11-12-2015

Bacterial Community Dynamics In Marine Sponge Cinachyrella kuekenthali Under Irradiance and Antibiotics

Nidhi Vijayan
Nova Southeastern University, nv215@nova.edu

NOVA SOUTHEASTERN UNIVERSITY OCEANOGRAPHIC CENTER

BACTERIAL COMMUNITY DYNAMICS IN MARINE SPONGE *Cinachyrella kuekenthali* UNDER IRRADIANCE AND ANTIBIOTICS

By

NIDHI VIJAYAN

Submitted to the Faculty of
Nova Southeastern University Oceanographic Center in partial fulfillment of the requirements for
the degree of Master of Science with a specialty in:

MARINE BIOLOGY

Nova Southeastern University

December 2015

TABLE OF CONTENTS

ACKNOWLEDGMENT

I would like to thank my advisor, Jose Lopez for all his support and guidance. Also, for his encouragment and trust to pursue the experiments to accomplish my thesis project. I have become more confident and responsible under his advisement.

I thank my committee members, Patricia Blackwelder and Cristina Diaz for having confidence in my work. Everything I have learnt about Electron Microscopy is due to Patricia Blackwelder. She has showed me the beauty and the importance of imaging microscopic structures. I would like to thank Cristina Diaz for imparting to me her passion for sponges and reminding me the importance of my work, when I needed it.

I would also like the thank Abby Renegar for teaching me skills and techniques to perform histological analyses on my samples. Thank you to Nick Turner for his advice on tank equipment and maintenance. Thank you to Sanda Lalicic for assisting me with sectioning of my samples for transmission electron microscopy analyses.

Thank you to my family and friends for their support throughout my Masters program. Thank you also to members of my lab Alex, Andia, Lauren, Renee, Cole and Jorie for their advice and support on my thesis project.

ABSTRACT

The Marine sponge *Cinachyrella sp* used in this study are commonly found in offshore South Florida and Caribbean waters and appeared to be resilient in closed system aquaculture. Marine sponges host diverse bacterial symbionts that are distinct compared to bacteria found in ambient seawater, however the roles of a large fraction of the bacterial community in marine sponges are unknown. Comparison of symbiotic to aposymbiotic (bacteria-free) sponges could provide information about interactions (metabolic and physiologic) between the bacteria and sponge. In this study, a single *Cinachyrella kuekenthali* individual was subsectioned into explants (N=240) in order to provide identical bacterial communities to perform comparative studies. Presence of photosymbionts was also analyzed by characterizing bacterial communities from varying light and dark conditions. Tools for characterization included transmission electron microscopy (TEM) and 16S rRNA sequence analysis obtained from Illumina Miseq. High throughput DNA sequencing revealed bacterial taxa belonging to phyla Thaumarchaeota, Chloroflexi, Nitrospira, Acidobacteria and Verrucomicrobia persist in the explants. This study also demonstrated that antibiotics (Ampicillin, Tetracycline, Penicillin-Streptomycin and combination of all) can alter the bacterial community in the marine sponge *C. kuekenthali* explants *in vitro*. Bacterial communities of explants treated with different antibiotics were statistically (Unifrac and Bray-Curtis analysis) different from controls (p-value < 0.001, R2=41%). Penicillin-streptomycin and cocktail of antibiotics treatment contributed to the highest difference in the bacterial communities. Also, bacterial communities of explants at difference time points treated with corresponding antibiotics were also statistically significant (p-value<0.05, R2=15%). TEM observations of denatured nucleic acid and osmotic lysis of bacteria, due to the effect of antibiotics were observed, creating a LMA mesohyl. However light versus dark conditions did not produce any statistically significant difference in beta diversity between bacterial communities. These interdisciplinary results indicate that while individual bacterial symbiont taxa may persist after community disruption, significant changes in the overall composition of the bacterial symbiont population can be created.

Key words: *Cinachyrella sp,* aposymbiotic, antibiotics, time, photosymbionts, HMA

List of Figures

List of Tables

List of Abbreviations

DNA- Deoxyribonucleic acid
rRNA- ribosomal ribonucleic acid
OTU- Operational Taxonomic Unit
QIIME- Quantitative Insight Into Microbial Ecology
NCBI- National Center for Biotechnological Information
BLAST- Basic Local Alignment Search Tool
CARD-FISH- Catalyzed Reporter Deposition Fluorescent in situ Hybridization
HMA/LMA- High/Low Microbial Abundant Sponge
NLR- Nod-like Receptor
SRCR- Scavenger Receptor Cystein Rich Domain

INTRODUCTION

Sponges are found in all biotopes from the tropics to the poles with an estimated diversity of 11,000, of which 8500 are considered valid (Van Soest et al., 2012). In marine ecosystems, sponges enable efficient recycling of dissolved organic matter and nutrients to higher trophic levels in nutrient poor coral reefs through the sponge loop (Goeji et al., 2013). Therefore, sponges play a vital role in the coral reef ecosystem.

Sponge-derived compounds have been used in biomaterial engineering and in pharmaceutical industries. For example, biosilica-producing enzymes from sponges have been applied in nanotechnology (Schröder et al., 2007) and avarol from *Dysidea avara* have *in vivo* antileukemic activity and inhibits *in vitro* replication of HIV (Muller et al., 2000).

Sponges may be composed of up to 40% bacterial cells (Schmitt et al., 2012). The role of bacteria in sponges has been highly debated (Webster and Blackall 2009) and while certain bacterial species have a proven impact on sponge health, the specific beneficial relationship with a majority of bacteria is yet to be demonstrated. Understanding the relationship between host and symbiont can reveal how the symbiosis occurs and persists. Symbiosis is not simply an interaction between organisms but an innovative mechanism of survival (Seckbach, 2002).

Sponges

Sponges belong to the phylum Porifera, which are among the most primitive multicellular organisms. These sessile organisms occur in all seas, wherever there are rocks, shells, submerged timbers, or corals to provide a suitable substratum. Their bodies vary in size and shape (Bergquist 1998). Sponges possess an active filtration system of incurrent openings, channels, chambers and excurrent opening to obtain food and oxygen from the surrounding water and remove waste from their body. In this system, flagellated, ameboid cells, collagen and skeletal elements that fill the internal space are essential components of this process (Müller 2003). The whip-like motion of the flagella of the choanocyte cells causes an unidirectional movement of water and other nekton through the pores called the ostium(a), into the body of the sponge. Water is lead to the

spongocoel by incurrent and excurrent channels collecting it from the choanocyte chambers, which in turn leads to excurrent opening, called the osculum (oscula) through which all waste products are released from the sponge body (Ruppert. et al., 2004).

Sponge Natural Products and Cultivation

There are approximately 7000 sponge derived novel molecules, however only hundreds are used in the industry due to the difficulty in obtaining sufficient biomass from natural populations (Blunt et al., 2009). The compounds derived from natural sources are generally developed by chemical synthesis However, the potency of these complex compounds have to be additionally assessed, before a potential drug is identified. This can be done with preclinical studies, which in turn, requires large amounts of the natural compounds. Since bioactive compounds are found in such small quantities in nature, large amounts of biomass needs to be harvested. Doing so could disrupt the ecosystem. For example, 7000 tons of *Lissodendoryx sp* were needed to obtain halichondrin B to treat 25% of all melanoma patients. However, only 300 tons exist in nature (Sipkema et al., 2005). This paradox of early drug development being dependent on biological production methods, is often referred to as "The Supply Issue" (Osinga et al., 1998, Faulkner 2000).

Sponge farming can be adopted to increase sponge biomass, however the methods of aquaculture varies from species to species. These include horizontal ropes, coral boulder and artificial substrates (Schiefenhövel and Kunzmann 2012). Information regarding optimal external environmental conditions and nutrient requirement is necessary for successful farming (Duckworth 2009).

One way the supply issue of sponges may be overcome is by *in vitro* culture techniques (Schippers et al., 2013). *In vitro* cultivation allows for control of culture conditions and manipulation to increase product concentrations. Loosely organized cellular structure and the presence of totipotent cells, appear to favour the initiation of sponge cell lines (Sipkema et al., 2005). However the presence of sponge associated microorganisms make it difficult to establish pure contaminant-free sponge cell lines, also referred to as *axenic* cultures.

Explant culture has proven to be successful in studies of cell proliferation and development of biomass (De Caralt et al., 2010). Carballo et al. (2010) have published results indicating fragment culture to be a viable method of producing metabolites. Different cultivation methods result in different sponge metabolite activity. For example, compared to wild sponges, cell culture results in a low concentration of lipids, loss of sterols and volatile compounds (De Rosa et al., 2002).

The presence of microbes presents a challenge to *in vitro* cultures of sponge biomass for the purpose of extracting metabolites (Lopez et al., 2002). The addition of a cocktail of antibiotics has proved ineffective in obtaining an axenic culture (Schippers et al., 2012). Thus, more documentation about changes in bacterial diversity and abundance in experiments would provide insight into the sponge microbial response to the antibiotics.

Cinachyrella sp

The genus *Cinachyrella* belongs to the class Demospongiae and family Tetillidae of the order Tetractinellida (Morrow and Cardenas 2015). These globe shaped sponges possess concave depressions (porocalices) that contain aggregations of microscopic incurrent pores. The commonly called "golf ball sponge" is yellow to orange–red externally and yellow-orange internally (Rutzler et al., 1992) (Appendix 7). Its geographic distribution ranges from shallow coastal waters of North Carolina to the south Atlantic waters of Brazil. Three common species of *Cinachyrella* found in waters off South Florida are *C. kuekenthali*, *C. alloclada* and *C. apion* (Cardenas et al., 2009). Sequence analysis is the optimal method to identify the sponge species. This is due to the structural similarities of the species in the genus making it difficult to distinguish the species of *Cincahyrella* by macroscopic observations (Table 1). The spiny microoxeas of *C. kuekenthali* viewed under the compound microscope can distinguish it from *C. apion*.

Table 1: Morphological description and Identification of *Cinachyrella* species (Smith et al., 2013, Master's thesis).

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

Some natural metabolites extracted from *Cinachyrella sp* have been found to be beneficial. For example, the first natural 6-hydroximino-4-en-3-one steroids were isolated from *Cinachyrella* sp by Rodriguez et al., in 1998. This molecule is an example of a metabolite that can be deployed against a specific type of tumor cells.

Another example of bioactive sponge compound is Enigmazole A and related compounds. It has been isolated from *Cinachyrella enigmatica* that selectively target aberrant c-Kit signalling. C-kit is a type of transmembrane protein- tyrosine kinase that is

important in the control of gametogenesis, hematopoiesis, mast cell development and function, and melanogenesis. This kinase has implications for cancer patients with a specific c-Kit genotype that undergo treatment with Gleevec (Novartis Pharmaceutical Corp: common name: imatinib mesylate). Enigmazole A also shows cytotoxic effects against IC-2 mast cells (Skepper et al., 2010).

In 2012, bioactivity-based screening of *Cinachyrella sp* extract identified an ancestral member of the galectin family based on its unexpected ability to positively modulate mammalian glutamate receptor function. They play important and diverse physiological roles, particularly in the immune system of sponges, and are thought to be critical metastatic agents for many types of cancer cells, including gliomas; a type of tumor that originates in the brain or spine, by inhibiting cell proliferation (Freymann et al., 2012).

Marine Sponge as Microbiome

The microbial symbionts and their genes make up the microbiome, that can provide traits not evolved by the host (Turnbaugh et al., 2013). The majority of the bacterial symbionts are unculturable. Using DNA sequencing of 16S ribosomal RNA (rRNA) gene of bacterial communities, new taxa can be identified in the environment (Patin et al., 2013). The 16S rRNA gene is weakly affected by horizontal gene transfer and is universally distributed in all mitochondrial genomes. This universality of 16S rRNA allows for phylogenetic and taxonomic classification (Wang and Qian 2009). The similarity of the 16S rRNA reads to a reference database is used to identify the bacterial taxa. The cluster of reads with 97% similiarity is also referred to as Operational Taxonomic Units (OTUs) (Chang et al., 2008).

Schmitt et al., (2012) compiled 11,000 16S rRNA sequences from sponge associated bacteria that included alpha-,beta-,gamma-, and deltaproteobacteria, Acidobacteria, Actinobacteria, Bacteriodetes, Chloroflexi, Cyanobacteria, Firmicutes and Poribacteria, which can constitute upto 40% of the biomass in some sponges. Sponges provide a suitable substrate for attachment and nutrients for microbial symbionts

(Schippers et al., 2012). The sponge hosts that possess a high concentration of bacteria in their mesohyl are called bacteriosponges or high microbial abundance (HMA) sponges,. Those with low concentrations are called low microbial abundance (LMA) sponges (Hentschel et al., 2006). HMA sponges possess a denser mesohyl, more complex aquiferous system with narrower and longer water canals that allows slower filtration rates (Weisz et al., 2008). δ^{15} N ratio metabolic differences were also observed in *Ircinia felix* and *Aplysina cauliformis* (i.e. LMA) compared to *Niphates erecta* (HMA) . Microbes in LMA sponges provide nitrogen while *N.erecta* obtained nitrogen solely from an external source (Weisz et al., 2007).

Sponge associated bacteria have been hypothesised to produce vitamin B12 utilized by the sponge, assist in ammonium assimilation and in the generation of reductive energy (Thomas et al., 2010). Recent evidence identifies specific prokaryotic genes involved in various metabolic pathways that could be activated by the sponge. For example, key enzymes for thiamin synthesis were found in metatranscriptome of *Xestospongia muta* and the genes for the activation of the pathway were found in the sponge transcript (Fiore CL et al., 2015). However, the beneficial functions of a large fraction of bacterial species associated with sponges have yet to be documented.

Sponge-Bacteria Dynamics

Sponge-microbe dynamics is still under scrutiny. Sponges with similar eco-evolutionary characteristics share more microbes between them than the hosts with different characteristics (Björk et al., 2013). Yet the factors that structure sponge-bacteria associations remain poorly understood (Noyer et al., 2014). In some cases, sponges maintain a stable bacterial community across spatial and temporal scales (Erwin et al., 2012, Friedrich et al., 2001) while others can be affected by many factors. These factors include environmental changes, geography (Taylor et al., 2005), pollution (Webster et al., 2001), temperature (Webster and Blackall 2009), transfer into aquaculture (Mohamed et al., 2008) or disease-related physiological changes (Webster et al., 2008).

Molecular Biology of Sponge-Bacterial Interaction

The capacity of sponge cells to distinguish between food and symbiotic bacteria has been discussed since Bergquist (1978) observed no particle selection feeding of sponge cells using latex beads and Indian ink. Wilkinson (1984) observed choanocytes and pinacocytes performed initial phagocytosis followed by amoebocytes in the mesohyl.

Genomes of symbiotic bacteria possess large numbers of mobile elements that are proposed to have an important role in the evolution of bacterial genomes for symbiotic relations (Thomas at el., 2010). Four eukaryote-like, ankyrin repeat proteins (ARP) found in uncultured gamma-proteobacterial sponge symbiont were made to express in *E.coli* using recombinant technique and it appeared to interfere with phagosome development of amoebal cells. This interference mechanism suggests a possible method by which bacteria escape digestion by sponge amoebocytes (Nguyen, Liu and Thomas 2014). Metatranscriptomics of sponge associated bacteria also suggest TPR (tetratricopeptide repeats) secrete extracellular protein which could present a mechanism by which they avoid digestion.

Sponges also use pattern recognition receptors, namely NLR (nucleotide-binding domain and leucine-rich repeat containing) that can recognize a broad spectrum of microbial ligands, critical in mediating animal-bacterial communication. For example, *Amphimedon queenslandica* genome encodes approximately 135 bona fide NLR genes (Degnan, 2014). Molecules containing scavenger receptor cysteine-rich (SRCR) domains have also been identified in some sponges and have high a similarity to mammalian surface antigen which suggests they could be involved in bacterial-cell recognition (Muller and Muller 2003, Steindler et al., 2007).

Microbiomes in Aquaculture

It is important to determine feasibility of growing sponges in *in vitro* and aquaculture systems to evaluate their effect on sponge health (Nayer et al., 2014). In order to extract compunds from unculturable bacteria in sponges, artificial culturing of sponges may be necessary. Some cases have found aquaculture of sponges, provide less perturbation of the bacterial symbiont community (Mohamed et al., 2008).

In other cases, there have been changes in the bacterial community in aquaculture environment. An increase in bacterial community in *Mycale laxissima* and an increase in alpha- and gamma- proteobacteria in *Ircinia strobilina* in aquaculture compared to wild were discovered (Mohamed et al., 2008). The diversity in *Ircinia strobilina* increased after 3 months in the tank and reduced to an intermediate level after 9 months, suggesting acclimatization. Bacterial community of Mediterranean sponge *Aplysina aerophoba* remained stable for the 11 day period, observed under starving conditions in recirculating seawater aquaria (Friedrich et al., 2001). A stable bacterial community was also observed in *Rhopaloeides odorabile* (Webster et al., 2011) and *Aplysina aerophoba* (Hausmann et al., 2006).

Effect of Light on Sponge Symbiocosm

The most prevalent photosymbionts in sponges are cyanobacteria (Erwin and Thacker, 2007) in addition to dinoflagellates and filamentous algae. The translocation of 9 to 17% of total photosynthate from a chlorella-like green alga to the freshwater sponge *Ephydatia fluviatilis,* a phototrophic sponge has been identified (Wilkinson 1979). This may not be the case in all sponges hosting phototrophic organisms such as cyanobacteria, filamentous algae and dinoflagellates. For example, coral reef sponges can comprise 25-50% cyanobacterial cells yet relatively little is known about metabolic exchanges between the organisms (Taylor et al., 2007). Studies suggest the relationship between the sponge and its photosymbiont varies with sponge species. For example, the marine sponge *Lamellodysidea chlorea* has a mutualistic relationship with the filamentous cyanobacterium *Oscillatoria spongeliae* whereas, marine sponge *Xestospongia exigua* was found to be commensalistic with the unicellular cyanobacterium *Synechococcus spongiarum* (Thacker 2005). *S. spongarium* may represent a generalist symbiont with the ability for widespread dispersal. In 2008, Erwin and Thacker revealed three clades (A, B and C) of *S. spongarium* that had variable nutritional benefits for *Neopetrosia subtriangularis*.

Sponges may be defined as phototrophic if the ratio of gross primary productivity to respiration exceeds 1.5, implying the sponge receives a significant amount of nutrition from photosymbionts (Wilkinson 1979). Determining the amount of chlorophyll-a is a

common technique for estimateing the percentage of cyanobacteria in the sponge, however it may only estimate the amount of phototrophic bacteria present in the sponge and does not demonstrate a symbiotic relationship. Cyanobacteria may in turn, benefit from the sponge through nutrition, UV protection, nitrogen fixation and production of secondary metabolite (Erwin and Thacker 2008).

Species Specific Bacterial Community

The presence of distinct microbial communities in different sponge species is still under debate. *Aplysina aerophoba* and *Theonella swinhoei* from geographically separated regions, share high similarity in bacterial communities distinct from that found in the ambient seawater (Hentschel et al., 2002). In contrast, comparative of *Cymbastela concentrica*, *Callyspongia* sp. and *Stylinos* sp from temperate Australia using denaturing gradient gel electrophoresis (DGGE) showed substantial differences, with little variability within each species (Taylor M. W. et al., 2003).

The bacterial communities in sponges collected from the Indian Ocean, Pacific Ocean, Mediterranean, Caribbean and Red Sea were compared and found large species-specific bacterial communities. That is, common Operational Taxonomic Units (OTU) were found in specific sponge species found in different locations (Schmitt et al., 2012).

Bacteria that were believed to be exclusive to low abundance sponges (eg. *Poribacteria*) were also present in seawater (Taylor et al., 2013). The bacteria detected in seawater could be in metabolically inactive dispersive phases. Only some members of a highly complex microbial system may be active at any particular time (Schaechter et al., 2006).

The consortium of microbes present in many sponges span the three Domains of life with the association ranging from mutualistic to commensalistic and exploitative (Taylor et al., 2007). The complexities of the sponge-microbe interaction can provide clues of origin, evolution and maintenance of sponge-microbe interaction.

Development of Aposymbiotic Model System

Aposymbiotic organisms are those that are symbiont-free that are most often induced or reared experimentally. For example, in order to study a cnidarian-dinoflagellate relationship, *Aiptasia* was chosen since it can exist without dinoflagellate symbionts, and was made aposymbiotic using cold-shocking and the photosynthesis inhibitor, diuron. The difference in gene expression was then analysed (Lehnert et al., 2012). A model for animal-microbe mutualistic association is the Hawaiian-bobtail squid and bioluminescent bacteria (McFall-Ngai 2014). Difference in proteome of uncolonized juvenile bobtail squid (aposymbiotic) and a colonized juvenile squid (symbiotic) was analyzed to understand the maintenance of this association (Bethany and Nyholm 2012). In another example, horizontal acquisition of *Burkholderia* bacteria by the bean bug (*Riptortus pedestris*) was also observed by rearing aposymbiotic bugs (Kikuchi et al., 2007). However, sponges have proved to be an experimental challenge to develop as aposymbiotic systems due to their complex microbial biota and the uncertain interactions between host and symbionts.

Action of Antibiotics on Sponge System

In 2012, Richardson et al., used sponge cell aggregates treated with different antibiotics to identify the difference between control and aposymbiotic sponge. This revealed different abundance in the bacterial community under different antibiotic treatment, although failed to create aposymbiotic sponge cell aggregates. Noticeable effects of the antibiotics (combinations of ampicillin and gentamycin) and starvation were not observed via TEM of *Aplysina aerophoba,* but there was a change in colony forming units (CFU) and difference in DGGE bands (Freidrich et al., 2001).

The experimental setup in this project used the following antibiotics: Penicillin-Streptomycin, ampicillin and tetracycline. These antibiotics were commonly used in sponge cell culture studies to control microbial contamination (De Rosa et al., 2003, Sun et al., 2007, Zhao et al., 2005). The mechanism of action of antibiotics include disruption of membrane structure, inhibition of enzymes involved in cell wall synthesis, nucleic acid metabolism or protein synthesis (Sigma-Aldrich).

Penicillin is a β-Lactam antibiotic that inhibits the formation of peptidoglycan link in the bacterial cell wall. This weakens the cell wall and osmotic pressure increases in the cytoplasm resulting in cell lysis (Van Bambeke et al., 1999). It mostly acts on majority of gram-positive bacteria. Ampicillin is derived from penicillin, and possess similar mechanism of cell wall disruption, afftecting gram-positive and gram-negative bacteria (AHFS 2006). These antibiotics are bactericidal and blocks the division of cyanobacteria, cyanelles, photosynthetic organelle of glaucophytes and chloroplasts in bryophytes (Kasten and Reski 1997).

Streptomycin inhibits protein synthesis by binding to S12 protein of 30S ribosomal subunit, thereby blocking translation, eventually leading to cell death (Sharma et al., 2007). Protein synthesis is inhibited by tetracycline by preventing binding of aminoacyl tRNA to acceptor site. Tetracycline is a broad spectrum bacteriostatic antibiotic, acting upon gram-positive and gram-negative bacteria (Chopra and Roberts 2001).

Antibiotics have been previously used to observe the effects of bacteria loss in sponges (Richardson et al., 2012). However, conclusive evidence suggesting the extent of dependence of the sponge on the bacterial community is yet to be established. This study hypothesises that antibiotics can be used to estimate the sponge-bacteria dependency. Once the success in demonstrating the change in sponge bacterial community due to antibiotics is established, difference in sponge bioactivity can be studied. Another study compared explants treated with antibiotics in 2 litres of seawater versus the whole sponge in a closed aquarium system without antibiotics with algae as food. It was found that the whole sponge had higher growth rates and explants showed long term survival and a loss in growth rate. Yet the explants continued to synthesize bioactive compounds (De Caralt et al., 2003).

Applications of Sponge- Associated Bacteria

Sponge microbial associates may provide a significant source of bioactive compounds. For example, antifungal activity was observed in the chitinase of *Strepromyces sp.* isolated from the marine sponge *Craniella australiensis* (family

Tetillidae). Compared with chitinase from terrestrial sources, marine chitinase has a higher pH and salinity tolerance which is useful for certain biotechnological applications (Han et al., 2009).

Marine sponges may provide novel compounds to prevent bacteria, viral, fungal and parasitic diseases that are gaining antibiotic resistance (Thomas et al., 2010). The therapeutic properties of secondary metabolites synthesised by microbial flora inhabiting sponges is a subject of great research interest and high biomedical potential.

HYPOTHESIS AND OBJECTIVES

H1 – *Cinachyrella sp* possess symbiotic bacterial community

- Compare 16S rRNA of bacterial communities in tank water and *Cinachyrella* sp explants, and baseline data.

H2 - *Cinachyrella sp* harbours photosymbiotic bacteria

- Sponge explants exposed to light and dark conditions are compared under electron microscopy, CARD-FISH and 16S rRNA metagenomics analysis

H3 - Antibiotic treatments possess the potential to develop aposymbiotic sponge explants

- Explants are treated with media containing cocktail of penicillin-streptomycin, ampicillin and tetracycline. Effects are observed under electron microscopy and CARD-FISH.
- Apply Metagenomic 16S rRNA analysis to compare and confirm the success of the treatments.

MATERIALS AND METHODOLOGY

Sample Collection

Cinachyrella sp was collected off the coast of Broward County, South Florida. They were then placed in the closed system aquarium with filtered seawater. The sponge for *in vitro* culture (day0) was kept in a closed system aquarium under starvation for 6 weeks. The *Cinachyrella sp* dead sponge(CinDead,CinDead2) was obtained when loss of

structure and discoloration was observed of after 6 months in closed aquarium under starvation.

The marine sponge made into explants was identified as *Cinachyrella kuekenthali* (Paco Cardenas's personal communication (Uppsala University)). It was yellow-orange sponge with a layer brownish-green filamentous algae, which was washed away prior to fragmenting. The sponge was subglobular shaped with an a diameter of 10cm approximately. *Cinachyrella kuekenthali* have concentrated pockmarks with circular cup-like depressions (porocalices) at the apical depression. They possess two main types of megascleres (Appendix 1): (1) straight rods with two pointed ends (oxea), sometimes kinked, rounded or stepped (2) Rods with one end pointed and other with 2-3 short pointed or blunt rays (Rutzler and Smith 1992).

Preparation of sponge explant culture

This protocol was adopted from Schippers et al., (2013). An individual *Cinachyrella* sp was in the tank for a period of 6 weeks under starvation to remove inactive bacteria in the sponge body. The sponge was processed by rinsing with filtered sea water and cut into pieces of approximately 15-20mm^3 in filtered seawater. The sponge pieces were placed in explant culture plates containing 8ml of artificial seawater (ASW) (Pomponi et al., 1997). Triplicates were made for each treatment specified below. A total of ten 24-well plates were prepared.

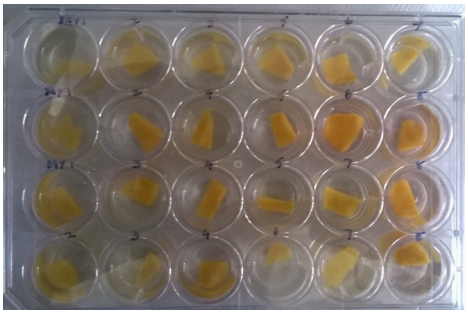


Figure 1: Sponge explant tissue culture plate at time point Day 0

Table 2: Sponge explants Experimental Plate Setup

Plate number	Culture Conditions
1	explant supplemented with combination of antibiotics under light
2	explant supplemented with 100IU/ml penicillin-streptomycin under light
3	explant supplemented with 100µg/ml Tetracycline under light
4	explant supplemented with 0.1mg/ml ampicillin under light
5	explant supplemented with combination of antibiotics under dark
6	explant supplemented with 100IU/ml penicillin-streptomycin under dark
7	explant supplemented with 100µg/ml Tetracycline under light under dark
8	explant supplemented with 0.1mg/ml ampicillin under dark
9	explant without antibiotic under light
10	explant without antibiotic under dark

Antibiotic treatment

Artificial sea water media was supplemented with a combination of 100IU/ml penicillin-streptomycin (Müller et al., 1999), 100µg/ml Tetracycline and 0.1mg/ml ampicillin (Rosa et al., 2003). Media was prepared and supplemented with each antibiotic

separately and in combination. The media was replaced with fresh media every 48 hours. The plates were kept in the incubator at 20°C.

Light treatment

The explants were cultured in tissue culture plates maintained in the light and dark (covered with foil). The transparent plates were illuminated throughout the experiment with 6W T5 10,000K and Actinic Bluelight, to simulate sunlight. The plates were kept in the incubator at 20°C with media replaced every 48 hours.

The explants were collected every 48 hours and stored in 2% gluteraldehyde in sodium cacodylate buffered seawater for electron microscopy. Samples were kept in 70% ethanol for DNA extraction, and 4% paraformaldehyde for 4 hours and then transferred to 70% ethanol for FISH. A total of 20 samples were prepared for EM and 30 samples for FISH experiments.

Transmission Electron Microscopy Preparation

Table 3: Samples used for TEM Ultrastructure study

Sample ID	Sample Description
day0	*Cinachyrella sp* explant day 0
CONTROLdark2	*Cinachyrella sp* control explant on day 2 under dark condition
CONTROLlight2	*Cinachyrella sp* control explant on day 2 under light condition
ABlight2	*Cinachyrella sp* cocktail of antibiotic treated explant on day 2 under light condition
ABdark2	*Cinachyrella sp* cocktail of antibiotic treated explant on day 2 under dark condition

CONTROLlight8	*Cinachyrella sp* control explant on day 8 under light condition
CONTROLdark8	*Cinachyrella sp* control explant on day 8 under dark condition
ABlight8	*Cinachyrella sp* cocktail of antibiotic treated explant on day 8 under light condition
ABdark8	*Cinachyrella sp* cocktail of antibiotic treated explant on day 8 under dark condition
CIN-W 2011	Wild *Cinachyrella sp* collected in 2011
CIN-T 2011	*Cinachyrella sp* kept in tank for 2 weeks in closed system aquarium in 2011

The sponge samples fixed and stored in 2% glutaraldehyde were rinsed with 3 changes of 0.05M sodium cacodylate buffer. After the third change of buffer samples were placed in 1% osmium tetroxide in buffer and samples postfixed in this solution for 45mins. The postfixative was removed with three changes of buffer. The pellet was then dehydrated in a graded series of ethanol (20%, 40%, 60%, 70%, 95% and 100%) by rinsing three times for 5 mins each. If necessary, samples were stored in 70% ethanol for an extended period.

Transmission Electron Microscopy

After dehydration, samples were embedded in Spurr^{TM} low viscosity resin after three changes for 5 mins each. The samples were then transferred to BEEM capsules and spurr resin was added to fill the capsule. Labels were prepared and placed in the capsules. The samples in resin polymerized for 48 hours at 60^{O}C in a stable temperature oven. After polymerization, the capsules were cut open and the solidified resin containing the sample was prepared for sectioning. Blocks were trimmed at the tip to a trapezoidal shape and

placed in a block holder in a Sorvall MT-2 Ultramicrotome. Sectioning presented challenges due to the many large spicules. However, it was decided to not de-silicify the sponge during fixation to avoid potential artifacts produced by this process. Sections were then stained with Reynolds lead citrate. The carbon and formvar coated grids with sections were observed in Philips 300 TEM at the NSU Oceanographic Center, and photographs taken on a CM-10 Philips TEM at the Miller School of Medicine at the University of Miami. In some cases spicule fragments were evident in sections, but were minimized by the utilization of a rapid microtome cutting speed and restriction to a single staining technique (Pb citrate only) to avoid spicule crystal disassociation from the sections during the staining washes.

Collecting Bacteria in Tank Water

One liter of tank water was filtered through 0.45µm filter in a filtration set-up using a vacuum pump. Bacterial DNA were isolated using PowerSoil® Powerlyzer DNA Isolation Kit (from MoBio) following the Earth Microbiome Project protocol.

16S rDNA sequencing

Table 4: The V4 region of the 16S rRNA sequence was sequenced for the following samples:

Sample ID	**Duration in treatment**	**Description/Treatment**
1. July2014	OUTGROUP	Wild *Cinachyrella sp* collected in July 2014 from South Florida
2. April2014	OUTGROUP	Wild *Cinachyrella sp* collected in April 2014 from South Florida

3. CinDead	6 months tank	Dead *Cinachyrella kuekenthali*
4. Water1		filtered seawater in closed tank containing *Cinachyrella sp*
5. Water2		
6. Water3		
7. Water4		Filtered sewater in closed tank not containing sponge
8. Day0	6 weeks tank	*Cinachyrella kuekenthali* collected in August 2014 transferred to closed tank system for 6 weeks
9. CONTROLlight2	48 hours	Control explant under light
10. CONTROLlight4	96 hours	
11. CONTROLlight10	240 hours	
12. CONTROLdark2	48 hours	Control explant under dark
13. CONTROLdark4	96 hours	
14. CONTROLdark10	240 hours	
15. ABlight2	48 hours	Explant in combination of antibiotics under light
16. ABlight4	96 hours	
17. ABlight10	240 hours	
18. ABdark2	48 hours	Explant in combination

19. ABdark4	96 hours	of antibiotics under dark
20. ABdark10	240 hours	
21. AMPlight2	48 hours	Explants in ampicillin under light
22. AMPlight4	96 hours	
23. AMPlight10	240 hours	
24. AMPdark2	48 hours	Explants in ampicillin under dark
25. AMPdark4	96 hours	
26. AMPdark10	240 hours	
27. PENSTREPlight2	48 hours	Explants in penicillin-streptomycin under light
28. PENSTREPlight4	96 hours	
29. PENSTREPlight10	240 hours	
30. PENSTREPdark2	48 hours	Explants in penicillin-streptomycin under dark
31. PENSTREPdark4	96 hours	
32. PENSTREPdark10	240 hours	
33. TETlight2	48 hours	Explants in tetracycline under light
34. TETlight4	96 hours	
35. TETlight10	240 hours	
36. TETdark2	48 hours	Explants in tetracycline under dark
37. TETdark4	96 hours	
38. TETdark10	240 hours	

The DNA sequences for samples ABdark10, AMPdark4, TETlight4 and TETdark10 were not considered for further analysis due to their poor quality reads.Tank water samples Water1, Water2, Water3 and water4 were from the same closed tank system. Water1, Water2 and Water3 were sampled with the presence of *Cinachyrella sp* in the tank. "water4" was sampled without any sponge in the tank and hence was not included in any statistical analysis (Table 4).

Genomic DNA of the bacteria were extracted by following the basic Earth Microbiome Project protocol (earthmicrobiome.org), except for the MoBio 96-well Manual Extraction Method. The primers used target the V4 region: 515F (5'-GTGCCAGCMGCCGCGGTAA- 3') and 806R (5'- GGACTACHVGGGTWTCTAAT-3') (Caporaso et al., 2011), which contains a unique barcode used to tag each PCR product. The presence of amplified product was confirmed by observations with gel electrophoresis (Sambrook and Russel et al., 2001).

The genomic DNA was prepared for the Illumina Miseq by following the Illumina 16S Metagenomic Sequencing Library protocol (http://web.uri.edu/gsc/files/16s-metagenomic-library-prep-guide-15044223-b.pdf). The protocol was modified to adjust for the low concentration of extracted genomic DNA; the template DNA was optimized to a concentration of 1.2ng/ul for a total volume of 10.5ul and 1ul of 5uM amplicon PCR forward/reverse primer. The genomic DNA was quantified using a Qubit fluorometer (Life Technologies, Oregon).

Sequence analysis

Raw DNA sequence reads were downloaded from Illumina BaseSpace platform and processed in Quantitative Insights into Microbial Ecology (QIIME v1.9.0) (Caparaso 2010). Operational taxonomic units (OTUs) were assigned using uclust (Edgar, 2010) with 97% similarity, using open reference OTU picking. Taxonomic assignments were made using the uclust taxonomic assigner with an 90% confidence cutoff and the greengenes 13_8 reference database The sequences were aligned using the greengenes reference alignment (DeSantis et al., 2006). Chimeric OTUs were detected and removed using the usearch61 (Haas et al., 2011) algorithm in QIIME. The resulting alignment was

used to create a phylogenetic tree with FastTree (Price, Dehal, and Arkin, 2010) and a resulting OTU table. The paired-end sequences of all samples were submitted to NCBI Sequence Read Archives (SRR2976095-SRR2976127).

Multiple rarefaction with lowest rarefaction depth of 10 and highest rarefaction depth of 100 at an 10 times increment using *multiple_rarefactions.py*. Alpha diversity was measured using shannon index of the rarefactions using *compare_alpha_diversity.py*

Beta diversity was analyzed using ordination plot and multifactorial non-parametric ANOVA (MANOVA) based on Bray Curtis and weighted Unifrac with NMDS and PCoA plots, on normalized dataset at 1061 reads per sample. Weighted Unifrac measures the difference between communities based on the shared evolutionary history and includes the difference in relative abundance (Lozupone et al., 2011). Both these factors are important when studying the effect of antibiotics as it may cause changes in relative abundance of bacterial symbionts that may share evolutionary lineage. On the other hand, Bray-Curtis provides differences in community composition based on OTU counts regardless of taxonomic relationship. Bray curtis distance measures uses an algorithm that uses measures distance between communities based on the number of species they share and total number of species (Galimanas et al., 2014). By contrast, unweighted Unifrac is based on presence/absence of OTUs, to show whether the community has shifted to a new community, i.e checking for contamination (Lozupone et al., 2011). Plots were visualized using *ordinate* command and ellipse at 95% confidence interval (default) using *stat_ellipse()* and ADONIS test from the R 'vegan' package was used to perform MANOVA (Oksanen et al., 2007). ADONIS test was performed to test the significance of differences between time points, treatment and light/dark condition. The statistical significance of the interaction of the factors: antibiotic-time, time-irradiance and irradiance and antibiotic were analysed. The test was repeated by interchanging the orders of the factors to confirm the results. The effect of each antibiotic used were also analysed using ADONIS test. This analysis uses a permutation test with pseudo-F ratios. The distance matrix was generated using Bray-Curtis and Unifrac indices with 999 permutation.

Community composition

Relative abundance of bacterial community composition of the top 100 OTUs which made up 97% of the data was used to construct barplot of all samples, based on antibiotics at the Phylum and Class level and based on time point at the Class level. This was performed using 'Phyloseq' package in R (McMurdie and Holmes 2013).

Preparing sponge explants for Fluorescent in situ hybridization

Sponge explant samples was embedded in paraffin wax using an automatic apparatus. The apparatus was used in order to immerse the cassettes containing the sponge in two 80% ethanol solutions, two 95% ethanol solutions, three 100% ethanol solution, three 100% xylene solutions and three paraffin wax solutions for a period of 30 minutes each. The gradual changes of percentages was required to prevent an extreme change in hydrophobicity that would damage the cells. Once the sponge was dehydrated and processed in paraffin, the cassettes were placed in a melted paraffin bath. Samples were taken out of the cassettes and cut at desired locations and placed with the cut side down into a mould which was filled with melted paraffin.

The paraffin-embedded sponge blocks was sliced into sections with an Accu-Edge low profile microtome blades (Sakura Finetek). Sponge sections for day 2, day 8, day 5 and day 14 were cut to a width of 10µM and placed on a warm water bath where the sections float on the top in order to smooth out the sections and to make it easier to mount. Sections were then floated on top of a glass slide. The slides were placed in an incubator for 12 hours at 37^{O} C. The slides were then dewaxed by placing in xylene and ethanol solutions and air-dried.

Catalyzed Reported Deposition- Fluorescent in situ hybridization (CARD-FISH)

The protocol was adopted from Department of Microbiology, Lab 016, Technische Universität München (http://www.environmental-microbiology.de/pdf_files/CARDFISH_2march2013.pdf). Permeabilization mix was used with lyzozyme for eubacteria and proteinase K for Archae. The deparaffinized slides dipped in low melting point agarose were treated with permeabilization mix (Lyzozyme

10mg/ml (or) Proteinase K 10.9mg/ml, 1M tris-HCl 0.1M, 0.5M EDTA 0.05M) for 1 hour at 37C. They were subsequently washed in water and ethanol and air dried. Slides were washed in 0.1% hydrogen peroxide in order to inactivate peroxidase enzymes which might be present in the tissue.

Biotin labelled EUB338 (5'- GCT GCC TCC CGT AGG AGT -3'), NONEUB338 (5'-ACT CCT ACG GGA GGC AGC-3') and ARCH915 (5'-GTG CTC CCC CGC CAA TTC CT-3') were diluted to 50ng/ml using molecular grade water. Slides were treated with hybridization buffer (Dextran sulfate 10%, 5M NaCl 900mM, 1M tris-HCl, 100% Triton X100 0.025%, Formamide 55% for EUB338, Formamide 20% for ARCH915, 1% of 10% Blocking buffer), biotin-probe and HRP-streptavidin for 12 hours under darkness at 35°C. The probe binds to the target bacteria, and biotin and streptavidin form a strong non-covalent interaction. Slides were then washed with wash buffer (13mM of 5M NaCl, 20mM of 1M TrisHCl, 5mM of 0.5M EDTA, 0.01% of 10% SDS) at 37°C for 10 minutes. Slides were air dried and washed with PBS-T (1X PBS and 100% TritonX100) at room temperature for 5-10 minutes.

Tyramide-Alexa fluor488 NHS ester (1mg/ml) (Life Technologies) prepared overnight was diluted with amplification buffer (10% Dextran sulfate, 2M of 5M NaCl, 0.1% of 10% Blocking reagent in 1XPBS) and 30% hydrogen peroxide (final concentration of 0.0015%) and loaded onto the slide and incubated at 37°C for 45minutes. The tyramide molecules along with Alexa Fluor deposits on the horse-radish peroxidase (HRP), which amplifies the fluorescence (Fig. 2). This eliminates background fluorescence. Slides were washed in PBS-T and 96% ethanol and air-dried. Vectashield was used as mounting agent.

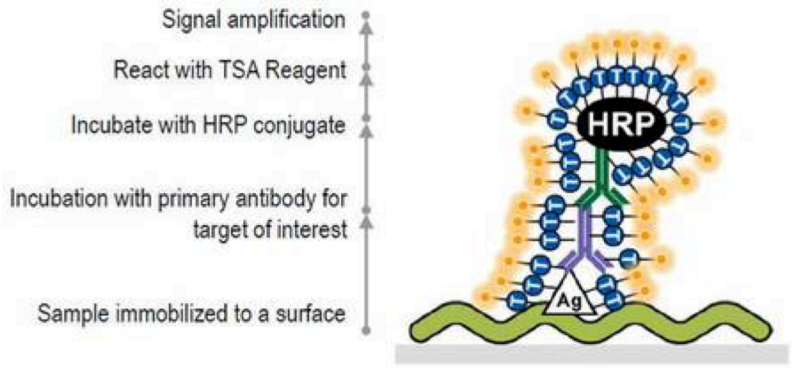
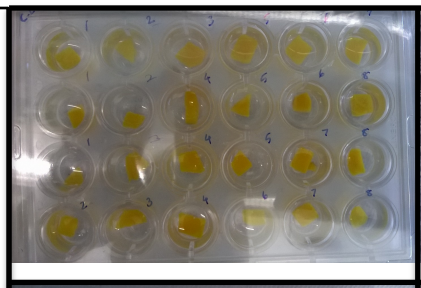
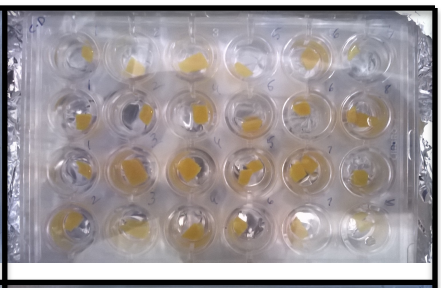
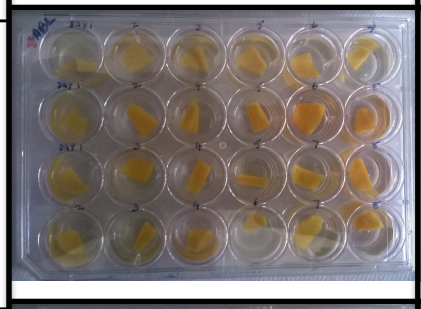
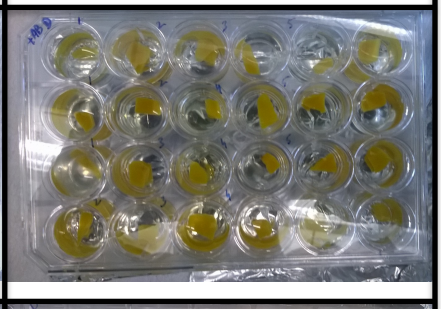
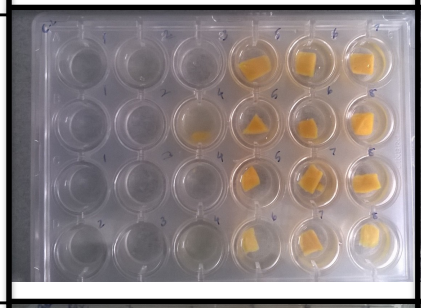
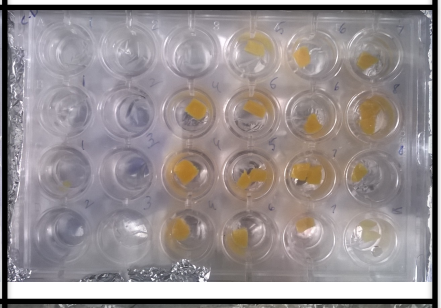
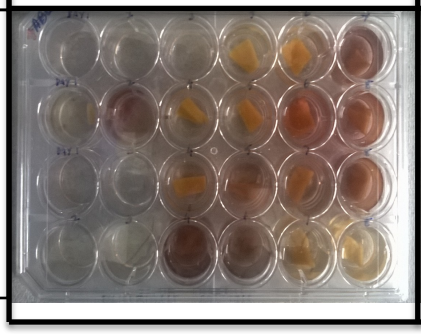
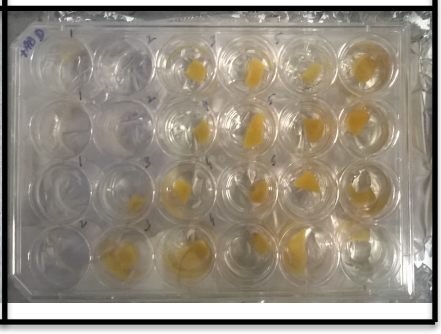


Figure. 2 : Tyramid signal amplification. Biotin-labelled probe binds to gene of interest. The HRP indirectly binds to biotin. Tyramide binds to Alexa Fluor binds to HRP. (Image credit: PerkinElmer)

RESULTS

Figure 3: Explant plate setup on Day 2 and Day 8 Control vs Antibiotics under Light/Dark Condition.

	Light	**Dark**
DAY 2 CONTROL		
DAY 2 COCKTAIL OF ANTIBIOTICS		
DAY 8 CONTROL		
DAY 2 COCKTAIL OF ANTIBIOTICS		

Transmission Electron Microscopy

Electron micrographs of *Cinachyrella sp* collected in February 2011 (CIN-W 2011) revealed a high microbial abundance (Fig. 4). The high abundance persisted after two weeks in closed aquaria (CIN-T 2011) (Fig. 5). Although this information is based on morphology, we can discern a high diversity of bacteria in the sponge mesohyl.

The *Cinachyrella kuekenthali* collected from the field in September 2014 (day0), kept in closed aquaria for 6 weeks, revealed a high abundance and diversity (Fig. 6). *Cinachyrella kuekenthali* after 6 weeks in tank was considered day 0 of the *in vitro* explant culture.

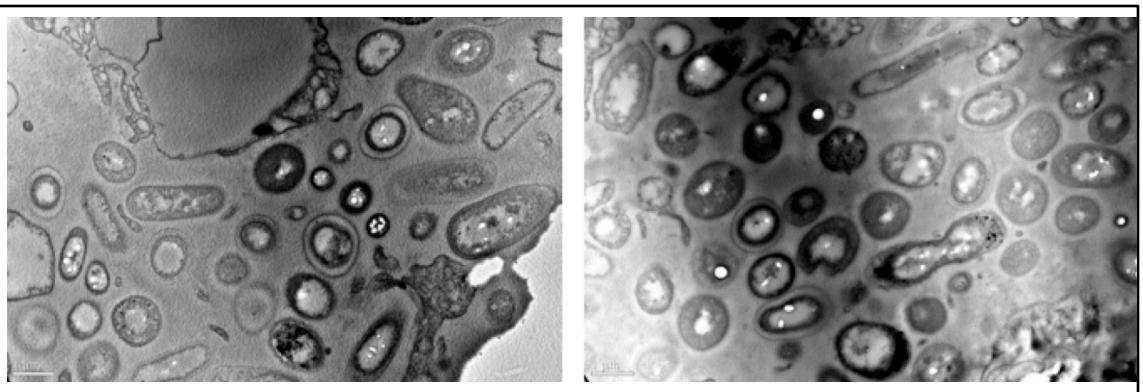


Figure 4: Electron micrographs of wild *Cinachyrella sp* (CIN-W 2011) mesohyl (scale = 1um)

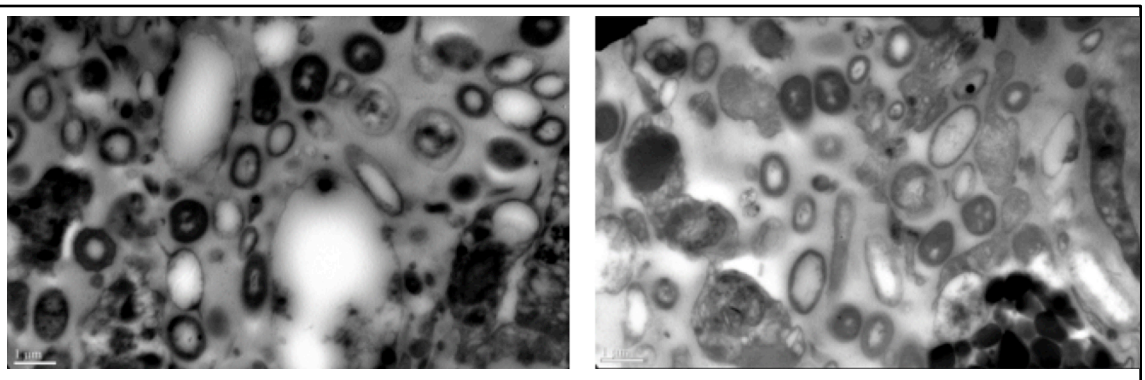


Figure 5: Electron micrographs of *Cinachyrella sp* (CIN-T 2011) mesohyl after a duration of 2 weeks in closed aquaria (scale = 1um)

Day Two Control Explants

Control explant observed in fixed samples for TEM after 48 hours under light conditions (CONTROLlight2) (Fig. 7) were not significantly different from day 0 in terms of bacterial abundance, although pronounced vacuoles were observed in the explant. Comparison of micrographs of the control explant 48 hours under dark conditions to those maintained under light, revealed marginal differences in bacterial morphology. A significant number of bacteria appear to possess a denatured nucleoid and cells appear bloated in the control explant under darkness (Fig. 8). There was no difference in macroscopic observation of the explants.

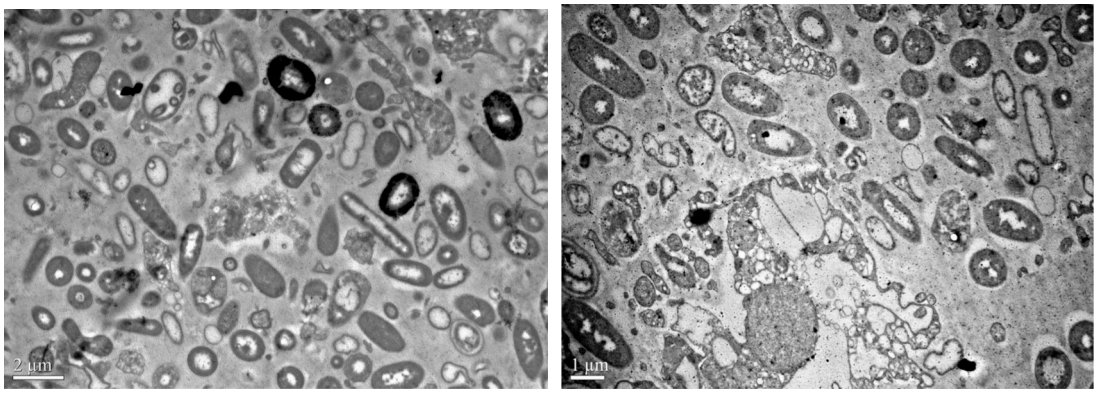


Figure 6: Representative micrographs of Cinachyrella kuekenthali mesohyl after 6 weeks starvation in closed tank system (day0).

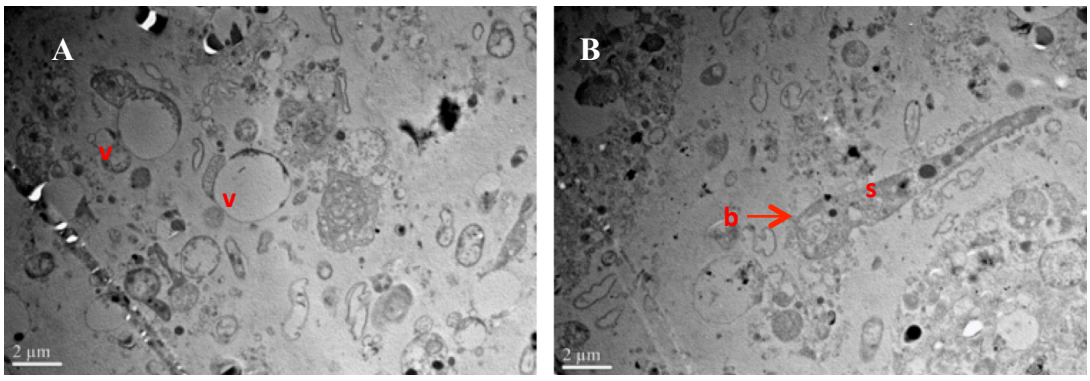


Figure 7: TEM of control explant (CONTROLlight2) mesohyl on day 2 under light condition (A) vacuoles *v* , sponge cells and bacteria *b* (scale bar=2um) (B) possible phagocytic activity of sponge cell *s* on bacteria *b*(scale bar=2um).

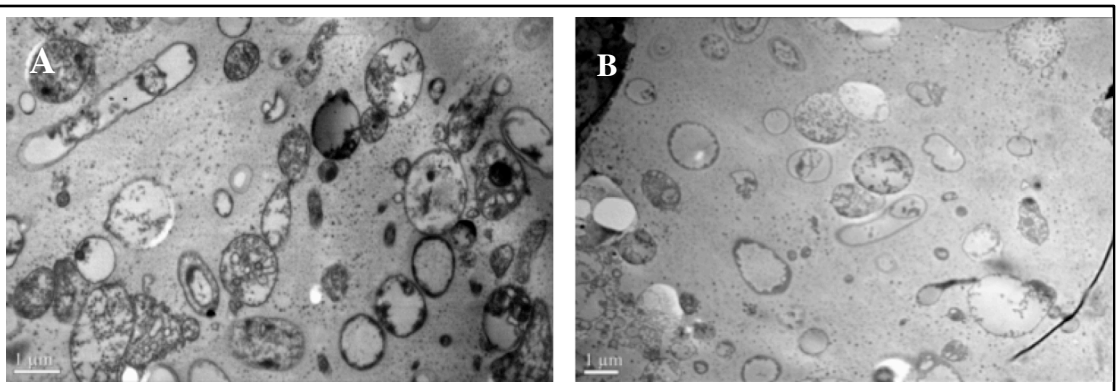


Figure 8: TEM of control explant mesohyl on day 2 under dark condition (CONTROLdark2) (A&B) bacterial cells in collagenous matrix (scale bar= 1um)

Day Two Cocktail of Antibiotic Treated Explants

Explants treated with antibiotics after 48 hours under light treatment showed discoloration in sponge and media (Fig. 3) Some bacterial cells appear to be affected by the antibiotics while a few have intact nucleoid and cell walls (Fig. 9). Abnormalities were observed in few sponge cells while intact sponge cells were also observed. Explants after 48 hour antibiotic treatment in dark showed no discolorations compared to the light and was not visibly different compared to control (Fig. 10). Sponge cells observed through electron microscopy showed no abnormalities, but the presence of vacuoles were observed (Appendix 2). It should be noted that few bacterial cells were observed.

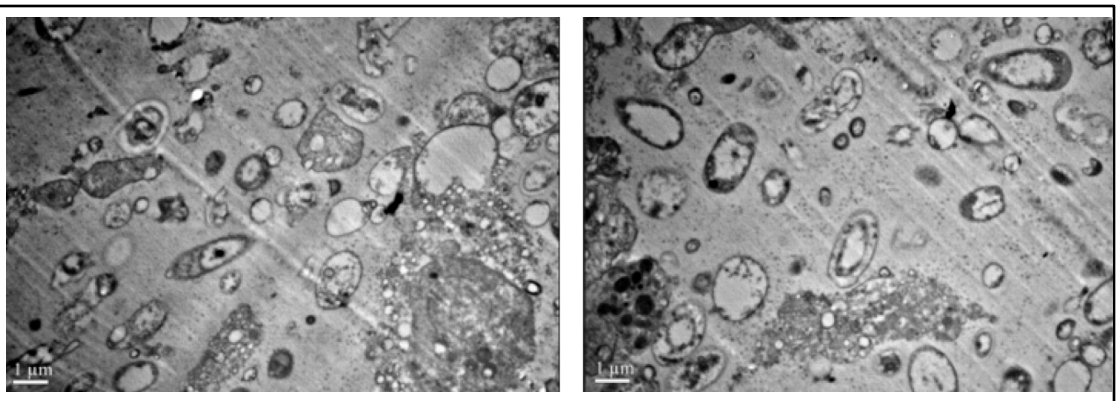


Figure 9: TEM of explant (ABlight2) treated with cocktail of antibiotics on day 2 under light condition. s= sponge cell (scale bar=1um)

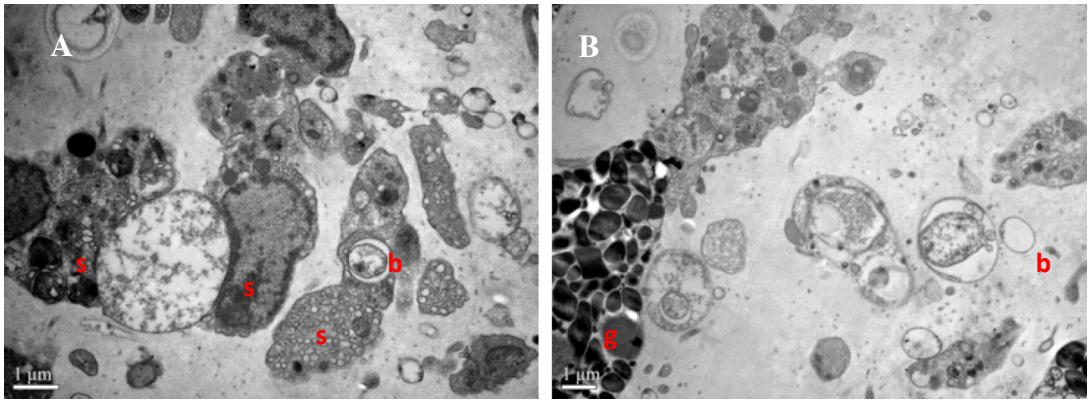


Figure 10: TEM of explant (ABdark2) treated with cocktail of antibiotics on day 2 under dark condition (A) sponge cells with vacuoles (scale bar=1um) (B) granulocyte *g*, sponge cells s and denatured bacteria *b* (scale bar=1um)

Other features found in antibiotic treated explants were characteristic "blebs" which were shared in both sponge and bacterial cells (Figs. 11). It can be hypothesized that they could be of sponge origin as they are present more commonly in sponge cells, however it cannot be discerned for certain.

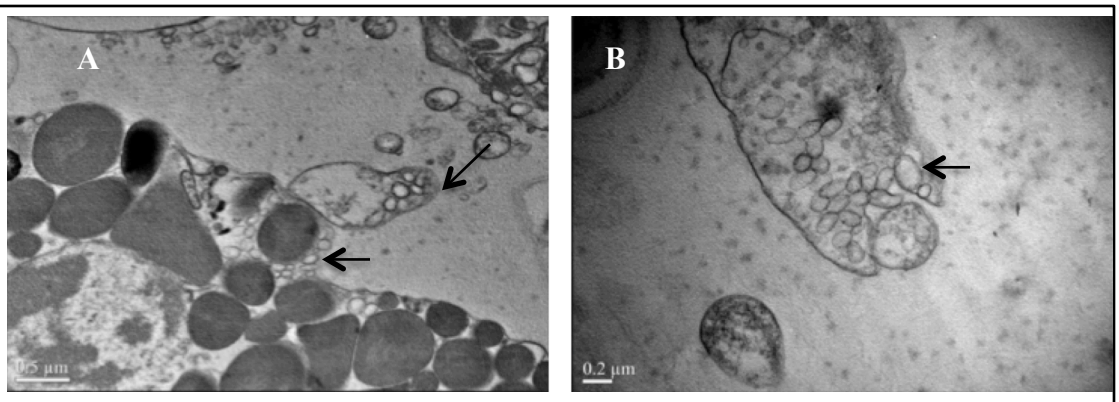


Figure 11: TEM of explant (ABdark2) treated with antibiotic cocktail on day 2 under dark condition. Interaction of sponge cell and bacteria (A) bleb-like structures observed in denatured bacteria and sponge cells (scale bar=0.5um) (B) bleb-like structures (arrow) observed in sponge cell (scale bar=0.2um).

Day 8 Control Explants

After 8 days of culture, control explants (CONTROLlight2, CONTROLdark2, CONTROLlight8, CONTROLdark8) exhibited no macroscopic differences under light and dark conditions. TEM micrographs revealed abundance of one specific morphotype under light treatment (Fig. 13B). This bacterial morphotype was ovoid with a central nucleoid with network of fibrils. Sponge cells were observed to have vacuoles filled with possible bacterial cells (Fig. 13A). Bacterial cell divisions were also observed (Figs. 13B&C). Dark treatment of controls showed reduced number of bacterial cells compared to light, however there were remote observations of possible phagocytosis of bacteria by ameboid sponge cells (Fig. 12B). Also, there were no visible abnormalities in sponge cells.

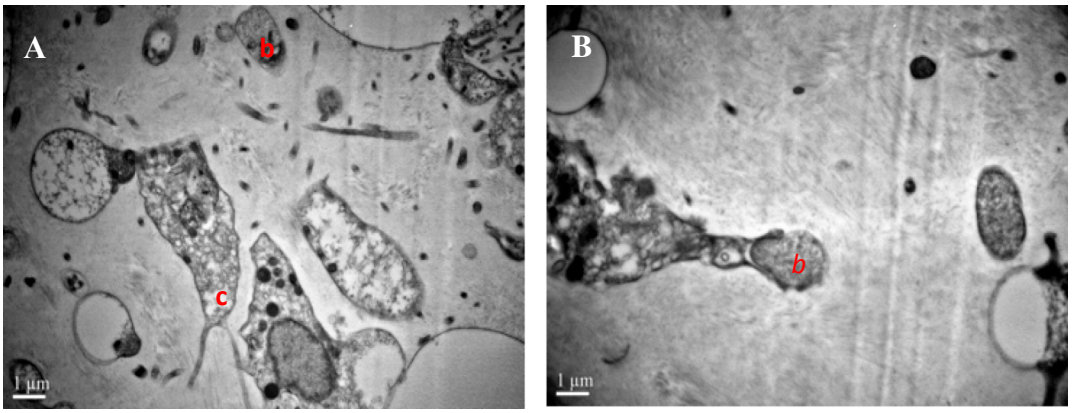


Figure 12: TEM of control explant (CONTROLdark8) on day 8 under dark condition (A) choanocytes *c* with motile flagella (scale bar=1um) (B) phagocytic activity of sponge cell (scale bar=1um)

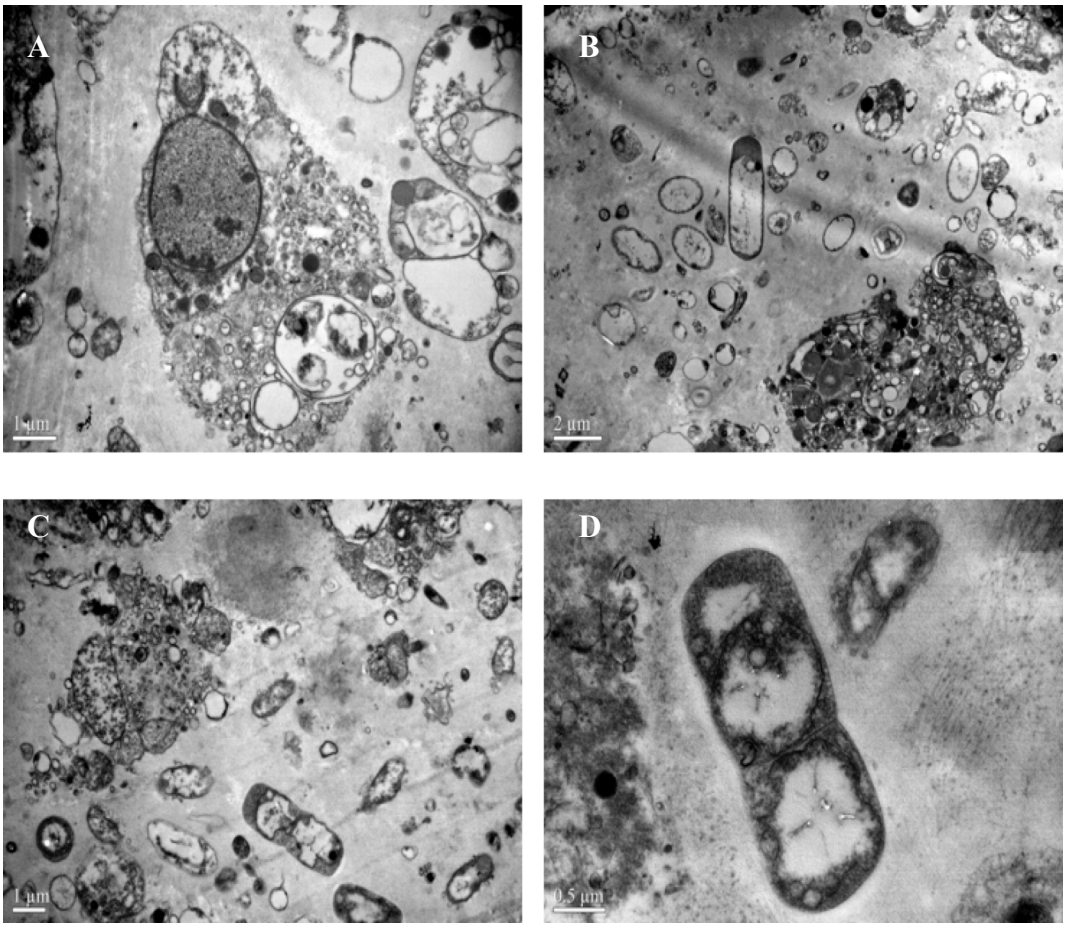


Figure 13: TEM of control explant (CONTROLlight8) on day 8 under light condition (A) sponge cell with vacuole (v) containing bacterial cells. scale bar=1um (B) common bacterial morphotype in the mesohyl (scale bar=2um) (C) bacteria diving in mesohyl (scale bar=1um) (D) dividing bacteria (scale bar=0.5um)

Day 8 Cocktail of Antibiotic Treated Explants

Antibiotic treated explants under light conditions 8 days into culture, showed more intense discolorations and the media turned pinkish in color (Fig 3). This was later learned to be due to phototoxic nature of tetracycline. On comparing experimental and control sponge cells, the antibiotic treated explants appeared to show swelling in a few granulocytes (Fig. 14B). Most bacterial cells appeared to have a degraded nucleoid and remnant cell membranes were observed (Fig. 14A&B). Under dark conditions, almost all bacterial cells appear to be similarly degraded (Fig. 15). Due to an insufficient number of observations, statistical conclusions cannot be drawn as to its significance.

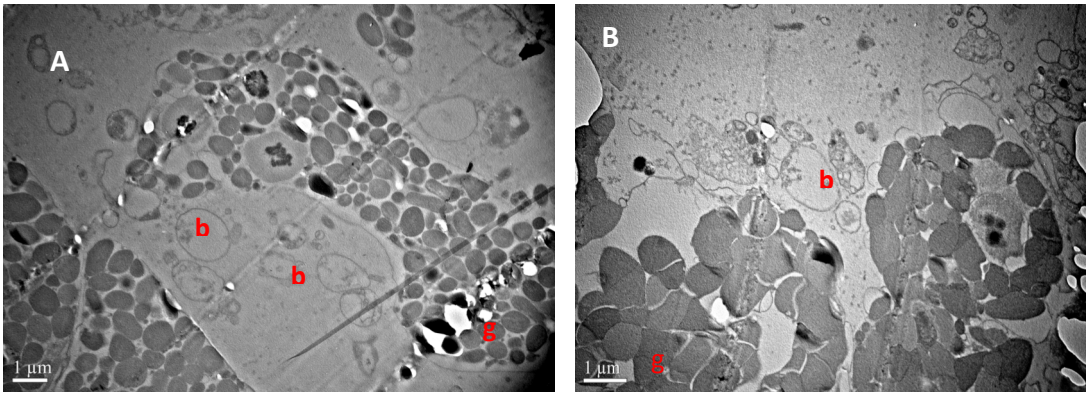


Figure 14: TEM of explant (ABlight8) treated with cocktail of antibiotics on day 8 under light conditions (A) granulocyte sponge cells *g* with denatured bacterial cells (scale bar=1um) (B) sponge cell and denatured bacterial cells *b* (scale bar=1um)

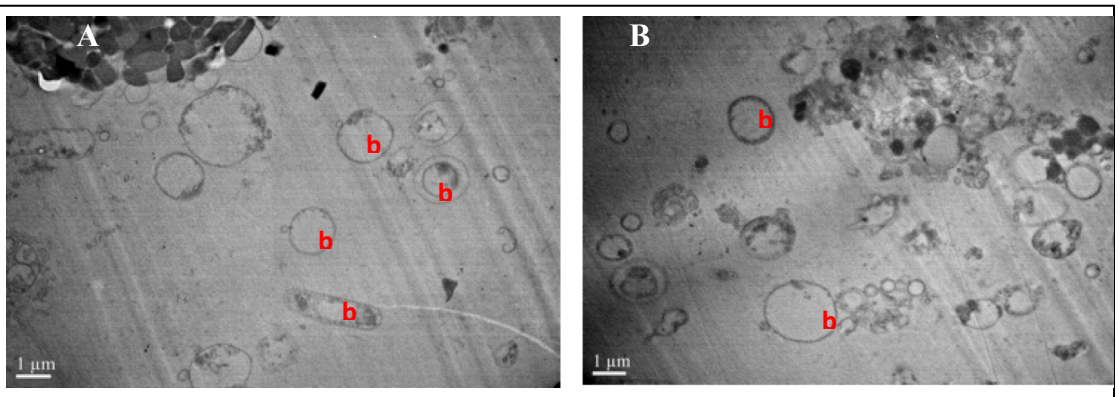


Figure 15: TEM of explant (ABdark8) treated with cocktail of antibiotics on day 8 under dark conditions (A) granulocyte sponge cells *g* with denatured bacterial cells (scale bar=1um) (B) sponge cell and denatured bacterial cells *b* (scale bar=1um)

16S rRNA Community Analysis

The total number of raw 16S rRNA amplicon sequences obtained with MiSeq was 270,381 reads. After filtering the chimeric sequences and singletons, 269,827 reads were generated. The average number of reads for the data set was 5191 reads with a standard deviation of 3911 reads. The cutoff quality score for all samples was Q25, the

default set in QIIME. The average length of the samples was about 250 base pairs. Certain samples (AMPdark4, TETlight4, ABdark10, TETdark10) did not successfully amplify by PCR, and were therefore unavailable for analysis.

A rank abundance plot was used to visualize the relative species abundance of the top 100 OTUs found in the sponge samples (Fig. 16). The X-axis is the rank of the species (table 5), that is, the most abundant is placed at rank 1 and Y-axis gives the log scale of the measure of abundance. A steep slope implies less evenness.

Figure 16: Rank abundance plot of all bacteria identified in the sponge explants (N=26). X-axis: rank of species, Y-axis: log measure of abundance.

Table 5: Top 10 Rank of bacterial phyla corresponding to X-axis of Rank abundance plot (Fig: 16).

Rank	Phylum	Class	Order	Total number of sequences
1	Crenarchaeota	Thaumarchaeota	Cenarchaeales	30254
2	Proteobacteria	Gammaproteobacteria	Alteromonadales	24149
3	Nitrospirae	Nitrospira	Nitrospirales	11588
4	Proteobacteria	Gammaproteobacteria	Vibrionales	11335

5	Chloroflexi	Anaerolineae	SBR1031	10274
6	Proteobacteria	Gammaproteobacteria	Alteromonadales	8320
7	Proteobacteria	Gammaproteobacteria	Chromatiales	7518
8	Acidobacteria	Acidobacteria-6	iii1-15	6363
9	Proteobacteria	Gammaproteobacteria	Vibrionales	3506
10	Verrucomicrobia	[Pedosphaerae]	NA(not available)	3472

In order to visualize the distribution of the phyla, a relative abundance plot was used (Fig. 17), where x-axis is the mean relative abundance of an OTU and y-axis is relative frequency of that OTU. The sequences were normalized to the minimum number of reads found in the data set (1061 reads) and plotted. Acidobacteria, Chloroflexi, Crenarchaeota, Nitrospirae and Proteobacteria were present in almost all sponge samples.

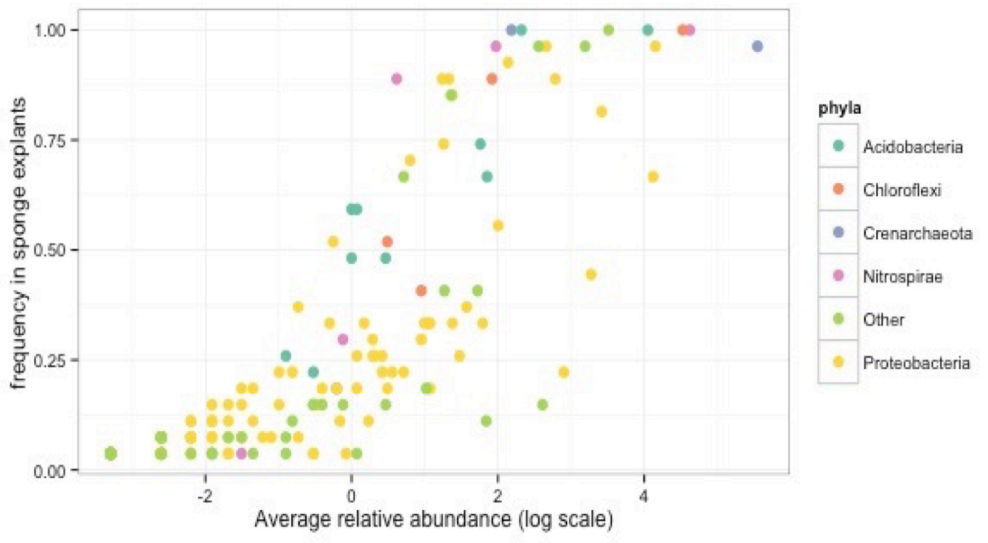


Figure 17: Distribution of bacterial phyla in the sponge explants. Data was normalized to 1061 reads/samples. Graph was plotted using *ggplot* in R.

Community Composition

Taxa summary bar plot of the top 100 OTUs compares all the samples at the phylum level. This plot represents 97.6% of all acceptable data. It should be noted that CinDead and CinDead2 were isolated from the same individual *C. kuekenthali* dead sponge.

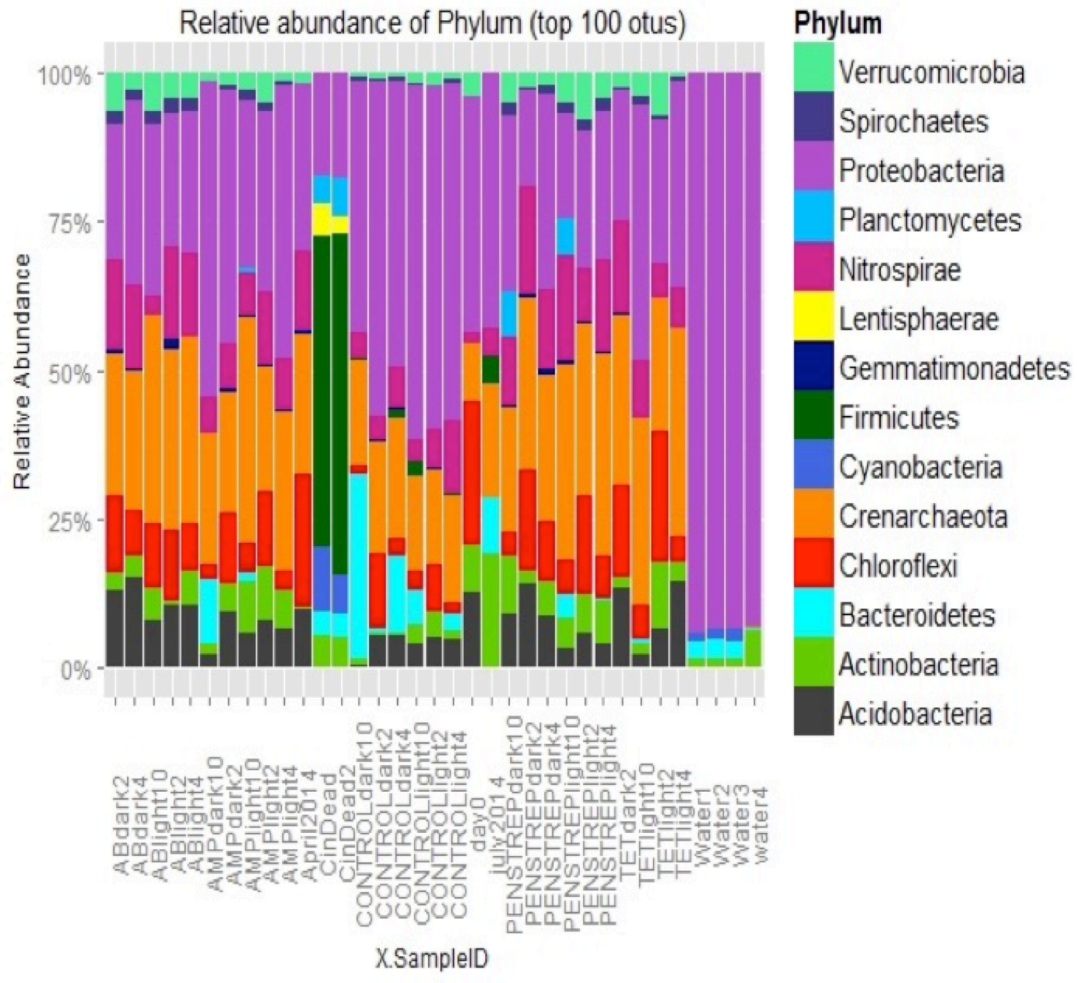


Figure 18: Relative abundance phyla of bacterial communities in all samples; explants (N=28) under all treatments and tank water sample replicates (water1, water2, water3, water4). The sample ID along with its description is listed in Table 4.

Bacterial taxonomic diversity in the explants was higher compared to tank water. Dominant phyla included Proteobacteria, Thaumarchaeota, Chloroflexi, Nitrospirae, Actinobacteria, Verrucomicrobiae and Acidobacteria (Fig. 18). Thaumarchaeota was introduced as a new phylum in 2008 however GreenGenes database has not indexed the phylum and is recognized as Crenarchaeota (Hong et al., 2014). Firmicutes appeared very high in the dead *Cinachyrella* sample, which was composed of class Clostridia, an anaerobic bacteria (Fig. 18). Cyanobacteria are only observed in the water and in dead *Cinachyrella sp.*

The overall diversity bacterial community diversity present in the 16S rRNA dataset showed a higher diversity in Proteobacteria, Bacteroidetes and Chloroflexi and a relatively lower diversity in Crenarchaeota, Nitrospirae, Acidobacteria, Verrucomicrobiae and Actinobacteria (Fig. 19). To better interpret results, the phylogenetic bacterial communities' Class and Order were analyzed by considering the top 100 OTUs of the sample based on type of treatment (Fig. 20 & Fig. 21).

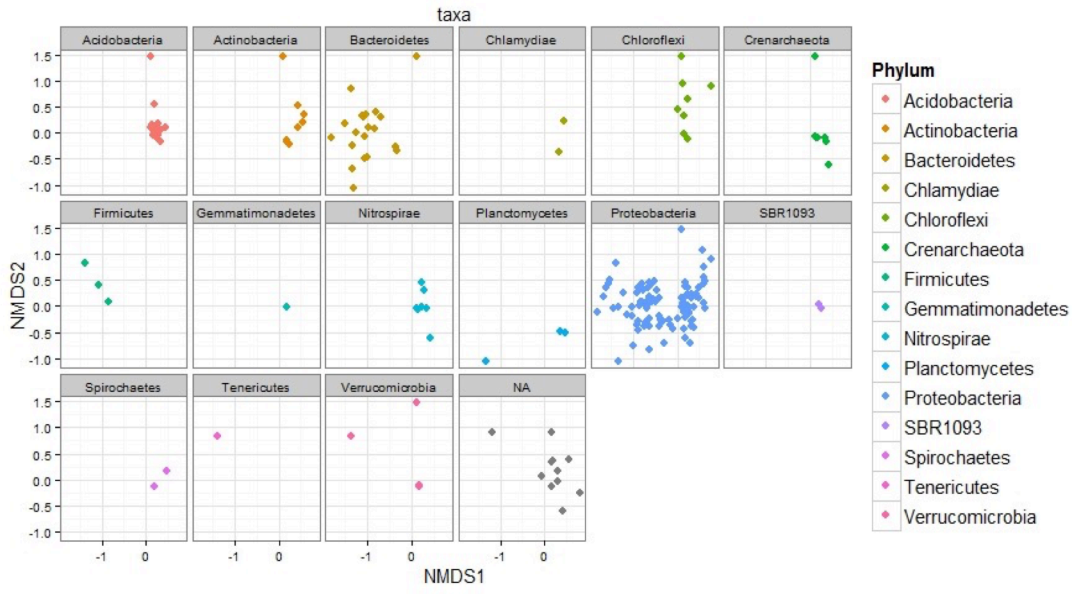


Figure 19: Bray Curtis NMDS plot of the bacterial community at the phylum level in the sponge explants (N=26)

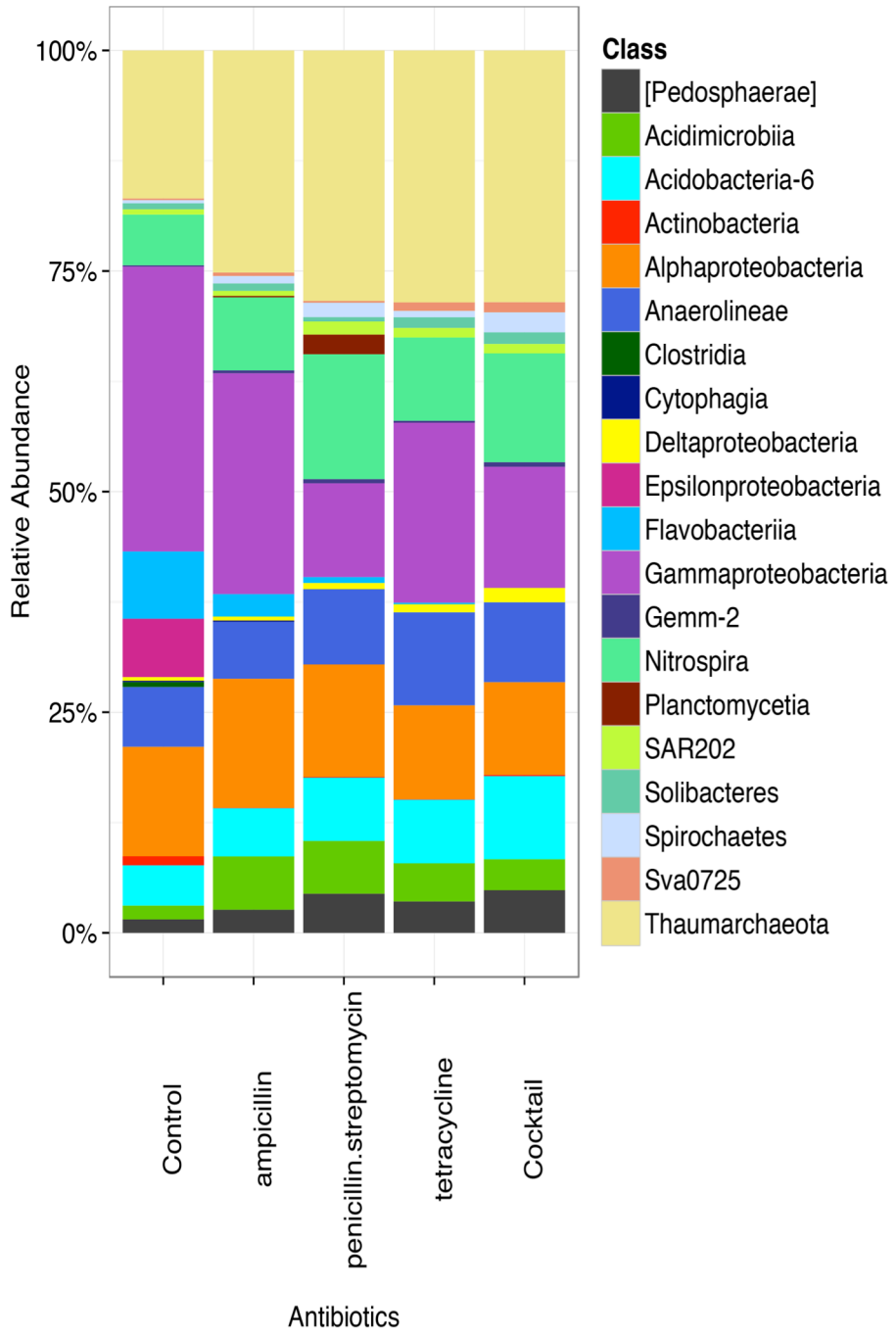


Figure 20: Relative abundance of bacterial communities at the Class level in the explants (N=26) based on the antibiotic treatment. The analysis of antibiotic effects on explant bacterial communities included the bacterial community at different time points.

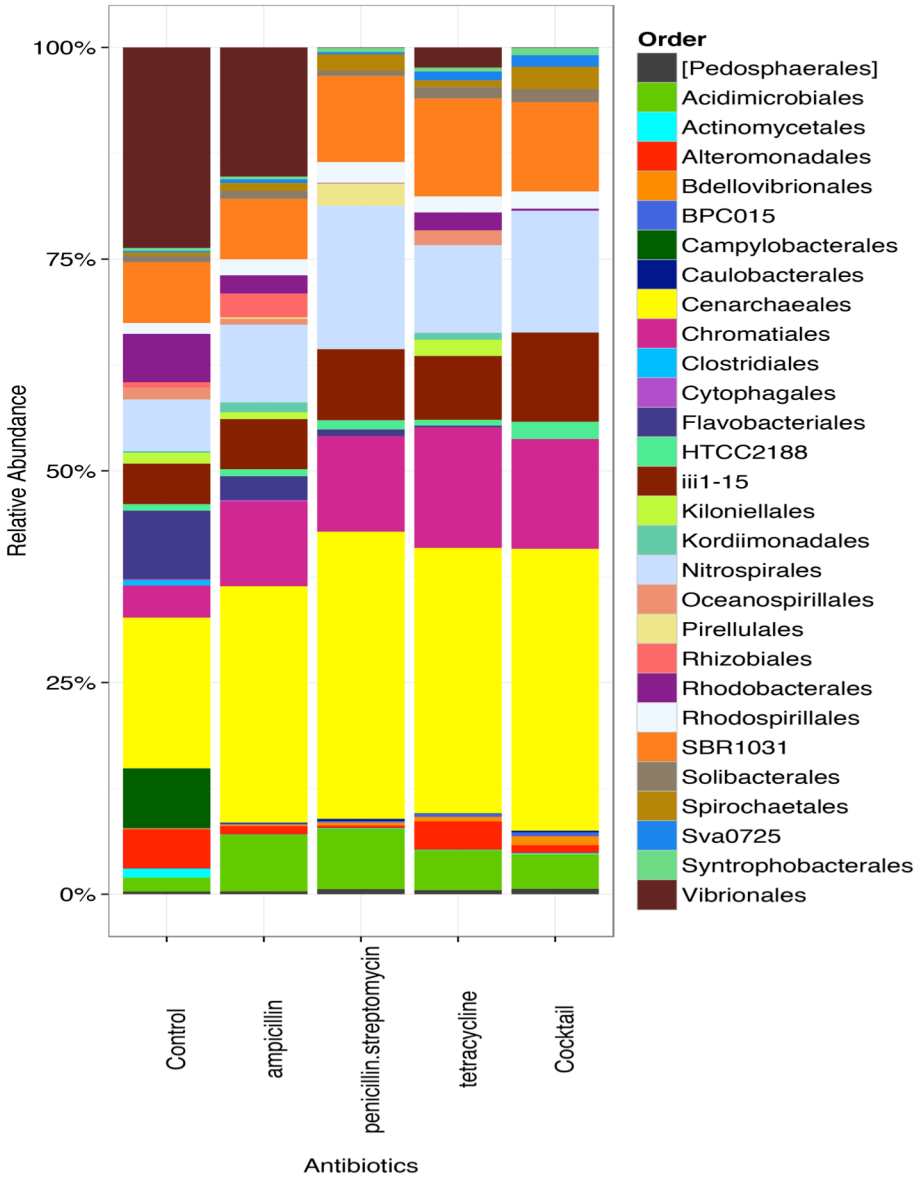


Figure 21: Relative abundance of bacterial taxa at the Order level in experimental explants (N=26) after antibiotic treatments. The analysis of antibiotic effects on explant bacterial communities included the bacterial community at different time points.

Tank water was dominated by Alteromonadales, a Gammaproteobacteria, which occupied a small portion (approx. 2%) of the total bacterial community in the sponge. Order Actinomycetales (Phylum: Actinobacteria) was present in the dead sponge and

tank water but not significant in the explants, however the explants have a significant presence of Acidimicrobiales (Phylum Actinobacteria). Bacterial orders Acidimicrobiales, Cenarchaeales (Phylum Thaumarchaeota), Chromatiales(Class: Gammaproteobacteria), iii1-15 (Phylum: Acidobacteria), Nitrospirales (Phylum: Nitrospirae), Rhodospirillales (Class: Alphaproteobacteria), SBR1031 (Phylum: Chloroflexi) and Spirochaetales were consistently present in all the explants with little variation in relative abundance (Fig 19 & 20).

Orders Campylobacterales (Class: Epsilonproteobacteria), Bacteroidales (Class: Bacteroidia) and Pasteurellales (Class: Gammaproteobacteria) were observed in the control explants but not in the antibiotic treated. The control and ampicillin treated explants appeared to have similar diversity compared to other antibiotic treated communities. Order Vibrionales was dominant in control and ampicillin but not in the remaining antibiotic treated explants (Fig. 21).

Considering the Class and Order of the bacterial community provides more information, however many bacteria have not been identified to Order level and hence have not been plotted and can give false information. In order to examine those, the following tabular columns lists the top 10 OTUs found in the different treatments.

Certain Orders were unidentified (NAi, NAii and NAiii) and hence not plotted. BLAST searches via NCBI revealed NAi to be 97% related to an uncultured Alphaproteobacteria also found associated with *Cinachyra cavernosa* and *Haliclona pigmentifera* in the Gulf of Mannar, east coast of India (Jasmin et al., 2015) and 97% identity with a partial sequence found in shallow marine hydrothermal vents off Papua New Guinea (Accession: JN838679.1, Unpubbl). NAii was identified to be 99% similar to uncultured Verrucomicrobiae in Saanich inlet off Vancouver island (Walsh et al., 2009). NAiii is an uncultured alpha-proteobacterium associated with the crystalline sponge *Astrosclera willeyana* of the Great Barrier Reef (Karlinska-Batres,K Unpubbl) and *Ancorina alata* (Kamke et al., 2010).

Change of bacterial community with time

To display the variability in sponge bacterial community with time, the phylogenetic Class of the top 100 OTUs at the Class level of explants based on time points were plotted (Fig. 22).

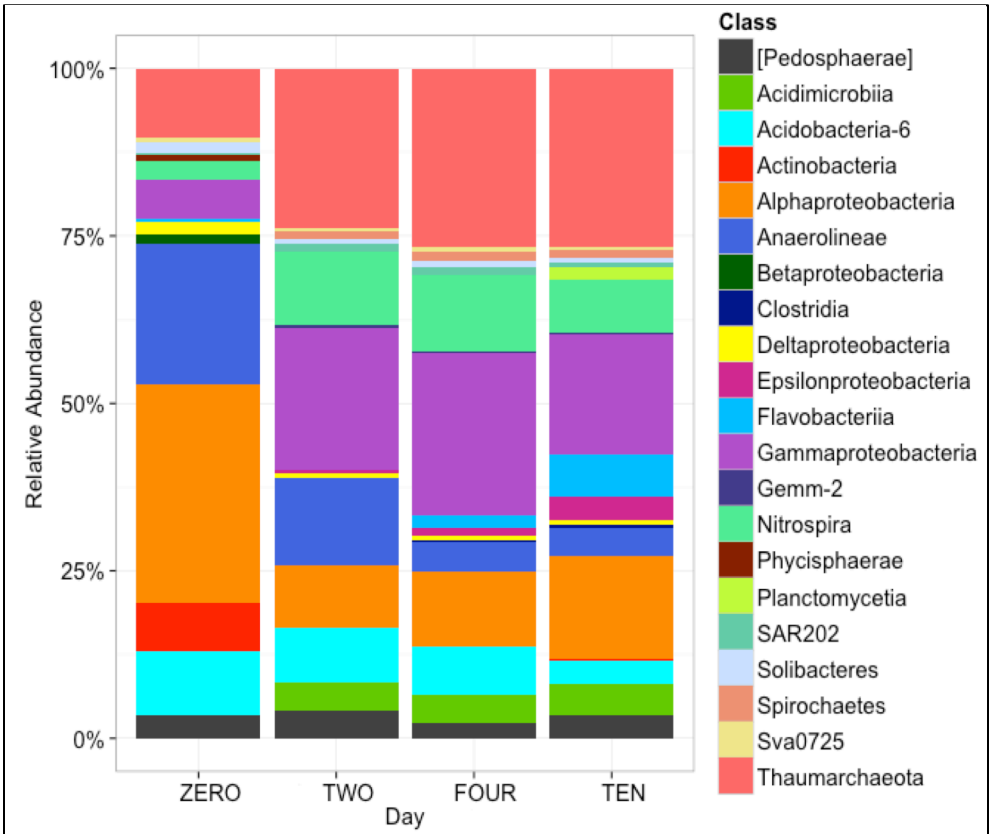


Figure 22: Relative abundance of phylogenetic Class of bacterial communities in explants based on time period

Class Thaumarchaeota, Nitrospira and Gammaproteobacteria were persistent in all the explants, with little variation in Acidobacteria-6. However, Class SAR202 (Phylum: Chloroflexi) and Anaerolineae (Phylum: Chloroflexi) bactreria appear to decrease from day 0 to day 10. Acidomicrobiia, Alphaproteobacteria, Flavobacteria and Epsilonproteobacteria increased to higher relative abundances at days 4 and 10 compared to day 0 and 2. This could have been due to the loss of bacteria, which resulted in an increased comparative abundance of other classes of bacteria.

Alpha Diversity

Alpha diversity using the Shannon and Simpson indices across all samples was visualized in QIIME. Tank water exhibited the lowest bacterial diversity and dead *Cinachyrella sp,* the highest diversity, while the alpha diversity of the explants was approximately constant (Fig. 23). This indicates the explants are similarly ecologically rich. The reference control samples included the primary *Cinachyrella sp* (day0) and two other *Cinachyrella sp* (collected in April and July 2014) individuals, which could explain the higher diversity. The alpha diversity of explants treated with the cocktail of antibiotics exhibits a narrow range, which indicates the smaller range of diversity.

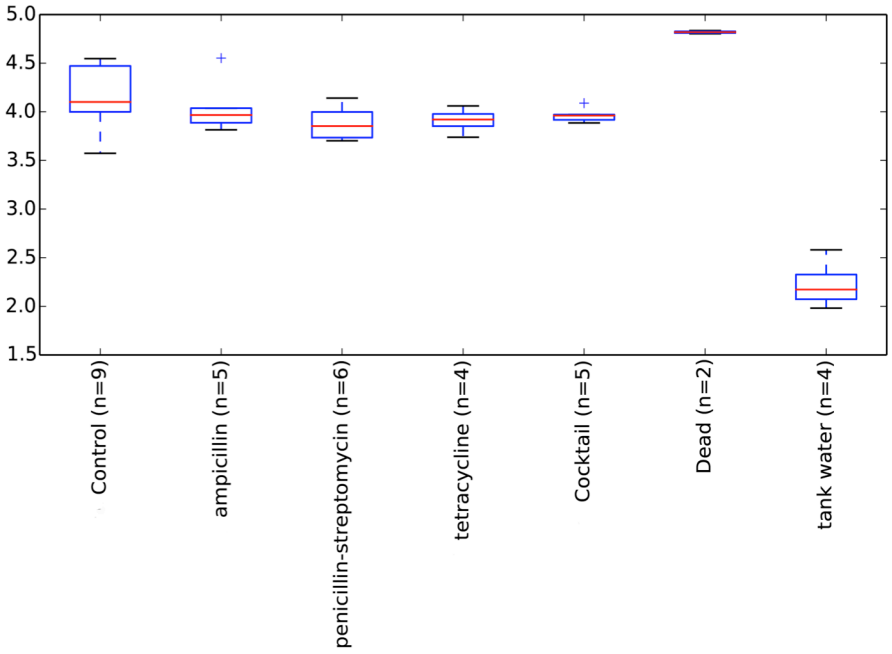


Figure 23: Explant alpha diversity using Shannon index of bacterial communities based on antibiotic treatment, Dead *Cinachyrella sp* (CinDead,CinDead2) and tank water

Beta Diversity

Beta diversity is the measure of diversity between sites, and was determined using Unifrac and Bray Curtis method illustrated with non-metric multidimensional scaling (NMDS) plots. Bray-Curtis distance measures clearly show the separation of bacterial community in the tank water, Healthy *Cinachyrella* sp and Dead *Cinachyrella kuekenthali* (CinDead, CinDead2) (Fig 24). The variability in the bacterial community of dead compared to healthy sponges can be a good indicator of the state of the sponge.

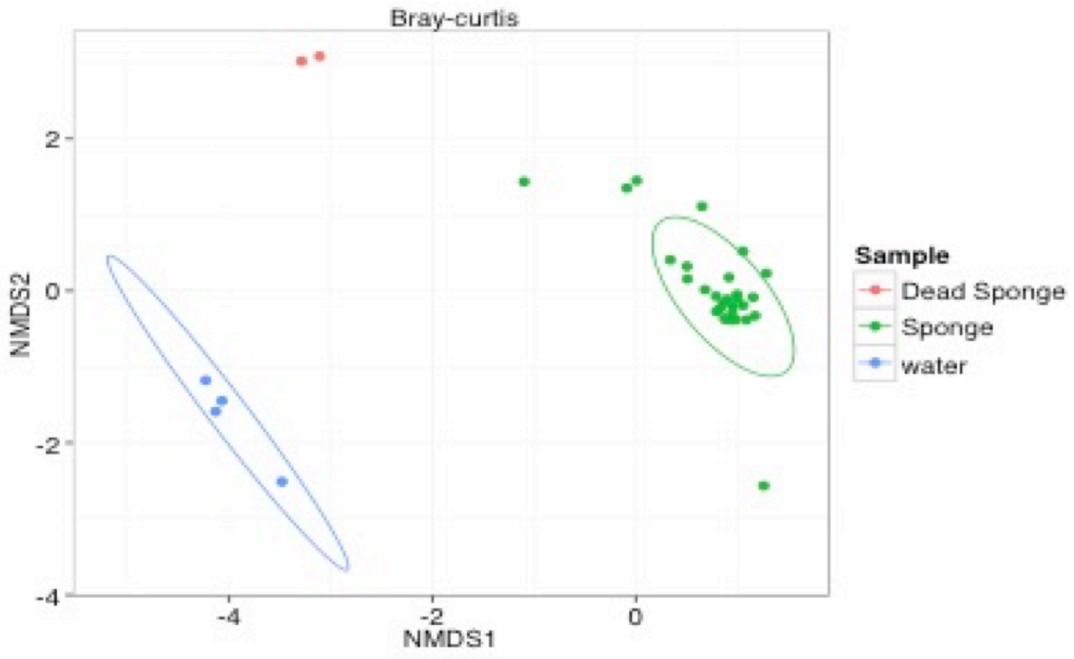


Figure 24: Bray -Curtis NMDS plot with statistical ellipse at 95% confidence interval of water= Tank water, Dead sponge= Dead *Cinachyrella kueknethali* and Sponge= Wild *Cinachyrella spp* (july2014 and April2014)+*C. kuekenthali* explants (N=26)

Bacterial Community Analysis Between Antibiotic Treatments

Bacterial communities of explants treated with different antibiotics were significantly different (ADONIS, p-value= 0.001, R2= 41.3%). The control was significantly different from all antibiotic treatments, ampicillin (bray curtis: p-value=0.004, unifrac: p-value=0.01), penicillin-streptomycin (p-value=0.001), tetracycline (Bray Curtis: p-

value=0.002, unifrac: p-value= 0.004) and cocktail of antibiotics (bray curtis: p-value=0.001, unifrac: p-value=0.003). There was significant difference between the cocktail of antibiotics and ampicillin (Bray Curtis: p-value=0.001 unifrac: p-value= 0.009) and between penicillin-streptomycin and ampicillin (Bray Curtis: p-value=0.001, unifrac: p-value=0.005). This was visualized using Unifrac via non-metric multidimensional scaling (NMDS). There was an overlap of clustering in the cocktail and penicillin-streptomycin treatments and a partial overlap of control and ampicillin treatment (Fig. 25). However the control was significantly clustered away from the cocktail of antibiotic treatment. This pattern can also be seen using Bray-Curtis method via principal coordinate analysis (PCoA) (Appendix 3).

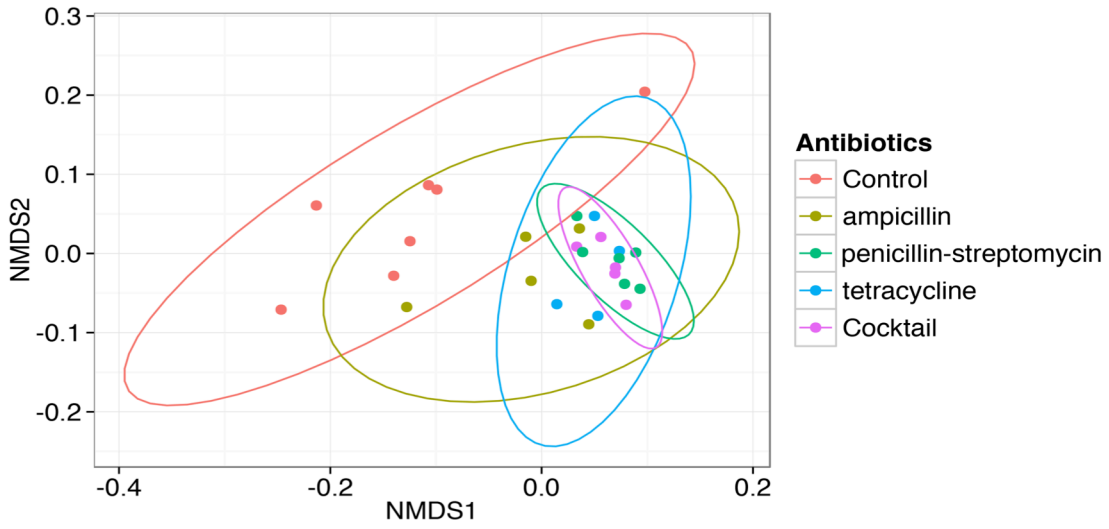


Figure 25: Weighted Unifrac NMDS plot of bacterial communities in the explants (N=26) based on Antibiotic treatment. Communities clustered within a statistical ellipse have a 95% confidence interval

Bacterial Community Analysis With Increasing Time Intervals

The bacterial community of the explants on different days was statistically different (ADONIS p-value=0.001, R2= 28%). For further analyses, UniFrac distance was analyzed via non-metric multidimensional scaling (NMDS) of bacterial communities of the explants on different days (Fig. 26). However, comparison of only

the controls from Fig. 25 with Fig. 26 indicates that the controls in different days are clustering significantly from each other. Similar graph was observed in Bray-Curtis analysis visualized with PCoA plot (Appendix 4).

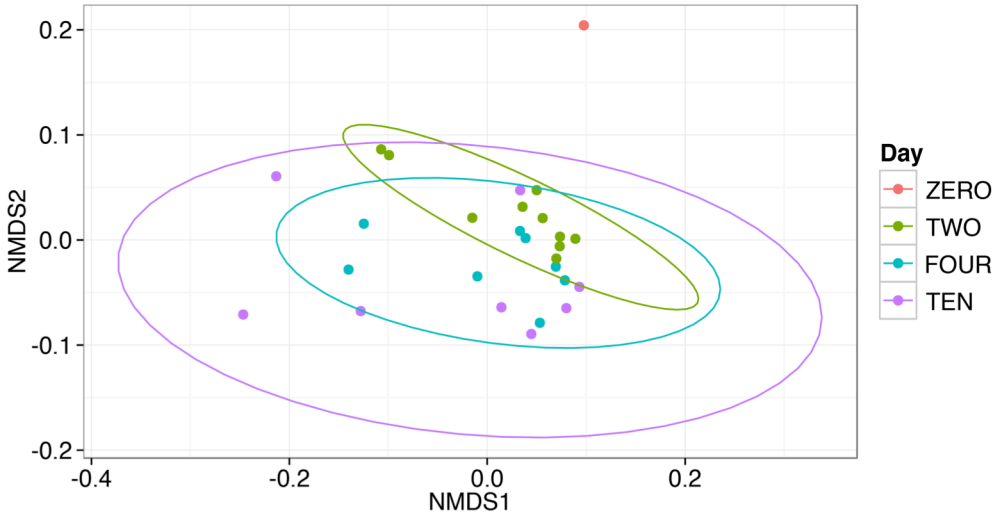


Figure 26: Weighted Unifrac NMDS plot of bacterial communities of the explants (N=26) on different days

The interaction of "antibiotics" and "temporal" factors results in bacterial communities that appears slightly statistically different (p-value= 0.043, R2= 15%). This confirmed that the bacterial communities of the explants treated with particular antibiotics, at different time intervals was significantly different.

Bacterial Community Analysis Between Light and Dark Conditions

Beta diversity based on irradiance did not show a significant difference (Fig. 27). Unifrac analysis performed on the dataset visualized via NMDS, resulted in a significant difference in irradiance gradients (p-value=0.006, R2=3.4%). However, there was no significant interaction between irradiance versus time period (p-value= 0.131) and irradiance versus antibiotics (p-value= 0.153).

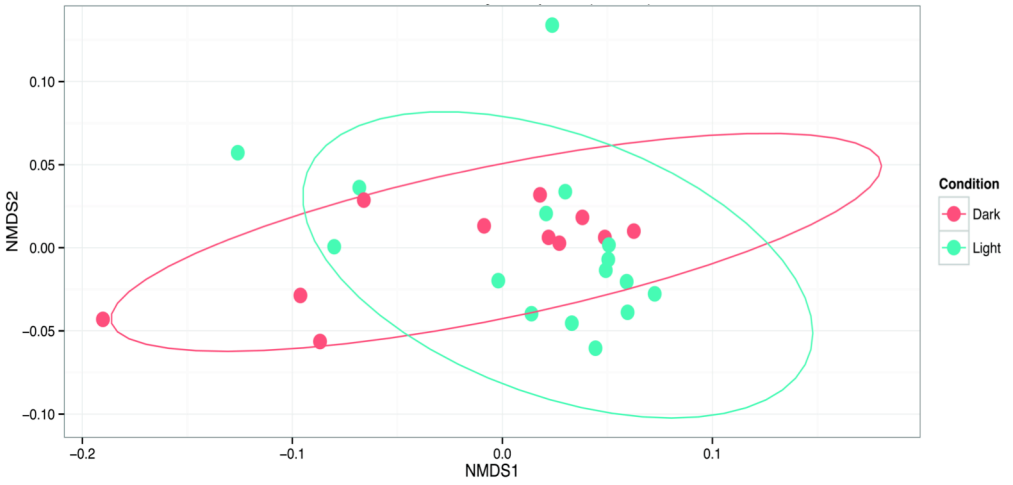


Figure 27: Weighted Unifrac of bacterial communities of explants(N=26) based on Light/Dark Conditions. Since Day 0 were kept in different conditions compared to explants, it is not categoried based on light/dark condition

Unweighted Unifrac of dataset was analyzed in order to confirm the shift in bacterial community was not due to introduction of new bacterial species or a change in bacterial community due to new species. There was significant overlapping of bacterial communities under different antibiotic treatments (Fig. 28) suggesting no new species was introduced. The partial overlap of the control with antibiotics treated explants suggests loss of bacterial species from control to antibiotics.

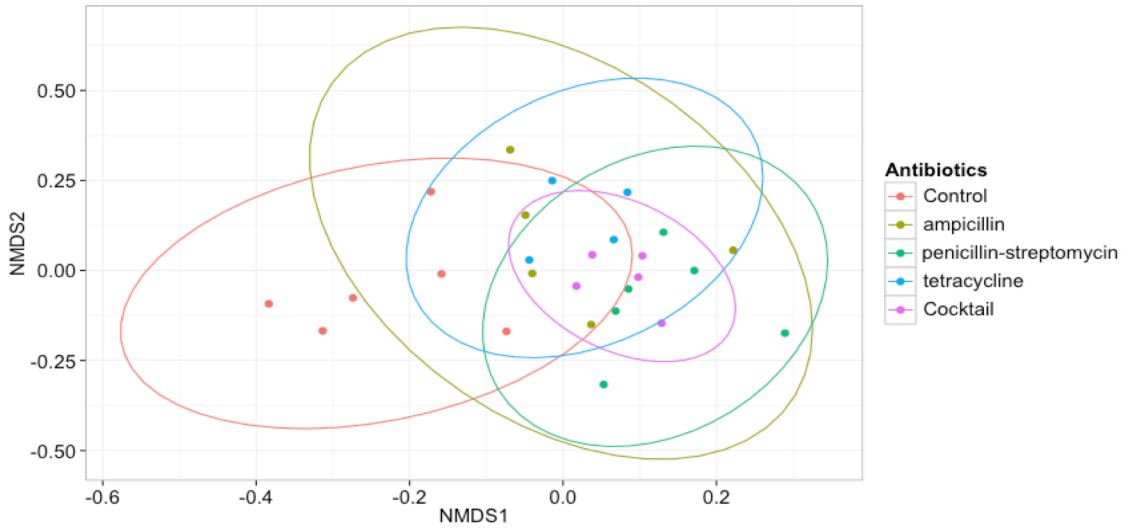


Figure 28: Uweighted Unifrac NMDS plot with statistical ellipse at 95% confidence interval of bacterial communities in explants (N=26) based on antibiotic treatment

The bacterial communities at different time points shared a common core bacterial community, which appeared evident in the NMDS plot of Fig. 28. The separation of day 0 could be a result of lack of replicates or a significant loss of bacteria.

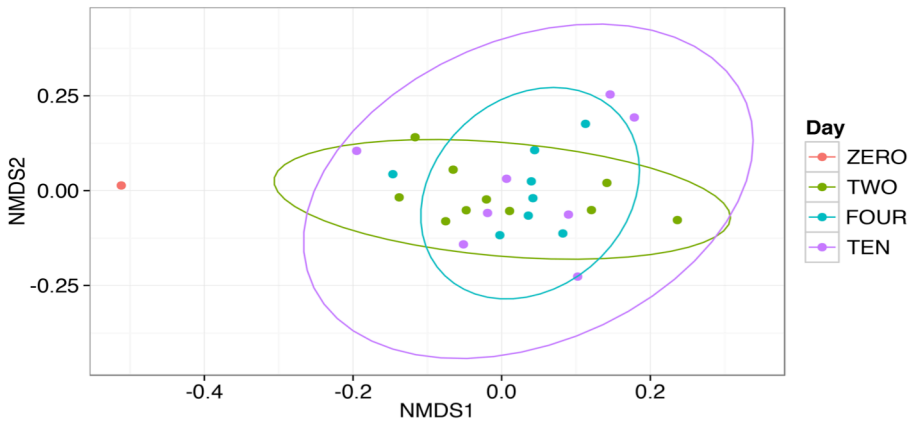


Figure 29: Unweighted unifrac of bacterial communities in explants (N=26) based on the time point (Day) the explant was sampled

Catalyzed Reporter Deposition Fluorescent in situ Hybridization

The hybrized bacterial and archae were visualized in the *Cinachyrella kuekenthali* explants AMPlight2 and CONTROLdark4 respectively (Figs 30 & 31). Unfortunately, a very low concentration of hybridized bacteria and archae were observed. To obtain an accurate estimate of the total bacteria present, one set of eubacterial probes may not be sufficient and could require EUB338I, EUB338II and EUB338III (Daims et al., 1999). Hence, low microbial density and insufficient probes could contribute to the infrequent hybridizations observed (Fig. 30). ARCH19 probe had a slightly higher number of hybridizations compared to EUB338I (Fig. 31). Further analysis was discontinued as it could not provide additional information for the purpose of this study.

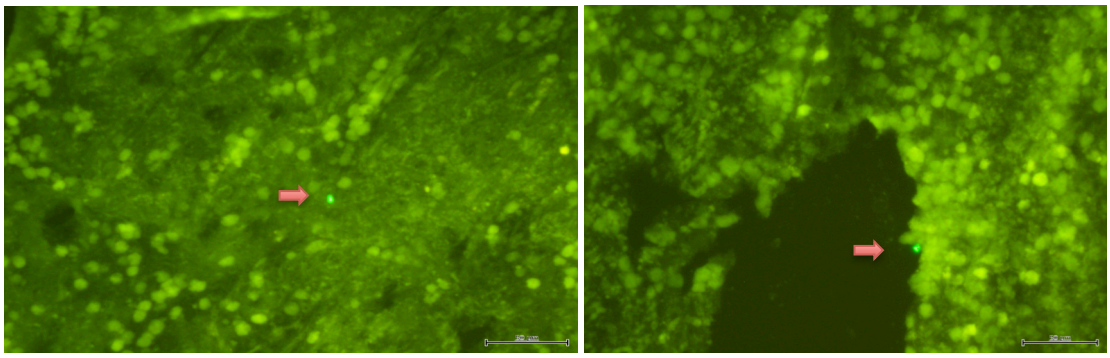


Figure 30: 10um section of Control *Cinachyrella kuekenthali* explant on Day 8 hybridized with EUB338 probe (arrow) with background autofluorescence.

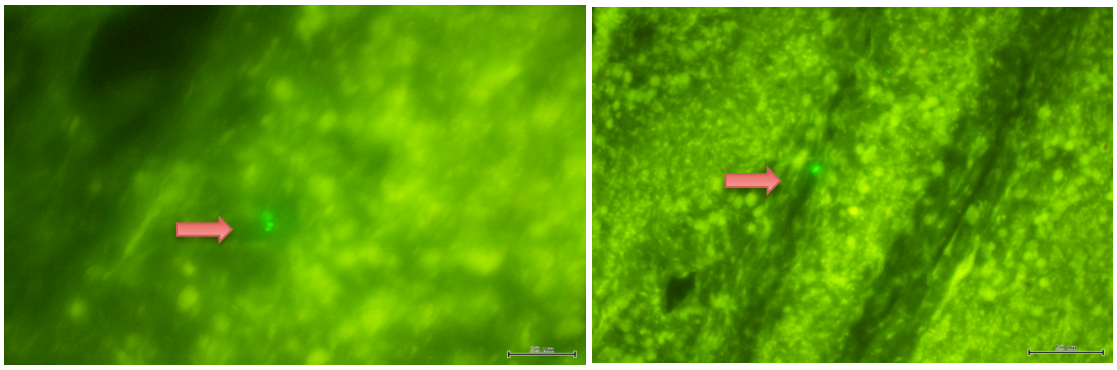


Figure 31: 10um section of Day 0 *Cinachyrella kuekenthali* with ARCH19 probe (arrow) with background autofluorescence.

DISCUSSION

The primary aim of this study was to investigate the potential to develop an aposymbiotic sponge in order to study the detailed interactions between sponge and bacteria. The change in gene expression between a symbiotic and aposymbiotic sponge could provide the metabolic interaction of the two organisms. Other aspects such as presence of photosymbionts and other bacterial symbionts were also investigated. Reverse engineering the process of introducing bacteria to an aposymbiotic sponge by quantifying the gradual elimination of identified bacterial taxa along with the change in sponge transcriptomics, could help provide more information regarding the sponge-bacterial interactions. To achieve this, the effect of antibiotics on sponge bacterial community was established in this project using electron micrographs and 16S rRNA metagenomics.

The sponge *Cinachyrella sp* was chosen because it has been developed as an experimental model sponge in our laboratory after the Deepwater Horizon oil spill accident (E. Blake NSU Masters Thesis, 2013; Cuvelier et al, 2014). However, verifying the taxonomy of the *Cinachyrella* genus has been more complicated than expected (Rutzler and Smith 1992). Preliminary analysis of the taxonomy of this sponge from South Florida region has identified possibly 2-3 closely related or visually similar taxa - *Cinachyrella kuekenthali,* and possibly two different *Cinachyrella alloclada*-like species (P. Cardenas, pers communication/unpublished).

Limitations in the Experiment

One problem in the current experiments appeared from hindsight, such as the lack of true replicates. Due to this, changes in bacterial composition of the explants due to antibiotic and time were analyzed separately. Using multiple identified sponges to make explants would provide true replicates. Multiple replicates would allow for the statistical power to combine the effects of antibiotics, time and light/dark condition in the analysis. However, it was discerned that light/dark condition did not significantly alter the bacterial community. Hence, the combined effect of antibiotics and time was studied using ADONIS test.

Another limitation, is the unidentified nature of the HMA *Cinachyrella sp* identified in 2011 (CIN-W and CIN-T 2011). The prior findings of *C. alloclada* as an LMA sponge (Gloeckner et al., 2014) could rule out that CIN-W and CIN-T were *C. alloclada* and were most likely *C. kuekenthali*. The sponges were collected in South Florida waters populated with *C. alloclada* and *C. kuekenthali.*

Shift in Microbial Abundance in Sponge Aquaculture

Several earlier studies have defined the criteria for high and low microbial abundance (LMA) sponge hosts via TEM (Gloeckner et al., 2014). TEM was a necessary tool to visualize the effects of experiments of antibiotics, since 16S rRNA sequencing can only identify and not quantify all possible bacteria present in the sample.

The preliminary analysis of the *Cinachyrella sp* specimens collected in 2011 (CIN-W 2011 and CIN-T 2011) showed possession of a high bacterial abundance (Fig.4 and Fig 5). Though we cannot identify the species of *Cinachyrella* collected in 2011, we can speculate that they were *C. kuekenthali.* Interestingly, a high microbial abundance was observed after 6 weeks of starvation in the closed tank system (Fig. 5). On the other hand, *Cinachyrella alloclada* has been previously reported to be of low microbial abundance (Gloeckner et al., 2014). This raises the question of what levels of symbiont species specificity exist in the microbial abundances of marine sponge of Genus *Cinachyrella*. This could correspond to previous findings of distinct microbial communities in different groups of *Cinachyrella sp* (Cuvelier et al., 2014).

TEM at the point of collection of the *Cinachyrella kuekenthali* (day 0) used in my experiments was not obtained and cannot provide evidence of the initial microbial abundance of the sponge. From the following evidence, we can speculate that the *Cinachyrella kuekenthali* are HMA sponges, in order to verify the loss of bacteria:

- Bacterial phylum Chloroflexi was found in high abundance (Fig. 20), which is characteristic of HMA sponge (Schmitt et al., 2011)
- The dead *Cinachyrella sp,* also identified as *Cinachyrella kuekenthali* harbored larger abundance of anaerobic bacteria (eg: Clostridia seen in Fig. 18). Steep concentration gradients are formed, on account of the denser HMA sponges, a

slower pumping rate through its complex aquiferous system and narrower canals. Deteriorating HMA sponges reduce pumping rates that reduces internal oxygen creating an anoxic internal environment (Weisz et al., 2008).

- There is evidence of HMA *Cinachyrella sp* from TEM of samples collected in South Florida waters in 2011 (CIN-W and CIN-T) (Fig. 4 and Fig. 5). The microbial abundance in *C. kuekenthali* after 6 weeks in tank possesses high microbial abundance in mesohyl.

The loss of bacteria in *C. kuekenthali* could be due to their uptake by the sponge by phagocytosis. Since there was no external food source supplied to the sponge, it could explain the decrease of bacteria in the sponge mesohyl. In addition, a large number of bacteria in the sponge could be transient, which were lost when transferred to the aquaculture.

Other Significant Structures Observed

Common bacterial morphotypes appeared unaffected by changes in environment (Fig 13B) , and some morphotypes have been observed in previous studies of *Stromatospongia micronesica* (Fuerst et al., 1998). It is highly likely this particular morphotype is an archae as it is consistent with the 16S rRNA analysis data. This morphotype also resembled *type 'a'* morphotype bacteria identified in *Oscarella malkhovi* (Vishnyakov and Erekovsky 2009) and *Aplysina fulva* (Hardoim et al., 2009).

Microbiome Analysis of Cinachyrella kuekenthali Explants

Community Composition

Although my aims differed from previous studies, this study complements recent microbiome characterizations of *Cinachyrella sp.* Previous findings suggest that the sponge used in my study (*C. kuekenthali*) played a significant role in its microbial community with little influence of geographic location and season (Cuvelier et al, 2014).

The OTUs with the highest abundance were identified by BLAST analysis to reveal the most abundant bacterial phyla present in the *Cinachyrella kuekenthali* explants throughout the experiement (Table 6). The bacterial community in the *Cinachyrella sp*

used in this experiment is more similar to Sponge Group 2 (Cuvelier et al., 2014) except Poribacteria, Deltaproteobacteria and PAUC34f were not observed.

Table 6: Bacterial symbionts of *Cinachyrella kuekenthali* and their significance. The OTU ID along with its FASTA sequence can be found in APPENDIX 6.

Taxonomic Class	**OTU# present in top 100**	**BLAST % Identity & e-value**	**Species and Location**	**Significance**
Acidobacteria	587581	100% & 0	Uncultured bacterium from Caribbean sponge Svenzea zeai (Lee et al., 2009, Accession: FJ529305.1)	Order iii1-15 of Class Acidobactera-6 and Phylum Acidobacteria are commonly found in soil. As they are largely unculturable, role of marine acidobacteria are less understood.
Bacteroidetes	New.ReferenceOTU35	97% & 5e-120	Uncultured bacterium from sponge tissue *Cymbastella coralliophila*, Great Barrier Reef (JX455299.1)	Phylum Bacteroidetes was found in sponge group 2 of *Cinachyrella spp*(Cuvelier et al 2014). Show obvious spatial specificity for the endosome (Yang and Li 2012)

Chloroflexi:	New.ReferenceOTU27	99% & 2e-140	uncultured bacterium isolated from *Cinachyra* sp in the Gulf Of Mannar (KC861135.1, Jasmin et al., 2015).	Chloroflexi found in different HMA sponges are similar whereas Chloroflexi in low microbial abundant sponges are more similar to seawater. Average OTUs observed in all explants were 433.2, which is characteristic of HMA sponge (Schmitt et al., 2011).
Actinobacteria:	213687	100% & 6e-150	uncultured bacterium isolated from coral *Porites lutea,* China on the Luhuitou fringing reefs (Kuang, W 2014, Unpubb, Accession KP305285.1).	Mutualistic actinobacteria found in insects have the ability to exploit wide variety of carbon and nitrogen sources and are hypothesized to engage in protective symbiosis (Kaltenpoth 2009). Due to its potential to produce important secondary metabolites, it is largely targeted for natural product and drug discovery.
Thaumarchaeota; Genus Cenarchaeum	New.ReferenceO	96% & 1e-127	Uncultured bacterium from Marine sponge *Stellata normani*	Vertical transmission and presence of AOA indicates possible role of ammonia detoxification in marine

	TU41		(Kennedy et al., 2014, Accession: KF597128.1)	sponge (Steger et al., 2008). Preliminary analysis confirms presence of ammonia-oxidizing archae using amoA primers for control and antibiotic treated explants. Due to the difference in structure of archae and bacteria, classic beta-lactam antibiotics directed against murein biosynthesis have no growth inhibition against archae. Antibiotics such as bacitracin and gardimycin, interfere with lipid cycle are inhibitory to different archae (Kandler and Konig 1998)

Alphaproteobacteria	New.ReferenceOTU28	96% & 1e-131	Uncultured bacterium from coralline sponge *Astrosclera willeyana* of the Great Barrier Reef (Karlinska-Batres,K 2014)	Alphaproteobacteria are found in sponges from several oceans and dominate the culturable community of the marine sponge *Rhopaloeides odorabile* (Webster et al., 2013). *Rhodobacteraceae* of the Order Rhodobacterales, was found common in *H. erectus* and *X. testudinaria* (Cleary et al., 2015).
Gammaproteobacteria	939811	99% & 2e-145	*Vibrio orientalis;* Pacific oyster hemolymph (Wendling et al., 2014 Accession: KJ507431.1) *Vibrio alginolyticus;* epidermal layer of stingrays (Luer et al., 2015, unpubb, Accession KP713657.1).	Members of this class have a broad range of trophism, temperature adaptations and morphologies (Williams et al., 2010). It is a common inhabitant of marine sponges (Webster et al., 2010) and nematodes (Woyke et al., 2006).

Epsilonproteobacteria	625724	100% & 6e-150	Uncultured bacterium from coral, *Porites lutea* (Roder et al., 2014 Accession KC527463.1).	Genus Arcobacter belonging to Order Campylobacterales was identified in the control explants and a lower abundance in the tank water. Genus Arcobacter is said to prefer microaerophilic conditions with an established sulfide-oxygen gradient flowing system (Wirsen et al., 2001). This could describe a sponge system, with an aquiferous system and lower levels of oxygen.
		99% & 9e-148	uncultured bacterium from sponge *Axinella corrugata* and surrounding seawater in Fort Pierce (Lopez et al., 2008 unpubb,Accession FJ215397.1).	

Nitrospirae	605566	99% & 7e-120	Unculutred bacterium from *Ircinia* spp in Bahamas (Pita et al., 2013. Accession JX280167.1), sponges in Great Barrier Reef (Erwin et al., 2012 Accession JX206652.1) and marine sponge *Stelleta normani* in Irish territorial waters (Kennedy et al., 2014 Accession: KF597114.1).	Nitrospirae is common in many sponges with varying abundance. White et al., (2012) found an abundance of Nitrospira in sediment but not in Broward seawater. They are mostly uncultured and diverse nitrite-oxidizing bacteria present in natural ecosystems and biological wastewater treatment. Nitrite oxidation is the second step in nitrification (Lucker et al., 2010). Hence it could play a role in removal of waste material in the sponge. Only few studies have addressed their physiology and ecology.
Actinobacteria:	New.ReferenceOTU13	99% & 7e-144	uncultured bacterium isolated from *Cinachyra* sp. from Gulf of	Mutualistic actinobacteria found in insects have the ability to engage in protective symbiosis

			Mannar, India (Jasmin et al., 2015)	(Kaltenpoth 2009). Due to its potential to produce important secondary metabolites, it is largely targeted for natural product and drug discovery.

Shifts in Bacterial Community

The bacterial community richness of the explants has a Shannon index ranging from 4.0-4.5 (Fig. 23), which is approximately within the range found in previous analysis (Cuvelier et al., 2014). However the bacteria in the control explants after 2 days appeared to be stressed, compared to that found in whole sponge (Fig. 8). Stressed bacterial morphology does not possess characteristic features such as a nucleoid similar to that of healthy bacteria. This shift in bacterial state could be due to change in environmental conditions. Artificial seawater does not possess sufficient nutrients to support growth of sponge-associated microbes. This could eliminate the bacteria that are not predominantly dependent on the sponge substrate and are transient. Also, the change in sponge activity from whole to explant state is not known and could contribute to the change in bacterial community. There was no significant difference in bacterial community of control explants versus wild in *Corticum candelabrum*, a HMA sponge (De Caralt et al., 2003). This could imply this manipulation could vary depending on sponge host.

a. Effect of Antibiotics

Comparing the TEM images of the control explant against the antibiotic treated, gives a visual confirmation of denatured bacterial cells and sponge cells under stress (Fig 13B). Bacterial communities treated with antibiotics have evidence of bacterial cells with cell envelope devoid of nucleoid and enlarged denatured bacterial structures (Figs 13&14). This is a characteristic of bactericidal antibiotics as they denature bacteria, whereas bacteriostatic will only prevent bacteria from further replication, leaving behind intact 16S rRNA.

This loss of bacterial species richness due to antibiotics is evident, with penicillin-streptomycin and cocktail with the lowest Shannon index value (Fig 23). The loss of Bacterial species was also observed in the community composition analysis of the bacterial community in explants based on phylogenetic Order (Fig. 22). The relative abundance of Vibrionales, Flavobacteriales, Campylobacterales and Alteromonadales is significantly reduced in the antibiotic treatments compared to that of Control. However,

the increase in relative abundance of certain Orders such as Cenarchaeales and Chromatiales is due to the loss of other bacterial species. The loss of particular species in a community will result in increase in the "relative" abundance of other species, even if their abundances are unaltered.

Variation in the bacterial species composition among explants under the effect of antibiotics was also revealed using Unifrac and Bray-Curtis analysis. The bacterial community in the control explants are not constant and are significantly different from the antibiotic treatments, except that of ampicillin. Ampicillin is a semi-synthetic derivative of Penicillin with an added amino group such that it targets both gram-negative as well as gram-positive bacteria (Sharma et al., 2013). The bacterial communities in explants treated with the antibiotic cocktail and penicillin-streptomycin overlapped in the Unifrac analysis (Fig 25). This indicates that both treatments resulted in similar bacterial communities. This similarity can also be discerned from the bacterial composition of the explants (Fig. 21). The relative abundance of bacterial Class in penicillin-streptomycin is similar to Antibiotic Coctail. Penicillin-streptomycin proves to be most effective as it has similar effect as the cocktail of antibiotics and clustered significantly away from control. It is a combination of synergistic antibiotics as the penicillin allows for the streptomycin to be taken up by the bacteria by damaging the cell wall (Farber and Mates 1986). Other studies using Penicillin-streptomycin treated sponge cell aggregates (SCA) of *Clathria prolifera* were more successfully maintained compared to control and other antibiotics: Nalidixic acid, trimethoprim and mixed (Richardson et al., 2012). Another example demonstrating effects of penicillin-streptomycin on bacterial community of *Corticum candelabrum* explants by TEM, where bacterial cells denatured after 24 hours but recovered after 2 months (De Caralt et al., 2003). While factors such as time, host and environment are important to consider, penicillin-streptomycin points to be a possible combination to use in order to remove non-symbiotic bacteria.

Broad-spectrum bacteriostatic tetracycline also causes a shift in bacterial community of the explants, but the true effect cannot be analyzed in this study due to the damage to the sponge tissue because of its phototoxicity. Phototoxicity can affect ribosomes and cell membranes of both prokaryotic and eukaryotic organisms (Chopra

and Roberts 2001).

b. Temporal effects

Another important factor to consider was time. Using explants from the same individual sponge for comparison confirms the effect of changing bacterial community, as the explants would initially harbor the same bacterial community. My experiments showed that the action of antibiotics on the bacterial community is gradual and more effective with increase in time. That is, replinishing the media with fresh antibiotics can gradually change the bacterial community. Weighted Unifrac analysis reveals the bacterial community in day 10 is similar to bacterial community in day 4 and day 2, but there is a change in the relative abundance. This accounts for the increasing and overlapping elliptical area enclosing the bacterial communties from day 2 to day 10, with day 2 at the center (Fig. 26). In addition, the change in the relative abundance of bacterial community with time is apparent in the community composition analysis of the top 100 OTUs in the explants (Fig. 23)

Changes in bacterial communities of control explants over time were also observed (Fig 26). This could indicate (1) loss of bacteria due to lack of nutrients/change in internal environment (2) change in sponge explant resulting in change in its bacterial community. All the changes seen could also be the result of contamination by an external bacterial agent. With the help of unweighted unifrac, which analyses data based of the presence or absence of bacteria, we observe significant overlapping of bacterial community in the explants, based on antibiotics and duration (Fig. 28 and Fig. 29). This implies that the explants share similar bacterial communities. The difference in ellipsoidal area encompassing the bacterial communities, at different times, would be due to difference in relative abundance. Hence, unweighted Unifrac refutes the possibility of change in bacterial community due to introduction of contaminants.

c. Effect of Irradiance

Cinachyrella kuekenthali is a dense sponge with a complex internal system that receives minimum light. Cyanobacteria are often found localized on the surface of all *Cinachyrella sp,* characteristic of marine sponge found on the trophic zone (Hentschel et

al., 2006). Apart from cyanobacteria, we focused on possible changes in phototrophic proteobacteria, chloroflexi and Anaerolinea, if present. Cyanobacteria have been found in the endosome of the golf-ball sponge of *Tethya aurantium,* which indicates that it is possible to be internally present (Yang and Li 2012).

Initial efforts with developing *Cinachyrella sp* cell line showed the presence of filamentous cyanobacteria in TEM (Appendix 5), but was not observed in explants. However, slightly higher bacterial abundance was observed in control explants exposed to light compared to dark condition after 8 days *in vitro* (Fig 12). This could be due to spatial difference in sections obtained from the explants. However, significant overlapping of bacterial communities in light and dark conditions were observed in the weighted unifrac analysis (Fig. 27). This indicates that explants placed in light and dark share majority of their bacterial communities. Resulting ADONIS test considering the factor "Irradiance" alone will result in false positives as other factors "time" and "antibiotics" play a significant role in altering the bacterial community. The combined effect of Irradiance with Time and Irradiance (p-value= 0.131) with Antibiotics was not significant (p-value= 0.153). Hence, sequence analysis showed no significant changes in bacterial community due to light compared to dark conditions.

It is possible that Cyanobacteria observed in the previous study of *Cinachyrella sp* (Cuvelier et al., 2014) was due to its presence on the surface/external layers of the sponge. This was significantly lost during the experiment and also in this study the experiment focused on internal symbionts. Absence of cyanobacteria in the sponge tissue is consistent with Erwin and Thacker's (2007) findings that identified very low levels of chlorophyll-a in *Cinachyrella alloclada* (<50ug/g).

CONCLUSIONS

Determining the stability of microbial associates of marine sponge is important in order to predict the success of aquaculture. Symbiotic microbial communities will be affected by artificial cultivation due to changes in environment (Webster et al., 2011). Explant culture of *Cinachyrella kuekenthali* has proved to alter the bacterial community of the control explants. However the community composition analysis reveals the

persistence of characteristic symbionts, which could suggest the loss of transient bacteria.

Based on the community composition analysis of the explants, Thaumarchaeota, Chloroflexi, Nitrospira, Acidobacteria, Actinobacteria and Gammaprtoebacteria are possible symbionts. Most of the bacteria identified in sponges are either yet to be cultured or difficult to culture (Sfanos et al, 2005). More recent studies are now pointing to the possibility that most species have a few "core", acclimated bacteria found in specific sponge hosts and thrive in their unique internal environment (Easson and Thacker 2014). The oxygen gradient, nutrients and a continuous flowing system provide the bacteria with desirable environment for growth. Yet the question remains, whether or not presence of bacteria is caused by or is the result of the morphological and physiological difference between high and low microbial abundant sponges (McDonald et al., 2002). *Cinachyrella kuekenthali* appeared to be metabolically active even with the loss of bacteria (Figs. 12 & 13), which could indicate that high microbial abundance is a result of its internal structure. To confirm a symbiotic role of the bacteria and sponge, interaction between the organisms will need to be identified.

This study has shown the bacterial community stability in the sponge *Cinachyrella* in aquaculture environment. This was observed via TEM images of *C. kuekenthali* after 6 weeks in a closed tank system supplied with only filtered seawater (Fig. 6). Ultrasctructure studies also show the change of bacterial abundance from whole sponge to explant state (Fig. 7 & Fig. 8).

Through rigorous statistical analysis using QIIME and R, the effects of antibiotics were also established with penicillin-streptomycin being the most effective after 10 days of treatment. This was established due to the significant overlapping of the bacterial communities in the exaplants treated with penicillin-strepromycin and the antibiotic cocktail using Weighted Unifrac analysis. The bacterial communities in the explants also appear to change with time, with respect to relative abundance. In addition, there was no significant difference of bacterial communities in the explants treated with light versus dark. The effect of antibiotics was also corroborated with TEM images of the mesohyl of the explants (Fig. 14 & Fig. 15).

Since it is challenging to determine the health and viability of explants, TEM was often used to check phagocytic activity. Evidence of phagocytosis of bacteria by sponge cell indicates metabolic activity of sponge cells . This verifies that the sponge cells were alive during the experiment (Fig 12).

FUTURE RESEARCH

To correspond 16S analysis with TEM, it is essential to determine the bacteial abundance, state and functional ultrastructure of the sponge microbiome, revealing information that might identify the LMA versus HMA condition of a partiular species and its ultrastructural stability. Analysing the TEM and 16S rRNA of bacterial community of *Cinachyrella* identified at the species level, would help discern the reason for the differences in bacterial communnity identified in previous findings (Cuvelier et al., 2014). In addition, it would be interesting to re-analyze the TEM of a larger sample size of *C. alloclada* and *C. kuekenthali* to confirm their LMA and HMA status respectively. Examination whether the differences between these sponges is consistent with previous findings comparing HMA and LMA sponges (Gloeckner et al., 2014), or the differences are largely contributed based on differences at the species level (Easson and Thacker 2014). This difference in abundance of bacteria within the same genus of sponge that are morphologically similar and found in the same geographic location, is particularly interesting. Latest research provides evidence of morphological and physiological differences between high and low microbial abundant sponges (Gloeckner et al., 2014).

Now that we have established the effect of antibiotics, the optimum time period, antibiotic to use and identified bacteria associated with the sponge, quantitating the identified bacteria in the sponge treated with penicillin-streptomycin with qPCR along with change in gene expression levels of the sponge could provide more information about their symbiosis. This study suggests that *Cinachyrella kuekenthali* could be a potential model HMA sponge to study this evolutionary relationship.

Developing an aposymbiotic sponge may not be possible as bacteria provide necessary nutirents for sponge development or as the sponge cells and the microbial

associates exist as a biological unit. However, analyzing change in gene expression of the sponge at different stages of loss of bacteria can provide more information on sponge-bacterial symbiosis. Possible genes to focus on would be scavenger receptor cysteine rich domains (SRCR) (Steindler et al., 2007), Nucleotide binding domains and Leucine rich repeats (NLR) (Degnan 2014). Isotope analysis of Carbon and Nitrogen could also be indicators of changes in the sponge physiology (Weisz et al., 2007).

The sponge explants were transferred to the closed system aquarium after in vitro treatment but regeneration was not observed by macroscopic observation. A higher concentration of marine bacteria in the order of 10^6 cells/ml could be necessary in order to support sponge nourishment (De Caralt et al., 2003) to predict the ability of *Cinachyrella sp* regeneration. Further analysis will also demonstrate the importance of specific bacterial symbionts for sponge development.

To summarize, a significant drop in bacterial abundance was observed in a high microbial abundance sponge under a stravation period of 6 weeks. This although a rare occurrence, suggests that the microbial abundance in a sponge might be altered by the microbial abundance of the sea water, soil or other organisms surrounding the sponge. This study demonstrates the utilization of antibiotics for manipulating the sponge-associated bacterial community and identifies the bacterial communities that persist.

References

Bergquist, P. R.,. ""Porifera"." *Invertebrate Zoology, Oxford University Press*, 1998: 10–27.

Bertin, Matthew J., Sarah L. Schwartz, John Lee, Anton Korobeynikov, Pieter C. Dorrestein, Lena Gerwick, and William H. Gerwick. "Spongosine Production by a Vibrio harveyi Strain Associated with the Sponge Tectitethya crypta." *Journal of natural products 78, no. 3*, 2015: 493-499.

Bertin, Matthew J., Sarah L. Schwartz, John Lee, Anton Korobeynikov, Pieter C. Dorrestein, Lena Gerwick, and William H. Gerwick. "Spongosine Production by a Vibrio harveyi Strain Associated with the Sponge Tectitethya crypta." *Journal of natural products 78, no. 3*, 2015: 493-499.

Björk, Johannes R., Cristina Díez-Vives, Rafel Coma, Marta Ribes, and José M. Montoya. "Specificity and temporal dynamics of complex bacteria-sponge symbiotic interactions." *Ecology 94, no. 12*, 2014: 2781-2791.

Blunt J.W., Copp, B.R., Hu, W.P., Munro, M. H. G., Northcote, P. T., and Prinsep, M. R. "Marine Natural Products." *Nat Prod Rep 26*, 2009: 170-244.

Brochier-Armanet C., Boussau B., Gribaldo S., Forterre P. "Mesophilic crenarchaeota: proposal for a third archaeal phylum, the Thaumarchaeota." *Nat Rev Microbiol 8*, 2008: 245–252.

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., ... & Knight, R. "QIIME allows analysis of high-throughput community sequencing data." *Nature methods, 7(5)*, 2010: 335-336.

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Lozupone, C. A., Turnbaugh, P. J., Fierer, N., and Knight, R. "Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample." *PNAS 108 Suppl 1*, 2011: 4516-4522.

Carballo, Jose L., Benjamin Yañez, Eva Zubía, Maria J. Ortega, and Cristina Vega. "Culture of explants from the sponge Mycale cecilia to obtain bioactive mycalazal-type metabolites." *Marine biotechnology 12, no. 5*, 2010: 516-525.

Cardenas, P., Menegola, C., Rapp, H., & Diaz, M. "Morphological description and DNA barcodes of shallow-water Tetractinellida (Porifera: Demospongiae) from Bocas del Toro, Panama, with description of a new species." *Zootaxa*, 2009: 1-39.

Chang, Ju Young, Dionysios A. Antonopoulos, Apoorv Kalra, Adriano Tonelli, Walid T. Khalife, Thomas M. Schmidt, and Vincent B. Young. "Decreased diversity of the fecal

microbiome in recurrent clostridium difficile—associated diarrhea." *Journal of Infectious Diseases 197, no. 3* , 2008: 435-438.

Chopra, Ian, and Marilyn Roberts. "Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance." *Microbiology and molecular biology reviews 65, no. 2*, 2001: 232-260.

Cleary, Daniel FR, Nicole J. de Voogd, Ana RM Polónia, Rossana Freitas, and Newton CM Gomes. "Composition and Predictive Functional Analysis of Bacterial Communities in Seawater, Sediment and Sponges in the Spermonde Archipelago, Indonesia." *Microbial ecology*, 2015: 1-5.

Crispino, A., A. De Giulio, S. De Rosa, and G. Strazzullo. "A new bioactive derivative of avarol from the marine sponge Dysidea avara." *Journal of natural products 52, no. 3* , 1989: 646-648.

Cuvelier, Marie L., Emily Blake, Rebecca Mulheron, Peter J. McCarthy, Patricia Blackwelder, Rebecca L. Vega Thurber, and Jose V. Lopez. "Two distinct microbial communities revealed in the sponge Cinachyrella." *Frontiers in microbiology 5* , 2014.

Daims, Holger, Andreas Brühl, Rudolf Amann, Karl-Heinz Schleifer, and Michael Wagner. "The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set." *Systematic and applied microbiology 22, no. 3*, 1999: 434-444.

De Caralt, Sonia, Javier Sánchez-Fontenla, María J. Uriz, and Rene H. Wijffels. "In situ aquaculture methods for Dysidea avara (Demospongiae, Porifera) in the Northwestern Mediterranean." *Marine drugs 8, no. 6*, 2010: 1731-1742.

de Caralt, Sònia, Gemma Agell, and María-J. Uriz. "Long-term culture of sponge explants: conditions enhancing survival and growth, and assessment of bioactivity." *Biomolecular Engineering 20, no. 4*, 2003: 339-347.

de Goeij, Jasper M., Dick van Oevelen, Mark JA Vermeij, Ronald Osinga, Jack J. Middelburg, Anton FPM de Goeij, and Wim Admiraal. "Surviving in a marine desert: the sponge loop retains resources within coral reefs." *Science 342, no. 6154*, 2013: 108-110.

De Rosa, S., G. Tommonaro, K. Slantchev, K. Stefanov, and S. Popov. "Lipophylic metabolites from the marine sponge Ircinia muscarum and its cell culture." *Mar. Biol. 140*, 2002: 465–470.

De Rosa, Salvatore, Salvatore De Caro, Carmine Iodice, Giuseppina Tommonaro, Kamen Stefanov, and Simeon Popov. "Development in primary cell culture of demosponges." *Journal of biotechnology 100, no. 2*, 2003: 119-125.

Degnan, Sandie M. "The surprisingly complex immune gene repertoire of a simple sponge, exemplified by the NLR genes: A capacity for specificity?" *Developmental & Comparative Immunology 48, no. 2*, 2014: 269-274.

DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber,. " Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB." *Appl Environ Microbiol 72*, 2006: 5069-72.

Dikow, Rebecca B., and William Leo Smith. "Genome-level homology and phylogeny of Vibrionaceae (Gammaproteobacteria: Vibrionales) with three new complete genome sequences." *BMC microbiology 13, no. 1*, 2013: 80.

Duckworth, A. "Farming sponges to supply bioactive metabolites and bath sponges: a review." *Marine Biotechnology, vol. 11, no. 6,*, 2009: 669–679.

Edgar, R.C. "Search and clustering orders of magnitude faster than." *Bioinformatics 26 (19)*, 2010: 2460-2461.

Erwin, Patrick M., and Robert W. Thacker. "Phototrophic nutrition and symbiont diversity of two Caribbean sponge-cyanobacteria symbioses." *Erwin, Patrick M., and Robert W. Thacker. "Phototrophic nutrition and symbiont diversity of two Caribbean sponge-cyanobacteria symbioses." Mar Ecol Prog Ser 362*, 2008: 139-147.

Erwin, Patrick M., and Robert W. Thacker. "Incidence and identity of photosynthetic symbionts in Caribbean coral reef sponge assemblages." *Journal of the Marine Biological Association of the United Kingdom 87, no. 06* , 2007: 1683-1692.

Erwin, Patrick M., Lucía Pita, Susanna López-Legentil, and Xavier Turon. "Stability of sponge-associated bacteria over large seasonal shifts in temperature and irradiance." *Applied and environmental microbiology 78, no. 20* , 2012: 7358-7368.

Erwin, Patrick M., Lucía Pita, Susanna López-Legentil, and Xavier Turon. "Stability of sponge-associated bacteria over large seasonal shifts in temperature and irradiance." *Applied and environmental microbiology 78, no. 20*, 2012: 7358-7368.

Faulkner, D. J. "Marine pharmacology." *Antonie van Leewenhoek 77*, 2000: 135-145.

Fiore, C. L., Labrie, M., Jarett, J. K., & Lesser, M. P. "Transcriptional activity of the giant barrel sponge, Xestospongia muta Holobiont: molecular evidence for metabolic interchange. ." *Frontiers in Microbiology, 6*, 2015: 364.

Freymann D, Nakamura Y, Focia P.J, Sakai R and Swanson G.T. "Structure of a tetrameric galectin from Cinachyrella sp. (ball sponge)." *Acta Crystallogr D Biol Crystallogr ; 68(Pt 9)*, 2012: 1163–1174.

Friedrich, Anja B., Isabell Fischer, Peter Proksch, Jörg Hacker, and Ute Hentschel. "Temporal variation of the microbial community associated with the Mediterranean sponge Aplysina aerophoba." *FEMS Microbiology Ecology 38, no. 2-3*, 2001: 105-113.

Fuerst, John A., Richard I. Webb, Mary J. Garson, Lani Hardy, and Henry M. Reiswig. "Membrane-bounded nucleoids in microbial symbionts of marine sponges." *FEMS microbiology letters 166, no. 1*, 1998: 29-34.

Fuhrman J.A., McCallum K., Davis A.A. "Novel major archaebacterial group from marine plankton." *Nature 356*, 1992: 148–149.

Garrity, G. M., J. A. Bell, and T. G. Lilburn. *Class III. Gammapro- teobacteria class. nov., p. 1.* Vol. 2, in *Bergey's manual of systematic bacteriology, 2nd ed.,* , by N. R. Krieg, J. T. Staley, and G. M. Garrity In D. J. Brenner. New York, NY: Springer, 2005.

Gloeckner, Volker, Markus Wehrl, Lucas Moitinho-Silva, Christine Gernert, Peter Schupp, Joseph R. Pawlik, Niels L. Lindquist, Dirk Erpenbeck, Gert Wörheide, and Ute Hentschel. "The HMA-LMA dichotomy revisited: an electron microscopical survey of 56 sponge species." *The Biological Bulletin 227, no. 1*, 2014: 78-88.

Grasela, James J., Shirley A. Pomponi, Buki Rinkevich, and Jennifer Grima. "Efforts to develop a cultured sponge cell line: revisiting an intractable problem." *In Vitro Cellular & Developmental Biology-Animal 48, no. 1* , 2013: 12-20.

Haas, B.J., Gevers, D., Earl, A.M., Feldgarden, M., Ward, D.V., Giannouko,s G., et al. "Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons." *Genome Research 21*, 2011: 494-504.

Han Y, Yang B, Zhang F, Miao X, Li Z. "haracterization of antifungal chitinase from marine Streptomyces sp. DA11 associated with south China sea sponge Craniella australiensis." *Marine Biotechnology 11*, 2009: 132-140.

Hardoim, C. C. P., R. Costa, F. V. Araujo, E. Hajdu, R. Peixoto, U. Lins, A. S. Rosado, and J. D. Van Elsas. "Diversity of bacteria in the marine sponge Aplysina fulva in Brazilian coastal waters." *Applied and environmental microbiology 75, no. 10*, 2009: 3331-3343.

Hausmann, Rudolf, Marco P. Vitello, Frank Leitermann, and Christoph Syldatk. "Advances in the production of sponge biomass Aplysina aerophoba—a model sponge for ex situ sponge biomass production." *Journal of biotechnology 124, no. 1*, 2006: 117-127.

Hentschel, U., Usher, K. M., Taylor, M. W. "Marine sponge as microbial fermenters." *FEMS Microbiol Ecol, 55*, 2006: 167-177.

Hentschel, Ute, Jörn Hopke, Matthias Horn, Anja B. Friedrich, Michael Wagner, Jörg Hacker, and Bradley S. Moore. "Molecular evidence for a uniform microbial community in sponges from different oceans." *Applied and Environmental Microbiology 68, no. 9* , 2002: 4431-4440.

Hong, Jin-Kyung, Hye-Jin Kim, and Jae-Chang Cho. "Novel PCR Primers for the Archaeal Phylum Thaumarchaeota Designed Based on the Comparative Analysis of 16S rRNA Gene Sequences." (PLoS ONE 9(5)) 2014: e96197.

Kaltenpoth, Martin. "Actinobacteria as mutualists: general healthcare for insects?." *Trends in microbiology 17, no. 12*, 2009: 529-535.

Kandler, O., and H. König. "Cell wall polymers in Archaea (Archaebacteria)." *Cellular and Molecular Life Sciences CMLS 54, no. 4*, 1998: 305-308.

Kasten, Britta and Reski, Ralf. "β-lactam antibiotics inhibit chloroplast division in a moss (Physcomitrella patens) but not in tomato (Lycopersicon esculentum." *Journal of Plant Physiology 150 (1–2)*, 1997: 137–140.

Kennedy, Jonathan, Burkhardt Flemer, Stephen A. Jackson, John P. Morrissey, Ferghal O'Gara, and Alan DW Dobson. "Evidence of a putative deep sea specific microbiome in marine sponges." *PloS one 9, no. 3*, 2014: e91092.

Kikuchi Y, Hosokawa T, Fukatsu T. " Insect–microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation." *Appl. Environ. Microbiol 73*, 2007: 4308– 4316.

Lee, Kuo-Chang, Richard I. Webb, Peter H. Janssen, Parveen Sangwan, Tony Romeo, James T. Staley, and John A. Fuerst. "Phylum Verrucomicrobia representatives share a compartmentalized cell plan with members of bacterial phylum Planctomycetes." *BMC microbiology 9, no. 1*, 2009: 5.

Lee, On On, Yong Wang, Jiangke Yang, Feras F. Lafi, Abdulaziz Al-Suwailem, and Pei-Yuan Qian. "Pyrosequencing reveals highly diverse and species-specific microbial communities in sponges from the Red Sea." *The ISME journal 5, no.4*, 2011: 650-664.

Lee, On On, Yue Him Wong, and Pei-Yuan Qian. "Inter-and intraspecific variations of bacterial communities associated with marine sponges from San Juan Island, Washington." *Applied and environmental microbiology 75, no. 11* , 2009: 3513-3521.

Lehnert, Erik M., Matthew S. Burriesci, and John R. Pringle. "Developing the anemone Aiptasia as a tractable model for cnidarian-dinoflagellate symbiosis: the transcriptome of aposymbiotic A. pallida." *BMC genomics 13, no. 1* , 2012: 271.

Longnecker, Krista, and Anna-Louise Reysenbach. "Expansion of the geographic distribution of a novel lineage of ε-Proteobacteria to a hydrothermal vent site on the Southern East Pacific Rise." *FEMS microbiology ecology 35, no. 3*, 2001: 287-293.

Lopez, J.V. Peterson, C.L., Willoughby, R., Wright, A.E., Enright, E., Zoladz, S., Pomponi, S.A. "Characterization of genetic markers for in vitro cell line identification of the marine sponge, Axinella corrugata." *Journal of Heredity. 93*, 2002: 27-36.

Lozupone, Catherine, Manuel E. Lladser, Dan Knights, Jesse Stombaugh, and Rob Knight. "UniFrac: an effective distance metric for microbial community comparison." *The ISME journal 5, no. 2*, 2011: 169.

Luer, C.A., Schwarz,M., Mueller,J., Skutas,J., Walsh,C.J. and Ritchie,K.B. "Innate Immunity Derived from Commensal Microbes in the Epidermal Mucus Layers of Stingrays." *unpublished*, 2015.

Müller, W. "The Origin of Metazoan Complexity: Porifera as Integrated." *Integrative and Comparative Biology 43 (1)*, 2003: 3-10.

Müller, Werner EG, and Isabel M. Müller. "Origin of the metazoan immune system: identification of the molecules and their functions in sponges." *Integrative and comparative biology 43, no. 2*, 2003: 281-292.

Müller, Werner EG, Markus Böhm, Renato Batel, Salvatore De Rosa, Giuseppina Tommonaro, Isabel M. Müller, and Heinz C. Schröder. "Application of cell culture for the production of bioactive compounds from sponges: synthesis of avarol by primmorphs from Dysidea avara." *Journal of natural products 63, no. 8*, 2000: 1077-1081.

McDonald, Justin I., John NA Hooper, and Keith A. McGuinnes. "Environmentally influenced variability in the morphology of Cinachyrella australiensis (Carter 1886)(Porifera: Spirophorida: Tetillidae)." *Marine and Freshwater Research 53, no. 1*, 2002: 79-84.

McFall-Ngai, Margaret J. "The importance of microbes in animal development: lessons from the squid-vibrio symbiosis." *Annual review of microbiology 68* , 2014: 177-194.

McMurdie, Paul J., and Susan Holmes. "phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data." *PLoS ONE 8(4)*, 2013: e61217.

Mohamed NM, Rao V, Hamann MT, Kelly M, Hill RT. "Monitoring bacterial diversity of the marine sponge Ircinia strobilina upon transfer into aquaculture." *Appl Environ Microbiol 74*, 2008: 4133–4143.

Morrow, Christine, and Paco Cárdenas. "Proposal for a revised classification of the Demospongiae (Porifera)." *Frontiers in zoology 12, no. 1*, 2015: 7.

Nguyen, Mary THD, Michael Liu, and Torsten Thomas. "Ankyrin-repeat proteins from sponge symbionts modulate amoebal phagocytosis." *Molecular ecology 23, no. 6*, 2014: 1635-1645.

Noyer, Charlotte, Emilio O. Casamayor, and Mikel A. Becerro. "Environmental Heterogeneity and Microbial Inheritance Influence Sponge-Associated Bacterial Composition of Spongia lamella." *Microbial ecology 68, no. 3*, 2014: 611-620.

Oksanen, Jari, Roeland Kindt, Pierre Legendre, Bob O'Hara, M. Henry H. Stevens, Maintainer Jari Oksanen, and M. A. S. S. Suggests. "The vegan package." *Community ecology package*, 2007: 631-637.

Osinga, R., Tramper, J. and Wijeffls, R. H. "Cultivation of amrine sponges for metabolite production: Application for biotechnology?" *Trends Biotechnology 16*, 1998: 130-134.

Pester, Michael, Christa Schleper, and Michael Wagner. "The Thaumarchaeota: an emerging view of their phylogeny and ecophysiology." *Current opinion in microbiology 14, no. 3*, 2011: 300-306.

Pita, Lucía, Susanna López-Legentil, and Patrick M. Erwin. "Biogeography and host fidelity of bacterial communities in Ircinia spp. from the Bahamas." *Microbial ecology 66, no. 2* , 2013: 437-447.

Price, M,N., Dehal, P,S., and Arkin, A.P. "FastTree 2-Approximately MaximumLikelihood Likelihood Trees for Large Alignments." *PLos One 5(3)*, 2010.

Radax, Regina, Thomas Rattei, Anders Lanzen, Christoph Bayer, Hans Tore Rapp, Tim Urich, and Christa Schleper. "Metatranscriptomics of the marine sponge Geodia barretti: tackling phylogeny and function of its microbial community." *Environmental Microbiology 14, no. 5*, 2012: 1308-1324.

Roder, Cornelia, Chatchanit Arif, Till Bayer, Manuel Aranda, Camille Daniels, Ahmed Shibl, Suchana Chavanich, and Christian R. Voolstra. "Bacterial profiling of White Plague Disease in a comparative coral species framework." *The ISME journal 8, no. 1*, 2014: 31-39.

Rodriguez J, Nunez L, Peixinho S, Jimenez C. "Isolation and synthesis of the first 6-hydroximino-4-en-3-one steroids from the sponge Cinachyrella spp." *Tetrahedron Lett 38*, 1997: 1833–1836.

Ruppert, E. E., Fox, R. S., and Barnes, R. D. *Invertebrate Zoology(7 ed.).* Brooks/Cole.pp 76-97, 2004.

Rutzler, K., & Smith, K. "Guide to Western Atlantic Species of Cinachyrella (Porifera: Tetillidae). ." *Proc. Biol. Soc. Wash.,*, 1992: 148-164.

Sambrook, J., and D.W. Russell. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, 2001.

Schaechter, Moselio, John L. Ingraham, and Frederick Carl Neidhardt. *Microbe.* ASM press, 2006.

Scheuermayer, Matthias, Tobias AM Gulder, Gerhard Bringmann, and Ute Hentschel. "Rubritalea marina gen. nov., sp. nov., a marine representative of the phylum 'Verrucomicrobia', isolated from a sponge (Porifera)." *International journal of systematic and evolutionary microbiology 56, no. 9*, 2006: 2119-2124.

Schiefenhövel, Karin, and Andreas Kunzmann. "Sponge Farming Trials: Survival, Attachment, and Growth of Two Indo-Pacific Sponges, Neopetrosia sp. and Stylissa massa." *Journal of Marine Biology. Article ID 417360*, 2012: 11.

Schippers KJ, Martens DE, Pomponi SA, Wiffels RH. "Toward development of a sponge cell line: comparison of cell proliferation in sponge cell and tissue cultures." *In Vitro Cell Dev Biol Anim.*, 2012: 105.

Schmitt, Susanne, Peter Tsai, James Bell, Jane Fromont, Micha Ilan, Niels Lindquist, Thierry Perez et al. "Assessing the complex sponge microbiota: core, variable and species-specific bacterial communities in marine sponges." *The ISME journal 6, no. 3*, 2012: 564-576.

Schröder, H., Brandt, D., Schlossmacher, U., Wang, X., Tahir, M., Tremel, W., Belikov, S., Muller, W. "Enzymatic production of biosilica glass using enzymes from sponges: Basic aspects and application in nanobiotechnology (material sciences and medicine)." *Naturwissenchaften 94*, 2007: 339-359.

Seckbach, Joseph, ed. *Symbiosis: mechanisms and model systems. Vol. 4.* . Springer Science & Business Media, 2002.

Sharma D, Cukras AR, Rogers EJ, Southworth DR, Green R. "Mutational analysis of S12 protein and implications for the accuracy of decoding by the ribosome." *Journal of Molecular Biology 374 (4*, 2007: 1065–1076.

Sipkema, Detmer, Ronald Osinga, Wolfgang Schatton, Dominick Mendola, Johannes Tramper, and René H. Wijffels. "Large-scale production of pharmaceuticals by marine

sponges: Sea, cell, or synthesis?" *Biotechnology and Bioengineering 90, no. 2*, 2005: 201-222.

Skepper, Colin K, Quach T, Molinski T.F. "Total synthesis of Enigmazole A from Cinacharylla enigmatica. Bidirectional bond construction with an ambident 2,4-Disbustituted Oxazole Synthon." *J. AM. CHEM. SOC 132*, 2010: 10286-10292.

Spang, Anja, Roland Hatzenpichler, Celine Brochier-Armanet, Thomas Rattei, Patrick Tischler, Eva Spieck, Wolfgang Streit, David A. Stahl, Michael Wagner, and Christa Schleper. "Distinct gene set in two different lineages of ammonia-oxidizing archaea supports the phylum Thaumarchaeota." *Trends in microbiology 18, no. 8*, 2010: 331-340.

Steger, Doris, Piers Ettinger-Epstein, Stephen Whalan, Ute Hentschel, Rocky De Nys, Michael Wagner, and Michael W. Taylor. ""Diversity and mode of transmission of ammonia-oxidizing archaea in marine sponges." *Environmental Microbiology 10, no. 4* , 2008: 1087-1094.

Steindler, Laura, Silvia Schuster, Micha Ilan, Adi Avni, Carlo Cerrano, and Sven Beer. "Differential gene expression in a marine sponge in relation to its symbiotic state." *Marine biotechnology 9, no. 5*, 2007: 543-549.

Sun, Liming, Yuefan Song, Yi Qu, Xingju Yu, and Wei Zhang. "Purification and in vitro cultivation of archaeocytes (stem cells) of the marine sponge Hymeniacidon perleve (Demospongiae)." *Cell and tissue research 328, no. 1* , 2007: 223-237.

Taylor MW, Schupp PJ, de Nys R, Kjelleberg S, Steinberg PD. "Biogeography of bacteria associated with the marine sponge." *Environ Microbiol 7*, 2005: 419-433.

Taylor, Michael W., Peter J. Schupp, Ingela Dahllöf, Staffan Kjelleberg, and Peter D. Steinberg. "Host specificity in marine sponge-associated bacteria, and potential implications for marine microbial diversity." *Environmental Microbiology 6, no. 2*, 2004: 121-130.

Taylor, Michael W., Peter Tsai, Rachel L. Simister, Peter Deines, Emmanuelle Botte, Gavin Ericson, Susanne Schmitt, and Nicole S. Webster. "Sponge-specific'bacteria are widespread (but rare) in diverse marine environments." *The ISME journal 7, no. 2*, 2013: 438-443.

Taylor, Michael W., Russell T. Hill, Jörn Piel, Robert W. Thacker, and Ute Hentschel. "Soaking it up: the complex lives of marine sponges and their microbial associates." *The ISME journal 1, no. 3* , 2007: 187-190.

Team, RStudio. "RStudio: Integrated Development for R. RStudio, Inc." *Boston, MA*, 2015.

Thacker, Robert W. ""Impacts of shading on sponge-cyanobacteria symbioses: a comparison between host-specific and generalist associations." *Integrative and comparative Biology 45, no. 2* , 2005: 369-376.

Thomas, Torsten, Doug Rusch, Matt Z. DeMaere, Pui Yi Yung, Matt Lewis, Aaron Halpern, Karla B. Heidelberg, Suhelen Egan, Peter D. Steinberg, and Staffan Kjelleberg. "Functional genomic signatures of sponge bacteria reveal unique and shared features of symbiosis." *The ISME journal 4, no. 12*, 2010: 1557-1567.

Thomas, Tresa Remya A., Devanand P. Kavlekar, and Ponnapakkam A. ""Marine drugs from sponge-microbe association—A review." *Marine Drugs 8, no. 4*, 2010: 1417-1468.

Turnbaugh, P. J., Ley, R. E., Hamady, M., Fraser-Liggett, C., Knight, R., & Gordon, J. I. " The human microbiome project: exploring the microbial part of ourselves in a changing world ." *Nature 449(7164)* , 2013: 804-810.

Van Soest, R. W. M., Boury-Esnault, N., Vacelet, J., Dohrmann, M., Erpenbeck, D., De Voogd, N. J., … Hooper, J. N. A. "Global Diversity of Sponges (Porifera)." *PLoS ONE, 7(4), e35105.*, 2012: doi:10.1371/journal.pone.0035105.

Vishnyakov, Andrey E., and Alexander V. Ereskovsky. "Bacterial symbionts as an additional cytological marker for identification of sponges without a skeleton." *Marine biology 156, no. 8*, 2009: 1625-1632.

Wang, Yong, and Pei-Yuan Qian. "Conservative fragments in bacterial 16S rRNA genes and primer design for 16S ribosomal DNA amplicons in metagenomic studies." *PLoS ONE 4(10)*, 2009: e7401.

Webster NS, Webb RI, Ridd MJ, Hill RT, Negri AP. "The effects of copper on the microbial community of a coral reef sponge." *Environ Microbiol 3*, 2001: 19-31.

Webster, Nicole S., and Linda L. Blackall. "What do we really know about sponge-microbial symbioses." *ISME J 3, no. 1*, 2009: 1-3.

Webster, Nicole S., Andrew P. Negri, Richard I. Webb, and Russell T. Hill. "A spongin-boring α-proteobacterium is the etiological agent of disease in the Great Barrier Reef sponge Rhopaloeides odorabile." *Marine Ecology Progress Series 232* , 2002: 305-309.

Webster, Nicole S., Rose E. Cobb, Rochelle Soo, Shelley L. Anthony, Christopher N. Battershill, Steve Whalan, and Elizabeth Evans-Illidge. "Bacterial community dynamics in the marine sponge Rhopaloeides odorabile under in situ and ex situ cultivation." *Marine Biotechnology 13, no. 2* , 2011: 296-304.

Weisz, Jeremy B., Niels Lindquist, and Christopher S. Martens. "Do associated microbial abundances impact marine demosponge pumping rates and tissue densities?" *Oecologia 155, no. 2*, 2008: 367-376.

Weisz, Jeremy B., Ute Hentschel, Niels Lindquist, and Christopher S. Martens. "Linking abundance and diversity of sponge-associated microbial communities to metabolic differences in host sponges." *Marine Biology 152, no. 2*, 2007: 475-483.

Wendling, Carolin C., Frederico M. Batista, and K. Mathias Wegner. "Persistence, seasonal dynamics and pathogenic potential of Vibrio communities from Pacific oyster hemolymph." *PloS one 9, no. 4*, 2014: e94256.

White, James R., Jignasa Patel, Andrea Ottesen, Gabriela Arce, Patricia Blackwelder, and Jose V. Lopez. "Pyrosequencing of bacterial symbionts within Axinella corrugata sponges: diversity and seasonal variability." *PLoS One 7, no. 6*, 2012: e38204.

Wilkinson, Clive R. "Nutrient translocation from green algal symbionts to the freshwater sponge Ephydatia fluviatilis." *Hydrobiologia 75, no. 3* , 1980: 241-250.

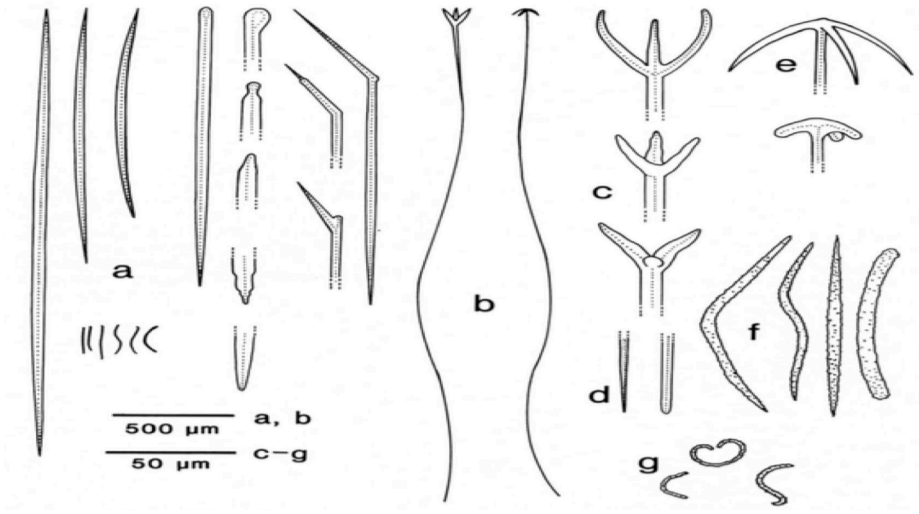
Williams, Kelly P., Joseph J. Gillespie, Bruno WS Sobral, Eric K. Nordberg, Eric E. Snyder, Joshua M. Shallom, and Allan W. Dickerman. ""Phylogeny of gammaproteobacteria." *Journal of bacteriology 192, no. 9*, 2010: 2305-2314.

Woyke, Tanja, Hanno Teeling, Natalia N. Ivanova, Marcel Huntemann, Michael Richter, Frank Oliver Gloeckner, Dario Boffelli et al. "Symbiosis insights through metagenomic analysis of a microbial consortium." *Nature 443, no. 7114*, 2006: 950-955.

Zhao, Q., Zhang, W., Jin M., Yu, X., and Deng, M. "Formulation of basal medium for primary cell culture of the marine sponge Hymeniacidon perleve." *Biotechnol. Prog. 21*, 2005: 1008-1012.

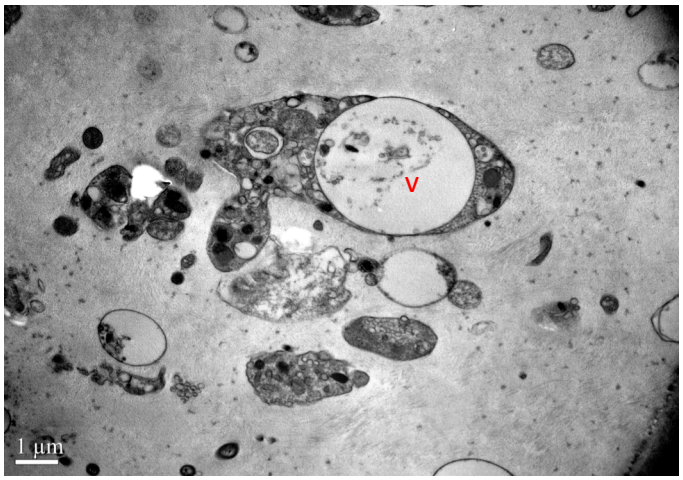
APPENDIX

APPENDIX 1



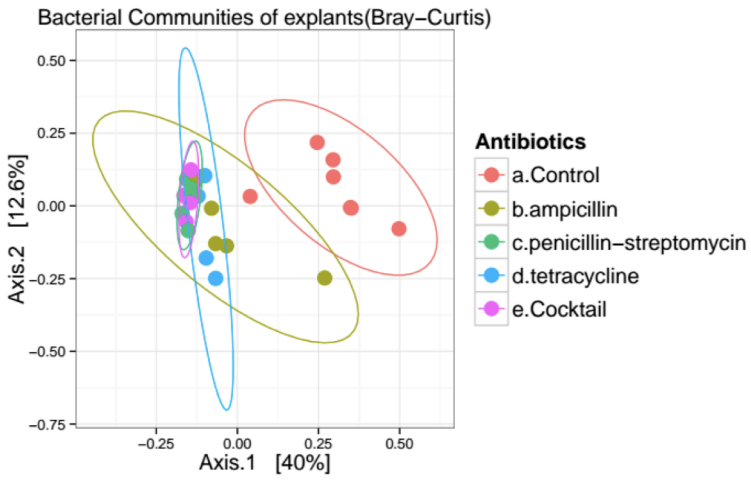
C. kuekenthali spiculation: a, Oxeas(large,meadium,small) and modifications; b, Tetractines; c, Protriaene cladomes and modifications; d, Protriaene shaft points; e, Anatriaene cladome and modification; f, Smallest (crenulate) category oxea enlarged; g, Sigmaspires. (Image credit: Ruetzler and Smith 1992)

APPENDIX 2



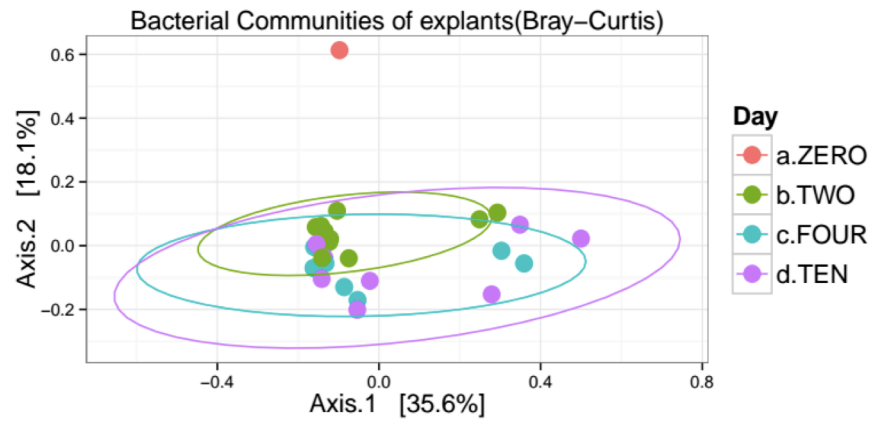
Bacterial community in Explants at day 2 treated with cocktail of antibiotics.(ABdark2) Vacuole (v) was observed in the sponge cell. (scale=1um)

APPENDIX 3



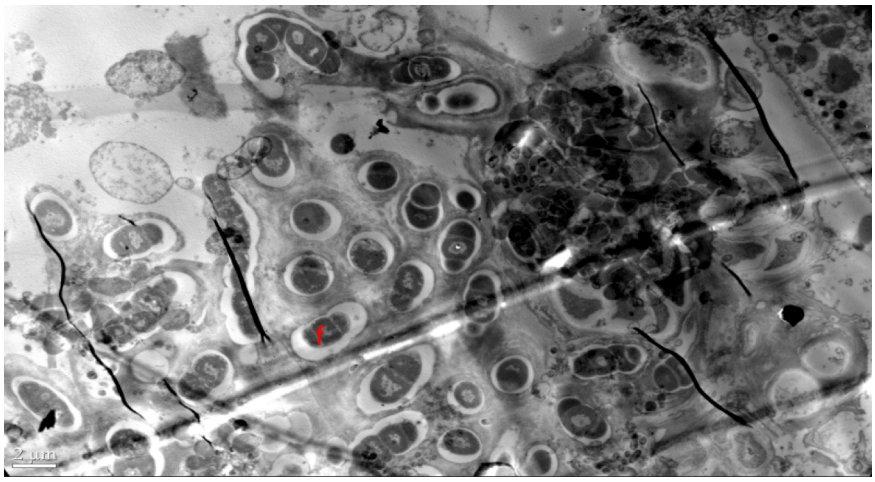
Bray Curtis analysis of bacterial communities based on antibiotic treatment. Bacterial communities are enclosed by an ellipse with 95% condifence interval. Clear separation of Control with Antibiotic treatment is observed.

APPENDIX 4



Bray- Curtis analysis of bacterial communities based on time period. Bacterial communities are enclosed in ellipse with 95% confidence interval. Day zero appears separate from day 2, 4 and 10 due to lack of replicates.

APPENDIX 5



Day 0 at attempting *Cinachyrella* sp cell cuture reveals filamentous cyanobacteria (f) in TEM (scale= 2um)

APPENDIX 6: OTU ID with its corresponding FASTA sequence

OTU ID	FASTA Sequence
New.ReferenceOTU41	GTGCCAGCAGCCGCGGTAAAACCAGCACCTCAAGTGGTCAGGAGGAT TATTGGGCCTAAAGCATCCGTAGCCGGCCGTGCCAGTCTTCGGTTAA ATCCATATGCTCAACATATGGGCTGCCGGAGATACTGCACAGCTAGG GAGTGGGAGAGGTAGACGGTACTTGGTAGGAAGGGGTAAAATCCTGT GATCTACTGATGACCACCTGTGGCGAAGGCGGTCTACTAGAACACGT CCGACGGTGAGGGATGAAAGCTGGGGGAGCAAACCGGATTAGAAACC CTTGTAGTCC
New.ReferenceOTU28	GTGCCGGCAGCCGCGGTAATACGGAGGGGGCTAGCGTTGTTCGGAAT TACTGGGCGTAAAGCGCGCGTAGGCGGTCTGGAAAGTCGGATGTGAA AGCCCGGGGCTCAACCCCGGAACTGCATTCGAAACTTCCAGGCTCGA GACTTGGAGAGGTGGGCGGAATTCCGAGTGTAGAGGTGAAATTCGTA GATATTCGGAAGAACACCAGTTGCGAAGGCGGCTCACTGGCCAAGTT CTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACC CTGGTAGTCC
939811	GTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAAT TACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAA AGCCCGGGGCTCAACCTCGGAATAGCATTTGAAACTGGCAGACTAGA GTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTA

	GAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATA CTGACACTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC CTGGTAGTCC
625724	GTGCCAGCAGCCGCGGTAATACGGAGGGCGCAAGCGTTACTCGGAAT CACTGGGCGTAAAGAGCGTGTAGGCGGGTTAATAAGTTTGAAGTGAA ATCCTATAGCTCAACTATAGAACTGCTTTGAAAACTGTTAACCTAGA ATATGGGAGAGGTAGATGGAATTTCTGGTGTAGGGGTAAAATCCGTA GAGATCAGAAGGAATACCGATTGCGAAGGCGATCTACTGGAACATTA TTGACGCTGAGACGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC CTGGTAGTCC
605566	GTGCCAGCAGCCGCGGTAATACGAAGGTGGCAAGCGTTGTTCGGATT TACTGGGCGTAAAGAGCACGTAGGCGGTTTAGTAAGCCCTTTGGGAA AGCTACGGGCTTAACCCGTAAAGGTCGAGGGGGACTGCTAAGCTAGA GGGCAGGAGAGGAGCGCGGAATTTCCGGTGTAGCGGTGAAATGCGTA GATATCGGGAAGAAGGCCGGTGGCGAAGGCGGCGCTCTGGAATGTCT CTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC CTTGTAGTCC
587581	GTGCCAGCAGCCGCGGTAATACGGGGGGGGCAAGCGTTGTTCGGAAT TACTGGGCGTAAAGGGCTCGTAGGCGGCCAGCCAAGTCGGACGTGAA ATCCCTCGGCTCAACCGGGGAACTGCATCCGATACTGGTTGGCTTGA AGCCGGGAGAGGGATGCGGAATTCCAGGTGTAGCGGTGAAATGCGTA GATATCTGGAGGAACACCGGTGGCGAAGGCGGCATCCTGGACCGGTC TTGACGCTGAGGAGCGAAAGCCAGGGGAGCAAACGGGATTAGATACC CTTGTAGTCC
New.ReferenceOTU13	GTGCCAGCCGCCGCGGTAACACGTAGGGCGCGAGCGTTGTCCGGATT TATTGGGCGTAAAGGGCTCGTAGGCGGTTCTGTAAGTCGGATGTGAA AACTCAGGGCTCAACCCGGAGATGCCATCCGATACTGCAGTGACTGG AGTCCGGTAGGGGAGCATGGAATTCCTGGTGTAGCGGTGGAATGCGC AGATATCAGGAGGAACACCAGTGGCGAAGGCGGTGCTCTGGGCCGGT ACTGACGCTGAGGAGCGAAAGCGTGGGGAGCAAACAGGATTAGATAC CCTTGTAGTCC
213687	GTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAAT TACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCGTTGTTCGTGAA AACCTGAGGCTTAACCTTGGGCGTGCGGGCGATACGGGCAGACTGGA GTACTGCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCA GATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCAGTAA CTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACC CTGGTAGTCC

APPENDIX 7

(A) *C. kuekenthali* (Image credit:http://www.nova.edu/ncri/sofla_sponge_guide/sp_78.html) (B) *C. alloclada* (Image credit :http://www.nova.edu/ncri/sofla_sponge_guide/sp_51.html)

APPENDIX 8

SAMPLE ID	**Total Sequence**
ABdark2	3113
ABdark4	3327
ABlight10	3141
ABlight2	3013
ABlight4	3225
AMPdark10	8308
AMPdark2	4806
AMPlight10	6385
AMPlight2	3555
AMPlight4	4412
April2014	42418
CinDead	7071
CinDead2	3537
CONTROLdark10	3357
CONTROLdark2	13487
CONTROLdark4	5496
CONTROLlight10	942

CONTROLlight2	5529
CONTROLlight4	5009
day0	2300
July2014	41834
PENSTREPdark10	7029
PENSTREPdark2	4412
PENSTREPdark4	4546
PENSTREPlight10	3947
PENSTREPlight2	1338
PENSTREPlight4	3378
TETdark2	4821
TETlight10	5531
TETlight2	3050
TETlight4	3608
Water1	4315
Water2	10937
Water3	19964
water4	3659