truly definitive statement, however, a future analysis incorporating more samples is planned to expand upon these findings.

4) **A culture independent analysis, using shallow shotgun sequencing, to determine the microbial (bacteria, archaea, and fungi) components of the tropical sponge *Hymeniacidon perlevis* (Chapter 6)**

Hymeniacidon perlevis is a common sponge along the rocky coasts of Europe and the United Kingdom. It is characterised by its bright orange colour and can frequently be found on rocks at low tide. *H. perlevis* is unique in that it can survive being exposed to air unlike other sponge species. *H. perlevis* is dominated mostly by Alpha-, Beta-, and Gammaproteobacteria and contains low levels of Actinobacteria, Firmicutes, and Cyanobacteria, which was surprising. Archaeal sequences are found in low abundance within *H. perlevis*. Major groups of eukaryotic sequences have been identified and include sequences from chordates, arthropods, cnidarians, and echinoderms. It is possible that some environmental DNA was detected within the sponge as sponges feed by filtering the water column and any organisms nearby could be detected (Mariani et al. 2019; Turon et al. 2020). Other microbial eukaryotes like fungi and protists were found in much lower numbers. Ascomycota groups, for example, only make up about 3-4% of the fungal reads within *H. perlevis* and Basidiomycota makes up an even smaller portion within the sponge at about 0.1%.

Despite the relatively low occurrence (<0.01%), some viral reads were also detected and yielded some incredible diversity with the majority belonging to Phydnaviridae, which is a group of viruses that infect algae. On the functional level, using KEGG Orthology (KO), the highest detected genes are those for metabolism as found in the previous chapter. Besides amino acid, energy, and carbohydrate metabolism being the dominant functions, low levels of terpenoid and polyketide metabolism have been identified. Interestingly, genes for xenobiotic metabolism were detected in *H. perlevis* and previous literature discusses using its use for natural bioremediation of microbial pollution. With xenobiotic genes present in the metagenome, such as those for aminobenzoate degradation, further investigations could reveal *H. perlevis*, its symbionts, and/or derived enzymes could be useful in remediation of contaminated areas. Overall, this aim of this chapter was to provide a profile of the microbial constituents and metabolic potential of *Hymeniacidon perlevis.* As cyanobacterial reads were not detected as expected, a follow-up could include a more targeted look at the symbionts using methods like qPCR or perhaps alternative extraction methods.

5) **A culture independent analysis, using shallow shotgun sequencing, to determine the microbial (bacteria, archaea, and fungi) components of the tropical sponge *Halichondria panicea* (Chapter 7)**

Halichondria panicea, known commonly as the breadcrumb sponge, is a species native to the rocky coasts of Europe and the United Kingdom. *Halichondria panicea* can be found attached to rocks at low tide, therefore, being exposed to air, often in the same places as *Hymeniacidon perlevis*. *H. panicea* is dominated by bacteria and

contains lower levels of Archaea and eukaryotic reads. Viral reads were not detected in high numbers withing *H. panicea*. Among the bacterial groups detected, Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria are among the highest groups identified which is a common characteristic of sponges identified as low microbial abundance. These results are similar to a previous 16S rRNA gene sequencing study of *H. panicea* from Iceland (Knobloch et al. 2019). Fungal groups were identified within *H. panicea* with the dominant groups belonging to Eurotiomycetes, Saccharomycetes, and Sordariomycetes, groups of yeasts belonging to the class Ascomycota. Unexpectedly, low levels of poriferan reads were detected. This is odd given that this sponge is considered an LMA sponge. A possible reason could be the extraction method which could have not lysed the sponge cells effectively. A different mixture of chemical lysis and better mechanical lysing may be needed to address the low host read count.

As seen in previous chapters, metabolism genes are the highest group of functional genes within the sponge. Most identified genes include those for amino acids, carbohydrates, energy, and nucleotides. Only a few encompass the metabolism of cofactors and vitamins and even fewer are identified as metabolizing terpenoids and polyketides. *H. panicea* contains genes for polyketide synthases and a small abundance of Actinobacteria reads, about 2%,1%, and 3% in sponge 1, 2, and 4, and previous research on *H. panicea* associated Actinobacteria shows it contains polyketide synthase genes and is responsible for 122 compounds with 88 yet to be identified and *H. panicea* serving as a potential source of antimicrobial compounds

(Schneemann et al. 2010; Rodriguez Jimenez et al. 2021). Overall, this chapter aimed to identify the taxonomic and metabolic makeup of the sponge, *Halichondria panicea*. Anticipated microbial groups were not detected in the reads suggesting the environment, the host, or possible extraction inefficiency could have influenced the results.

8.2 CONCLUDING REMARKS

This research has begun to show the microbial makeup of marine sponges from a completely culture-independent scope. Coupled with future research, this could have great impliations on sponge microbial ecology, mariculture, health of aquatic environments, and natural product research. This research and future research must be pursued in order to understand how sponges interact with their environment and how their microbial symbionts interact and respond to environmental changes or even static environments, like what might be found in most tank and raceway mariculture setups. This work highlights the need for the integration of multiple approaches to identify abundant and rare symbionts, and highlight overlooked and understudied groups like symbiotic eukaryotes. Further, many results are unexpected and inconsistent with prior works and future efforts incorporating larger sample sizes and alternative extraction methods are necessary for resolving these inconsistencies. Also, to note, further research into sponge metagenomes has the potential for the discovery of novel microorganisms and/or natural products.

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