IDENTIFICATION OF CULTURABLE BACTERIA ISOLATED FROM SPONGES AFFECTED IN THE 2016 MASSIVE DIE-OFF AT THE FLOWER GARDEN BANKS NATIONAL MARINE SANCTUARY (FGBNMS)

by

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Dedication

I would like to dedicate my achievements and success to my parents Mr. Amitkumar Pandya and Mrs. Vandana Pandya. They were and are always there for me when I need mental support, positivity and encouragement. Without them it would have been very difficult to attain all these accomplishments.

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Lastly, I am grateful to Michael Grimes and Shiv Annapareddy for having my back during this whole project.

ABSTRACT

IDENTIFICATION OF CULTURABLE BACTERIA ISOLATED FROM SPONGES AFFECTED IN THE 2016 MASSIVE DIE-OFF AT THE FLOWER GARDEN BANKS NATIONAL MARINE SANCTUARY (FGBNMS)

Dhatri Amitkumar Pandya
University of Houston-Clear Lake, 2017

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This project was conducted to identify culturable bacteria isolated from sponges affected in the 2016 massive die-off at the Flower Garden Banks National Marine Sanctuary (FGBNMS). The FGBNMS was considered a healthy reef system until sport divers reported on July 25th 2016 green, hazy water with huge patches of white mats on corals, sponges, other vertebrates and dead animals littering the bottom of East Flower Garden Bank. To help elucidate the root cause of this event, affected and unaffected sponge samples of *Agelas clathrodes* and *Xestospongia muta* sponges from East bank were examined for their associated microbial communities using culture-dependent methods. MALDI – TOF – MS and 16S rDNA sequencing were used to identify representative isolates. MALDI – TOF – MS and 16S rDNA sequencing analysis showed the presence of *Bacillus firmus, Pseudovibrio* spp., *Halanaerobium* spp., *Microbulbifer*

variabilis, and *Microbulbifer* spp. in unaffected samples. *Bacillus firmus* could help sponges by nitrogen fixation and producing secondary metabolites that protects sponges from predators and *Halanaerobium sehlinense* is fermentative bacteria. The role of *Halanaerobium sehlinense* in sponges is not yet clear. Affected samples showed the presence of *Vibrio* spp. and *Halanaerobium sehlinense*. *Vibrio* spp. which occurs naturally in sea water as opportunistic pathogenic bacteria exhibits stronger proteolytic (caseinase), phospholipase and hemolytic activities. These potential pathogens may have contributed into massive die-off at East bank of FGBNMS.

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CHAPTER I

Introduction

Flower Garden Banks National Marine Sanctuary (FGBNMS)

Flower Garden Banks National Marine Sanctuary (FGBNMS) is located 175 km southeast of Galveston, Texas and has three banks: East, West and Stetson. FGBNMS supports diverse corals, sponges, and vertebrates, including several species of fishes, sharks and rays. Corals dominate this sanctuary. The National Oceanic and Atmospheric Administration's (NOAA) manages and protects East and West Flower Garden banks [1].

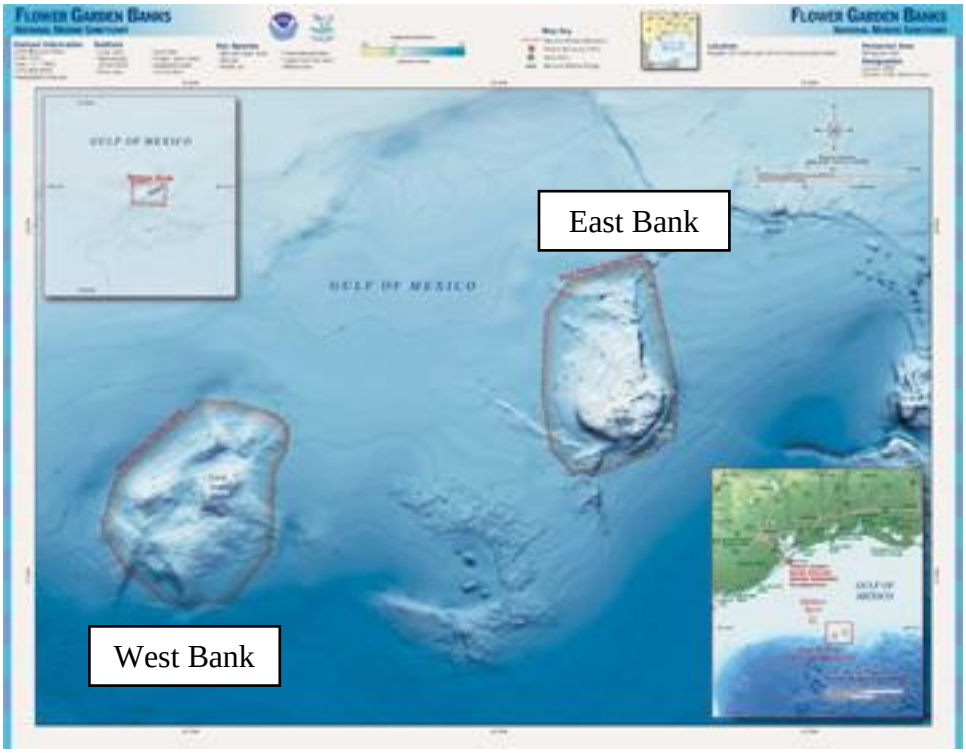


Figure 1: Map of East and West Flower Garden Banks

(https://flowergarden.noaa.gov/about/about.html)

The coral reefs of Flower Garden Banks National Marine Sanctuary (FGBNMS) are composed of hard corals, which are also known as stony corals, reef-building corals, hexacorals, hermatypic corals and scleractinian corals. Large number of sponge species located in coral cap region also contribute towards biodiversity of FGBNMS including *Agelas clathrodes, Xestospongia muta, Ectyoplasia ferox, Ircinia felix, Niphates erecta* [2].

Sponges

Sponges are porous sessile filter – feeding organisms that filter large volumes of seawater and accumulate diverse symbiotic microbial communities within their tissue. These communities make up 40% - 60% of the total sponge volume [17, 18]. Bacteria uniquely found in sponges belong to phylum 'Poribacteria' [18]. Sponges benefits from mutualisms by acquiring nutrients, protection from UV radiation, nitrogen fixation, nitrification and production of secondary metabolites [17]. Symbionts also help and stabilize sponge skeletons [20]. Sponges show potential as a new source of novel bioactive compounds like antibiotics, antifungal, cytotoxins, anti-inflammatory, antiviral etc. which are of pharmaceutical importance [18, 20].

Figure 2: Sycon cillatum sponge (0.05 mm) (http://www.storyofsize.com/sponges/)

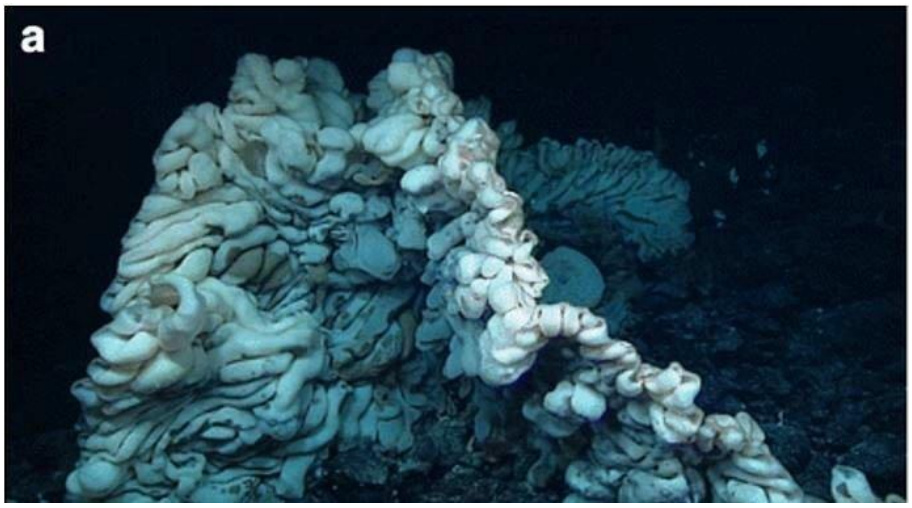


Figure 3: World's largest sponge found at Papahānaumokuākea Marine National Monument off Hawai. (http://www.npr.org/sections/thetwo-way/2016/05/26/479621399/deep-sea-explorers-discover-a-sponge-the-size-of-a-minivan)

Symbiotic microbes are located in both intra- and extra cellular spaces. Symbionts present in extra cellular spaces are of two types: exosymbionts (located in outer layers) and endosymbionts (located in mesohyl layer) while intracellular symbionts are found to harbor host cells or nuclei. Sponges provide habitat to symbionts and in return they provide sponges with nourishment. They also play important role in sponge defense mechanism against predators and biofouling [20].

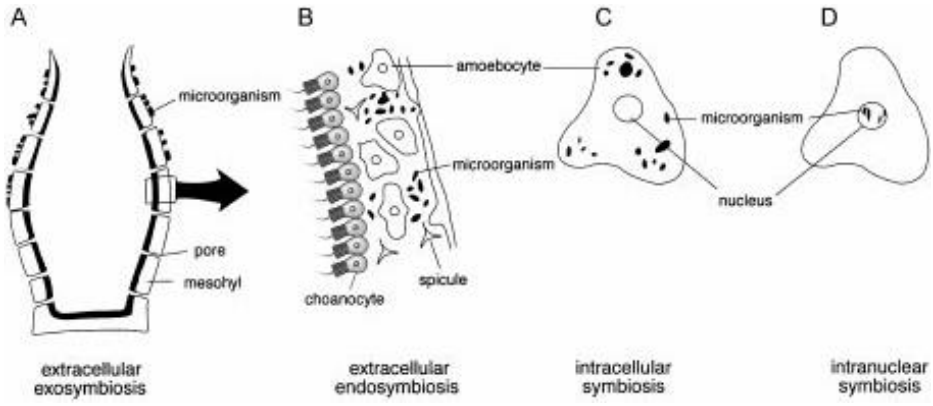


Figure 4: Diagram of symbiotic relationship between sponges and microorganisms [20]

During filtration, sponges are also exposed to large populations of opportunistic pathogens and fouling microorganisms, particularly from the release of sewage waste released into sea.. Most of the times, sponges defend themselves from such pathogenic and fouling microbes using cellular as well as morphological defense mechanisms [19]. *Vibrio* spp., are naturally present in seawater are one of the most commonly found pathogenic species. These microbes are dormant when water temperature fall below 15° C. During summer their population peaks [21]. Other pathogenic species found in

seawater include *Aurantimonas coralicida*, *Cytophaga* spp., Desulfovibrio spp. and *Serratia marcescans* [17].

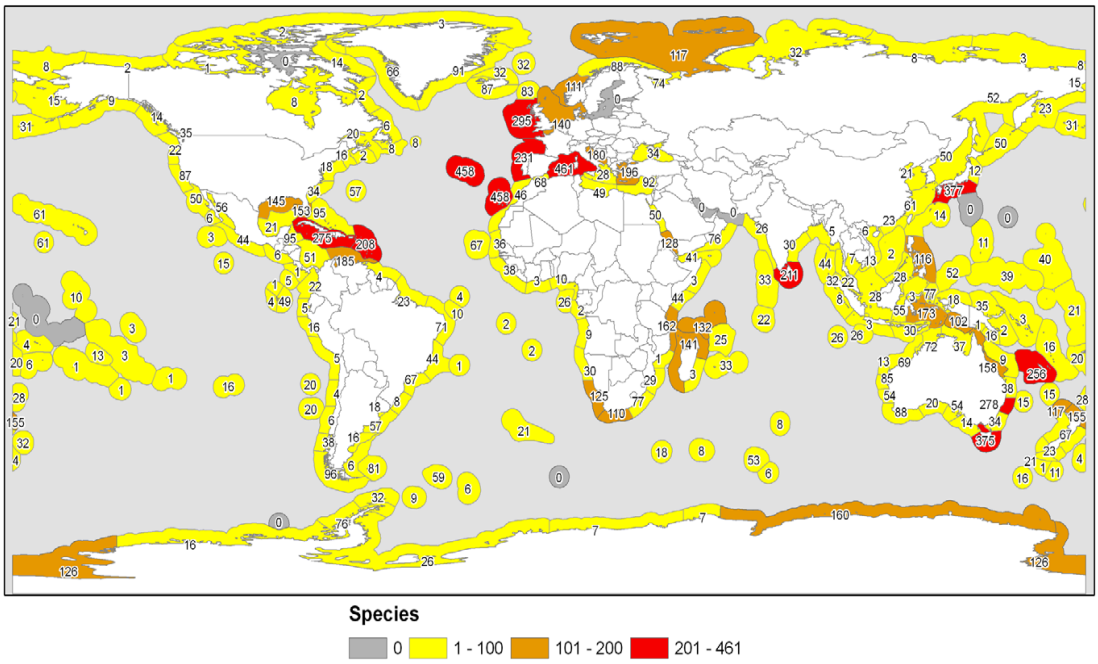


Figure 5: Picture indicating Sponge diversity around the Globe [29]

Agelas clathrodes

Orange Elephant Ear Sponge is generally found over coral reefs or walls and flourishes in quiet areas with dim light and at the depth of 35 to 130 feet [5, 6]. These sponges contribute to large demosponge populations in tropical and subtropical reefs and are widely distributed in Bahamas, Caribbean, and Florida. Structure wise *A. clathrodes* are massive, narrow base and of thickness ranging from 1.5 to 10 cm [5, 6]. These bright reddish orange colored sponges have thin lining of organic pinacoderm which is

supported protruding spicules [3, 5]. Other characteristic of this sponge includes thickly encrusting, massive, globular, branching, fan shaped and tubular [3].

A. clathrodes is a rich source of different secondary metabolites like oroidin and 4, 5-dibromopyrrol – 2 – carboxylic acid. These metabolites act as antipredator defense chemical and protect these sponges against predatory reef fishes [4]. *A. clathrodes* are oviparous hermaphrodite and they release male and female gametes in periodic manner annually. But it is seen that reproductive activity of these sponges is low [7].

Figure 6: Agelas clathrodes

(https://www8.nos.noaa.gov/onms/Park/Parks/SpeciesCard.aspx?refID=4&CreatureID=1343&pID=9)

Xestospongia muta

Barrel Sponge or Redwood of the Reef are thick walled, large solitary barrels having width that can be as big as six feet and live up – to 100 years. They generally grow on steep reef slopes of depth ranging from 50 and 130 feet. *X. muta* is widely found in Bahamas, Caribbean, and Florida and dominates Caribbean waters [8, 10]. These sponges can be found in various colors from purple to red - brown externally and tan internally. *X. muta* is brittle and crumbly and this often leads to injury of the sponge. Such injuries are caused by fish predation or anthropogenic or natural disturbances. Most of the times small injuries are repaired or broken sponge piece gets reattached to substrate and grow as a new individual sponge [5, 9].

X. muta forms a mutualism with *Synechococcus* species. This unicellular cyanobacterial group carries out photosynthesis thus benefiting the host sponge [11, 12]. These species of sponge undergo two types of bleaching: cyclic bleaching and fatal bleaching. Sponges recover from cyclic bleaching but not from fatal bleaching [11]. Both type of bleaching leads to loss of reddish-brown coloration. Fatal bleaching causes complete disintegration of tissue. During fatal bleaching distinctive sponge orange band (SOB) separates bleached and non - bleached portions of tissue [13]. Generally such bleaching events occur when temperature of seawater is at the highest i.e. mainly during summer or autumn [14]. Along with increase seawater temperature, other factors like virulence of pathogens or reducing host resistance or resilience also causes bleaching in these sponges [11].

Figure 7: Xestospongia muta

(https://www8.nos.noaa.gov/onms/Park/Parks/SpeciesCard.aspx?pID=9&refID=4&CreatureID=1339)

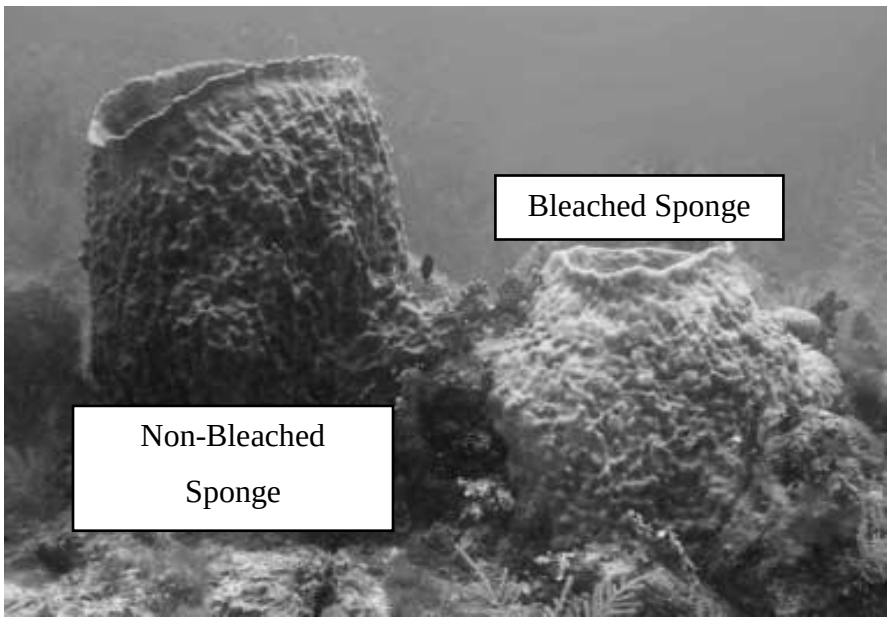


Figure 8: Non – bleached and bleached Xestospongia muta at Conch Reef, Key Largo, Florida [13]

Mysterious Mortality Event at East Flower Garden Bank

The Flower Garden Banks National Marine Sanctuary (FGBNMS) was considered healthy reefs system until sport divers reported green, hazy water with huge patches of ugly white mats on corals and sponges, and dead animals littering at the bottom of East Flower Garden Bank on July 25th 2016 [15].

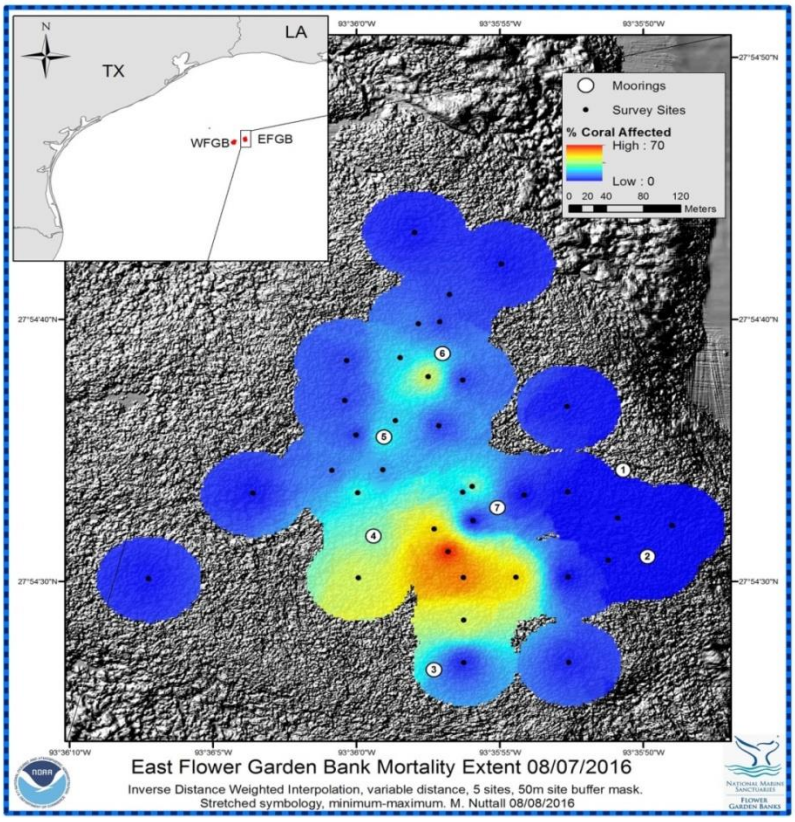


Figure 9: Map indicating mortality that occurred at East Flower Garden Bank. Mortality impacts range from 0% mortality near buoys no. 1 and no. 2 to 70% mortality between buoys no. 4 and no. 7 (https://flowergarden.noaa.gov/newsevents/massmortalityresponsearticle.html)

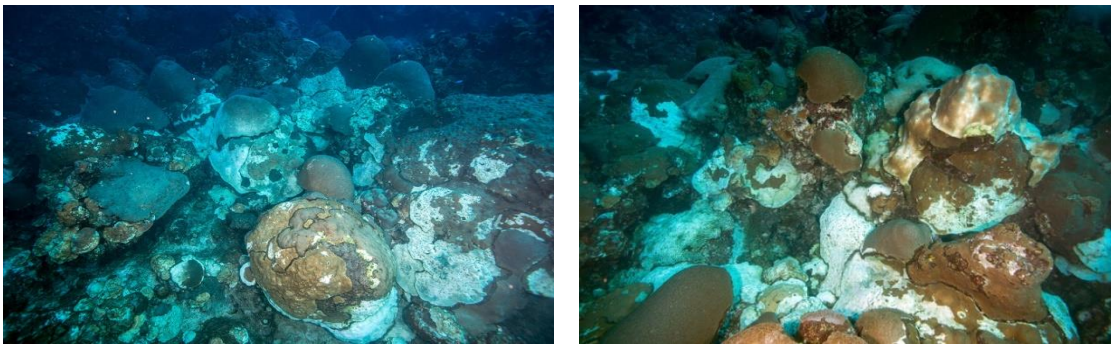


Figure 10: Corals reefs affected by the mortality event at East Flower Garden Bank (https://flowergarden.noaa.gov/newsevents/massmortalityresponsearticle.html)

Human activities or environmental disturbances could have caused such sudden massive mortality of reefs. Physical damage to reefs, chemical changes, sudden climate changes, loss of nutrients, sedimentation, overfishing, deep water horizon oil spill or bacterial or fungal or viral infection can be the few reasons for this die off [16]. To explain this event in reef system samples of *Agelas clathrodes* and *Xestospongia muta* from affected (East Bank) and unaffected (West Bank) banks were examined for any pathogenic microbial community. Isolates were identified by MALDI – TOF – MS and 16s rDNA sequencing.

MALDI – TOF – MS

Hillenkamp and Karas introduced Matrix – Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI – MS) in 1988. This method is widely used to analyze peptides, proteins, and most other biomolecules (oligonucleotides, carbohydrates, natural products, and lipids) [23]. MALDI basically allows vaporization and ionization of any size of nonvolatile biomolecules without causing any damage or destruction of them [23,

25]. To perform MALDI – MS, first the analyte is co-crystalized using matrix and then it is exposed to laser radiation causing evaporation of matrix along with the analyte. These vapors of matrix and analyte are then analyzed by mass analyzers. [23]. Exposure to laser radiation leads to ionization of the analyte (i.e. single – charged ions, z=1) thus mass to charge ratio (m/z) of the analyte represents mass value [25].

The matrix is a weak organic energy-absorbent compound which crystallizes on drying and thus entrapping analyte [26]. Substance used as matrix in this technique must have few qualities like: high molar extinction coefficient at given wavelength, solubility in the solvent analyte is mixed, stable in vacuumed tubes, of proper chemical composition; have lattice structure etc. [27]. Solid matrices are the most widely used matrix out of both solid and liquid ones. They are derived from weak organic acid such as α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), and 2, 5-dihydroxybenzoic acid (2, 5-DHB) [28].

Ions produced after exposure of laser radiation are analyzed by mass analyzers. Generally 3 types of mass analyzers are used and they are: linear time-of-flight (TOF), a TOF reflectron, and a Fourier transform mass analyzer. The most widely used mass analyzer is linear time-of-flight (TOF). It is the simplest of all the 3 analyzers and has been used with MALDI since beginning. In ionized form, all ions of analyte have same energy but they are different in mass thus time taken by each ion to reach the detector is different. Ions that are smaller travels faster due to higher velocity and larger ions take more time due to lower velocity. Thus to sum up, when ions will arrive on detector depends on mass, charge, and kinetic energy (KE) of the ion [23].

In recent years, Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI –TOF MS) has widely been used for identification of bacteria and fungi [24]. MALDI –TOF MS reduces time for identification of microbial communities to few minutes and is very simple to perform. Single isolated bacterial colonies are picked up from agar media using sterile tip and are transferred to ground steel MALDI target plate. Bacterial colonies are then covered with either CHCA or DHB matrix and at the end targeted by the laser for analysis [25].

Identification of fungal community using MALDI – TOF MS is bit different than that of bacterial community because they have thick cell wall which is difficult to breakdown thus adding few more steps. Fungal colonies picked up from media are suspended in 70% ethanol, pelleted down and then re-suspended in 70% formic acid and acetonitrile. After centrifugation, 1µL of supernatant is transferred to ground steel MALDI target plate, dried, covered with matrix and analyzed [25].

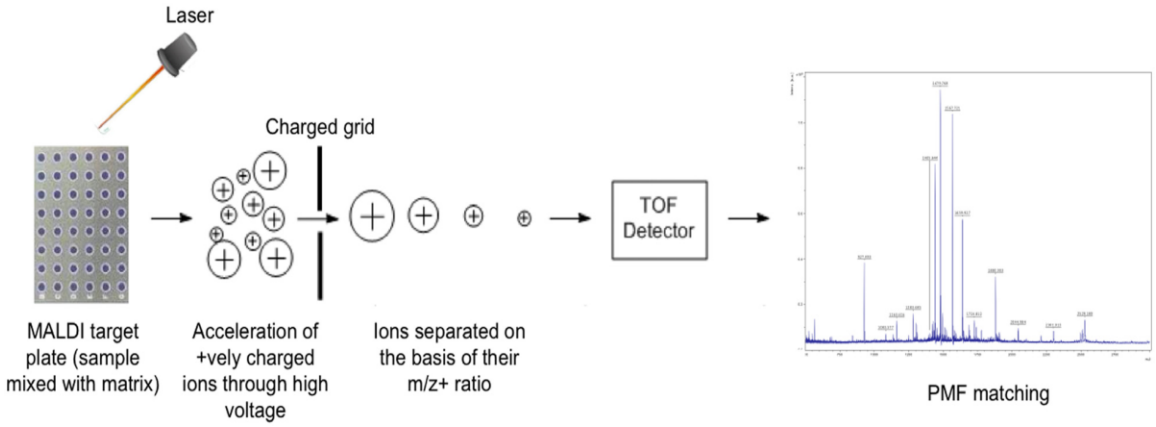


Figure 11: Schematic diagram of MALDI – TOF MS [26]

MALDI – TOF MS is fast, accurate and less expensive when compared to traditional molecular and immunological-based detection methods and also it doesn't required trained person to perform it. There is only one disadvantage for this technique and it is high initial cost of buying initializing the equipment [24, 26].

16S rDNA Sequencing

16S rDNA sequencing is the most widely used molecular technique for identification of bacteria [31]. Before this method was introduced, bacterial colonies were identified in clinical microbiology laboratories by performing phenotypic tests (Gram staining), biochemical tests and keeping an account of culture requirements and growth characteristics. Thus there was limitation in identification of uncultivable organisms or anaerobes and mycobacteria that required additional equipment and expertise. But with 16S rDNA sequencing it has become easy to identify unusual phenotypic profiles like rare bacteria, slow growing bacteria, uncultivable bacteria and culture-negative infections [33]. This molecular technique also helps in deducing evolutionary relationships among different microbes such as bacteria, archaebacteria, and eukaryotic organisms [32].

This "housekeeping gene" is used for identification due to many reasons such as: (A) it is present in all archaea and bacteria; (B) has low mutation rate i.e. highly conserved and (C) the length of this gene (~1500 bp) is appropriate and long enough for informatics purposes [31, 34].

It is not always necessary to have 1500 bp of sequence to identify an organism. Initial 500 bp of 16S rDNA sequence can provides adequate differentiation for

identification. Amplification and identification of ~500 bp is easier and cheaper than 1500 bp [34]. V1–V9 variant regions of 16S gene are used for identification. Primers designed for conserved region are generally of 15 – 20 nucleotides long and flank target region used for phylogenetic analysis. The very first set of primers for 16S rDNA sequence was developed using *Escherichia coli* 16S rDNA and named accordingly. For example: primer E685 was designed for P4 region of eubacterial primer A344 corresponds to the archaeal H339 region [35].

16S rDNA sequencing technique has proved to be very useful for identification bacterial communities. This technique too has limitation that is only short lengths of nucleotides are sequenced thus there is a possibility of error [43].

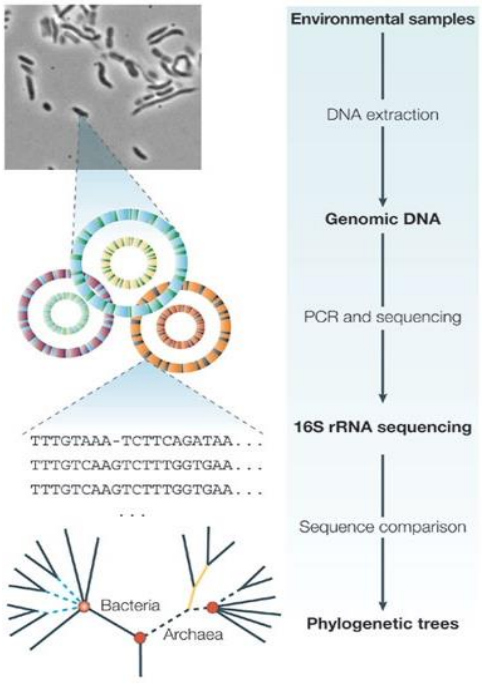


Figure 12: Schematic diagram if 16S rDNA sequencing (http://www.nxt-dx.com/metagenomics/16s-rrna-sequencing/)

Gram Staining

This technique was developed by Christian Gram while he was trying to stain *Schizomycetes* from tissue cells and published his work in 1883. Crystal violet is primary dye used in gram staining and is basic in nature. Iodine is used as mordant which helps in differentiation of colored cells. Decolorizer is used in this method to remove crystal violet from background. Ethanol is most widely used decolorizer. Use of counter stain is necessary to differentiate cells. Safranin is used as counter stain. [44]

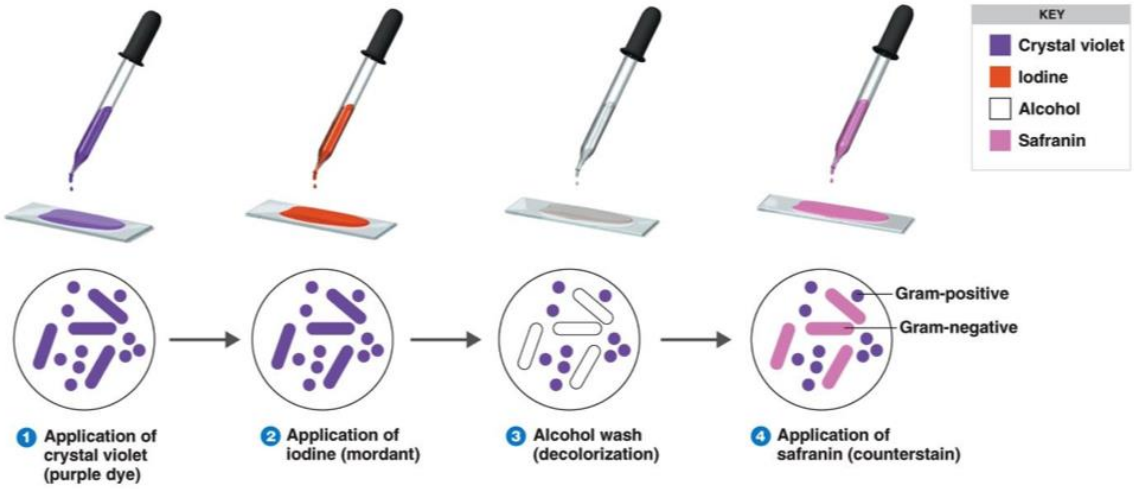


Figure 13: Gram staining procedure (http://www.medicalonline1.com/2017/03/16/gram-staining-principle-procedure-interpretation/)

Hypothesis

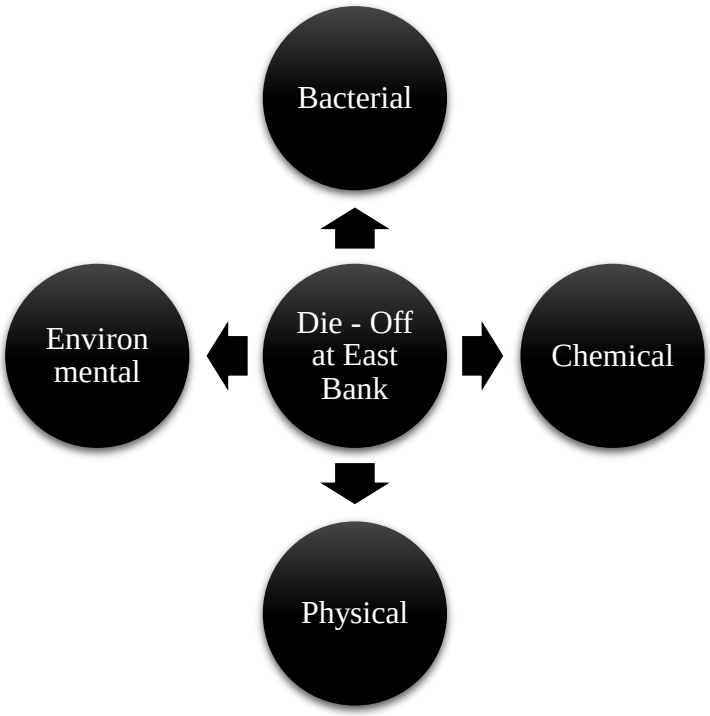


Figure 14: Possible reasons for die – off at East Bank of FGBNMS

Agelas spp.: Bacterial phylum that are reported to have endophytic relationship with *Agelas* spp. include *Cyanobacteria*, *Proteobacteria*, *Chloroflexi*, *Firmicutes*, *Actinobacteria*, *Acidobacteria*, *Planctomycetes*, *Bacteroidetes* and *Gemmatimonadet*es. Presence of these bacterial communities has proven very beneficial to sponges [55, 57].

Xestospongia spp.: Prominently found endophytes in *Xestospongia* spp. belong to phylum *Chloroflexi*, *Acidobacteria*, *Actinobacteria* and *Cyanobacteria*. These bacteria have beneficial effects on these sponges [55, 56].

Potential pathogens: Sudden mortality of *A. clathrodes* and *X. muta* that occurred at East Bank of Flower Garden Banks National Marine Sanctuary (FGBNMS) in July 2016 resulted mainly from infection of bacteria from phylum *Proteobacteria*. This

phylum includes *Vibrio* spp., *Aurantimonas coralicida*, *Desulfovibrio* spp., and *Serratia marcescans*. *Vibrio* spp. occurs naturally in sea water as opportunistic pathogen. Other bacterial phylum that has pathogenic impact on marine flora and fauna is Bacteroidetes (*Cytophaga* spp.) A white matt covering invertebrate is result of *Beggiatoa* spp. growing on dead flora and fauna [17, 21].

16S rDNA sequencing: All of cultured bacteria will be identified using this technique because it is a traditional method for identification of microbial community and have well developed databases.

MALDI – TOF – MS: Few of cultured bacteria will be identified using this technique. It is a new method for identification of microbial community and thus has limited data in its database.

Data comparison: Bacterial community which are identified by MALDI – TOF – MS technique will correlate with data obtained from 16S rDNA sequencing

Project Goals

1. Isolation of single bacterial colonies from sponge homogenates
2. Identification of isolated colonies by:

   (A) 16S rDNA sequencing

   (B) MALDI – TOF – MS analysis

   (C) Gram staining and morphological analysis

CHAPTER II

Materials and Methodology

All plastic ware, glassware, general lab chemicals and laboratory supplies were obtained from Fischer Scientific Company. The matrix and reagents used for MALDI – TOF – MS were from Sigma. Samples for MALDI – TOF – MS analysis were send to PennState Huck Institutes of the Life Sciences. Microbial DNA Isolation Kit from Qiagen, Valencia CA was used for DNA isolation. Sample for 16S rDNA sequencing were send to Eurofins Genomics.

Field Collection

Live specimens of *Agelas clathrodes* and *Xestospongia muta* were collected from both East and West Bank by FGBNMS diver Marissa Nuttall and coral researcher Dr. Sarah Davies (UNC). Only East bank was affected by mortality event while West bank remained healthy. Divers collected sponge samples from both the banks. Divers who collected samples were not researchers; they collected samples by hand and placed them on ice. Once all samples were collected, they were transported to lab and stored at -80° C without any cryoprotective agents.

Figure 15: FGBNMS diver Marissa Nuttall and coral researcher Dr. Sarah Davies (UNC) collecting samples

Homogenate Preparation

Sponge samples were present in -80° C for 8 months. Frozen sponge samples from -80° C were first thawed by keeping them at room temperature. Then approximately 0.5 g to 1 g of each sponge sample was weighed and chopped into small pieces. These chopped sponge pieces were then aseptically added to 25 ml culture tubes containing 5 ml marine broth and incubated at room temperature for 24 hours [36]. After incubation, each culture tube was individually emptied into sterile motor and sponge pieces along with marine broth were crushed properly. Obtained homogenate was aseptically transferred to 15 ml tube. 200 µl of DMSO and 200 µl 100% glycerol were added to homogenate as cryoprotective agents and it was stored at -80° C until further use.

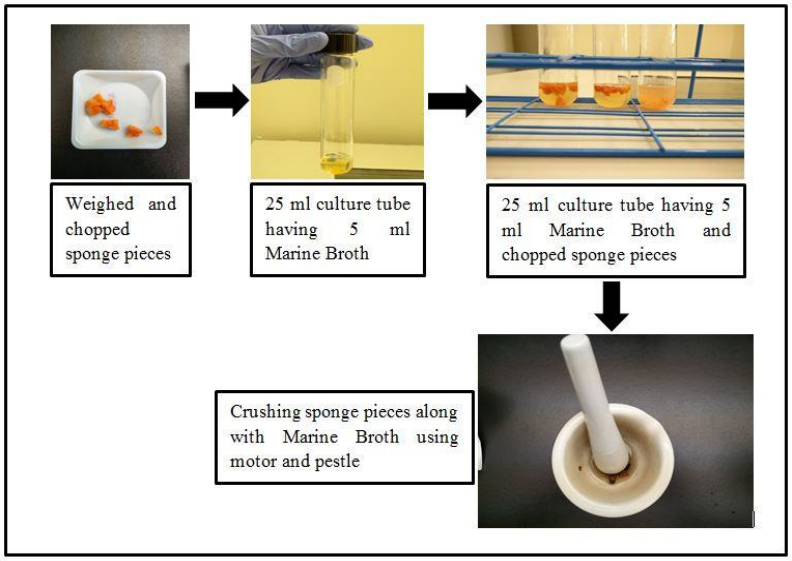


Figure 16: Homogenate Preparation from sponge samples

Sr. No.	Samples	Weight of Samples (g)
1	ACU – 1	0.6462
2	ACU – 2	0.4116
3	ACU – 3	0.4771
4	ACU – 4	0.4244
5	ACU – 5	0.4533
6	ACU – 6	0.5426
7	ACU – 7	0.5586
8	ACU – 8	0.5283
9	XMU – 1	0.7053
10	XMU – 3	0.6240
11	ACA – 1	0.2380
12	ACA – 2	0.4045
13	ACA – 3	0.5781
14	ACA – 4	0.4992
15	ACA – 5	0.6320
16	ACA – 6	0.2815
17	ACA – 7	0.5952
18	XMA – 1	0.7877
19	XMA – 2	0.9629
20	XMA – 3	0.8241
21	XMA – 4	0.6869
22	XMA – 5	0.5463

Table 1: Sponge samples and their respective weights during homogenate preparation

Preparation of Media Broth and Agar plates

Marine broth and agar plates (full strength) were prepared in DI water. Low nutrient broth and agar plates (10% strength of marine broth) were prepared in artificial sea water (ASW) to maintain the salinity. The pH and salinity of media was not checked. Cycloheximide (10 mg/l) was added when the media cooled to 55° C after autoclaving to inhibit fungi.

Sr. No.	Name of Broth/Agar	Weight of Marine broth (g)	Weight of Agar (g)	Volume
1	Marine Broth	20.125	10	500 ml of DI water
2	Low Nutrient Broth	2.0125	10	500 ml of ASW

Table 2: Preparation table for both media

Inoculation of Homogenate on Marine and Low Nutrient Agar plates

Microbes were pre-incubated by adding 30 µl of homogenate was aseptically added to 2 ml tubes having 1 ml to Marine broth and Low Nutrient broth. These tubes were then incubated at room temperature. For *A. clathrodes* unaffected (ACU) samples, tubes were incubated for 48 hours whereas for *A. clathrodes* affected (ACA), *X. muta* unaffected (XMU) and *X. muta* affected (XMA) tubes were incubated for 24 hours. After this pre-incubation, sponge homogenates were serially diluted using sterile artificial sea water. 50 µl of 10^{-4} dilution of *A. clathrodes* unaffected (ACU) samples and of 50 µl of 10^{-6} dilution of *A. clathrodes* affected (ACA), *X. muta* unaffected (XMU) and *X. muta* affected (XMA) were spread on both media plates. Plates were incubated at room temperature until growth was observed on plates.

Obtaining Single Isolated Colonies and Sub – culturing

Once growth was observed on the plates, colonies were screened morphologically. Single colonies with morphological difference were carefully picked up using sterile loop and streak on new media plate. A total of 39 single colonies were isolated from both Marine agar and Low Nutrient agar (LNA).

Sr. No.	Samples	Media	Sr. No.	Samples	Media
1	XMA – 1	Marine agar	21	ACU – 7	Marine agar
2	XMA – 2	Marine agar	22	ACU – 8.1	Marine agar
3	XMA – 3.1	Marine agar	23	ACU – 8.2	Marine agar
4	XMA – 3.2	Marine agar	24	ACU – 8.3	Marine agar
5	ACA – 1.1	Marine agar	25	XMU – 3.1	Marine agar
6	ACA – 1.2	Marine agar	26	XMU – 3.2	Marine agar
7	ACA – 2	Marine agar	27	XMU – 1	LNA
8	ACA – 3.1	Marine agar	28	XMU – 3	LNA
9	ACA – 3.2	Marine agar	29	ACU – 1	LNA
10	ACA – 6.1	Marine agar	30	ACU – 4	LNA
11	ACA – 6.2	Marine agar	31	ACU – 6.1	LNA
12	ACA – 6.3	Marine agar	32	ACU – 6.2	LNA
13	ACA – 7.1	Marine agar	33	XMA – 1.1	LNA
14	ACA – 7.2	Marine agar	34	XMA – 1.2	LNA
15	ACU – 1.1	Marine agar	35	XMA – 5	LNA
16	ACU – 1.2	Marine agar	36	ACA – 1.1	LNA
17	ACU – 2.1	Marine agar	37	ACA – 1.2	LNA
18	ACU – 2.2	Marine agar	38	ACA – 3.1	LNA
19	ACU – 5	Marine agar	39	ACA – 3.2	LNA
20	ACU – 6	Marine agar			

Table 3: Samples isolated on 2 different media plates

MALDI – TOF – MS

A two ml tube containing one ml of media and a loop full of single isolated colony was used to prepare protein samples for MALDI - TOF - MS. Tubes were

centrifuged and media was removed. Cell pellet was washed using 70% ethanol (HPLC/MS grade) twice. Samples were spin alone to remove excess ethanol. Pellets were re – suspended into 70% formic acid and incubated for 5 min at room temperature. Acetonitrile was added to all tubes and mixed properly by brief vortexing. Tubes were centrifuged and were stored at -20° C till further use. 1 µl of each: 2 *E-coli* protein standards and 2 blanks were loaded on clean steel target. 1 µl of supernatant of each sample was spotted and was allowed to air dry. 1 µl of matrix was spotted on air dried samples and then send for analysis [37].

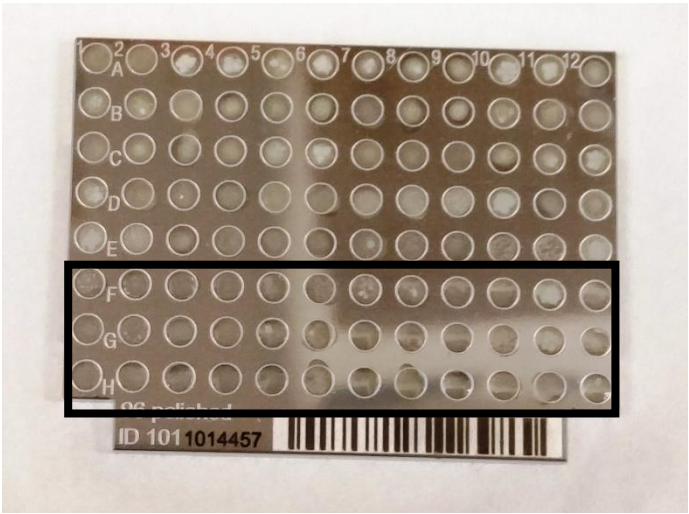


Figure 17: MALDI – TOF steel plate loaded with samples and matrix from F1 to H12

	1	2	3	4	5	6	7	8	9	10	11	12
F	1	2	3	4	5	6	7	8	9	10	11	12
G	13	14	15	17	19	20	21	22	24	25	26	27
H	28	29	30	31	32	33	34	36	35	37	38	39

Table 4: Worksheet of plate as per sample loaded on each spot

16S rDNA Sequencing

(A) DNA Isolation

Samples for DNA isolation were prepared by inoculation a loop full of single isolated colony into one ml of liquid media. Isolation of DNA from all samples was done using Microbial DNA Isolation Kit (Mo Bio UltraClean®) and protocol followed was given by manufacturer (Qiagen, Valencia, CA). Once DNA from all samples was isolated, readings of concentration and purity of samples were taken using NanoDrop.

(B) Touchdown PCR

Isolated DNA was subjected to touchdown PCR using 1055F and 1392R universal primers. Both the primers used in PCR were of 10 µM concentration

Sr. No.	PCR Reagents	Volume (µl)
1	GO Taq	25
2	1005F primer (10 µM)	2
3	1392R primer (10 µM)	2
4	Nuclease free water	19
5	Isolated DNA	2
6	Total	25

Table 5: PCR reaction for isolated DNA samples

Sr. No.	PCR Event		Temperature	Time
1	Initial denaturation		98° C	5 min
2	Denaturation	20X	98° C	5 sec
3	Annealing		65° C with drop of 0.5° C per cycle	20 sec
4	Elongation		72° C	20 sec
5	Denaturation	10X	98° C	5 sec
6	Annealing		55° C	20 sec
7	Elongation		72° C	20 sec
8	Final Elongation		72° C	10 min
9	Hold		4° C	∞

Table 6: Program for Touchdown PCR

(C) Agarose Gel Electrophoresis and NanoDrop

PCR products were run on 0.8% agarose gel prepared in 1X TBE buffer. Gel was run at 120 V for 30 min. 1 µl of ladder and 5 µl of samples were loaded in wells. Readings of concentration and purity of PCR products were taken using NanoDrop.

(D) Re – PCR on 1st PCR Products

PCR Products obtained from 1st PCR reaction were again subjected to PCR using same primers, volumes and PCR program as 1st one. Again an agarose gel was run for re – PCR products as mentioned above and readings of concentration and purity of re – PCR products were taken using NanoDrop.

(E) Purification of final PCR Products

PCR products were purified using GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Scientific) to remove green dye from products. Readings of concentration and purity of purified PCR products were taken using NanoDrop.

(F) 16S rDNA Sequencing

Purified PCR products were diluted to 5 ng/µl concentration using nuclease free water. 1 µl of each primer was added to each tube. Samples were send to Eurofins. After data analysis, sequences were submitted with GenBank with accession numbers MG597120 – MG597141. The evolutionary history was inferred using the Maximum

Parsimony method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed.

Gram Staining

A loop – full of each colony was re – suspended into 30 µl of nuclease free water. A smear was made on slide and was heat fixed by passing it over heat source. Smear was flooded with crystal violet and was allowed to stay for a minute. After a minute slide was rinsed off with DI water and then flooded with iodine solution. After 1 minute of incubation, slide was washed with DI water. 70% ethanol was used to decolorize slide. At the end slide was flooded with safranin for 30 seconds and was rinsed off. Slide was air dried and observed under microscope.

CHAPTER III

Results

Growth of colonies on Marine and Low Nutrient agar

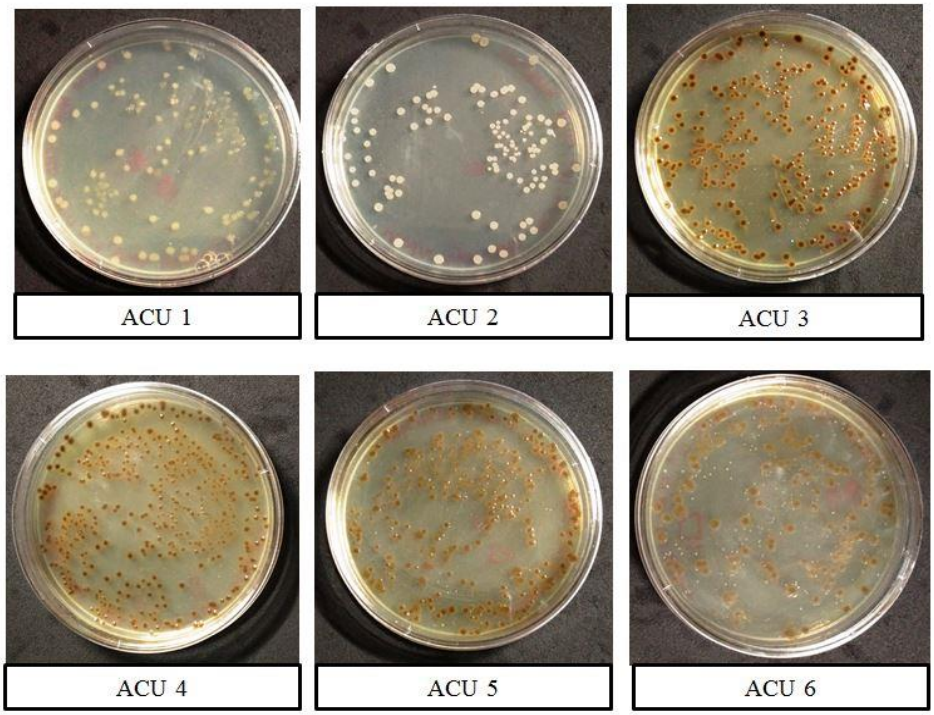


Figure 18: Microbial growth from unaffected A. clathrodes homogenate on Marine agar

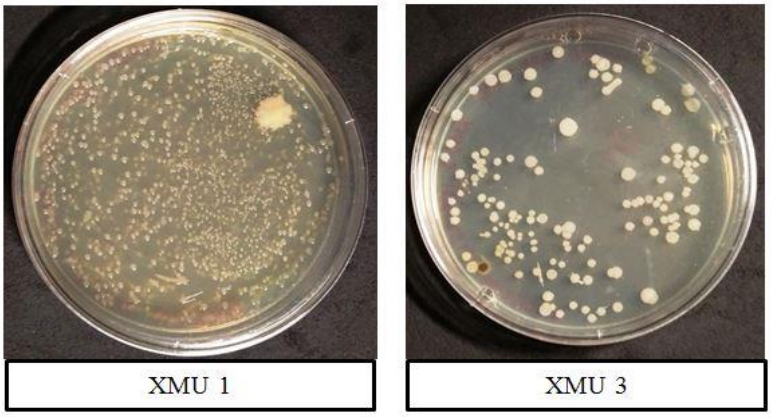


Figure 19: Microbial growth from unaffected X. muta homogenate on Marine agar

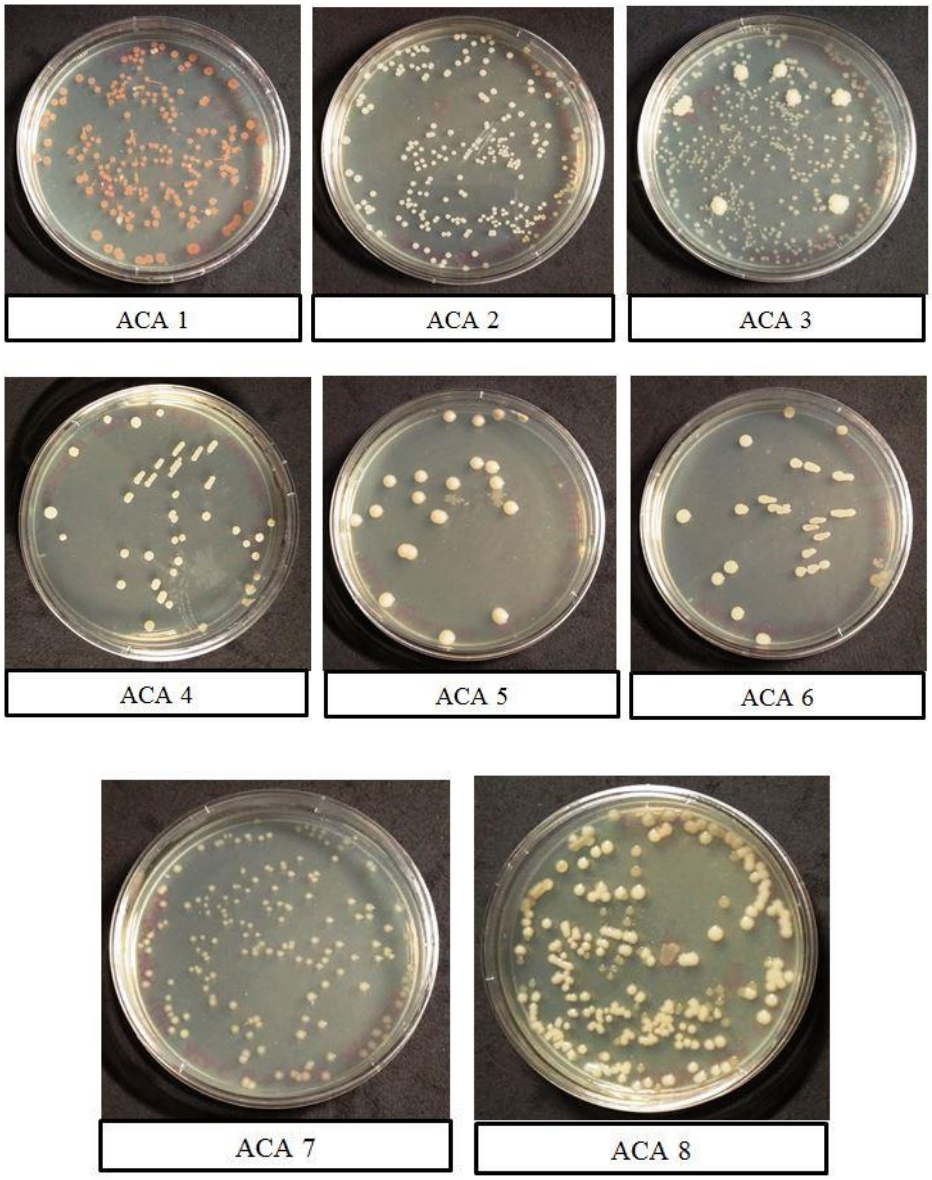


Figure 20: Microbial growth from affected A. clathrodes homogenate on Marine agar

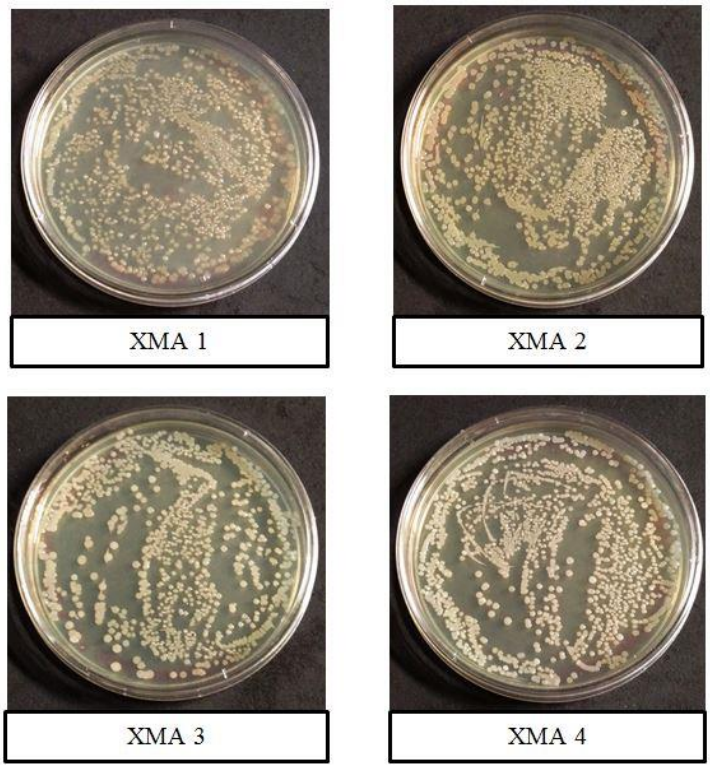


Figure 21: Microbial growth from affected X. muta homogenate on Marine agar

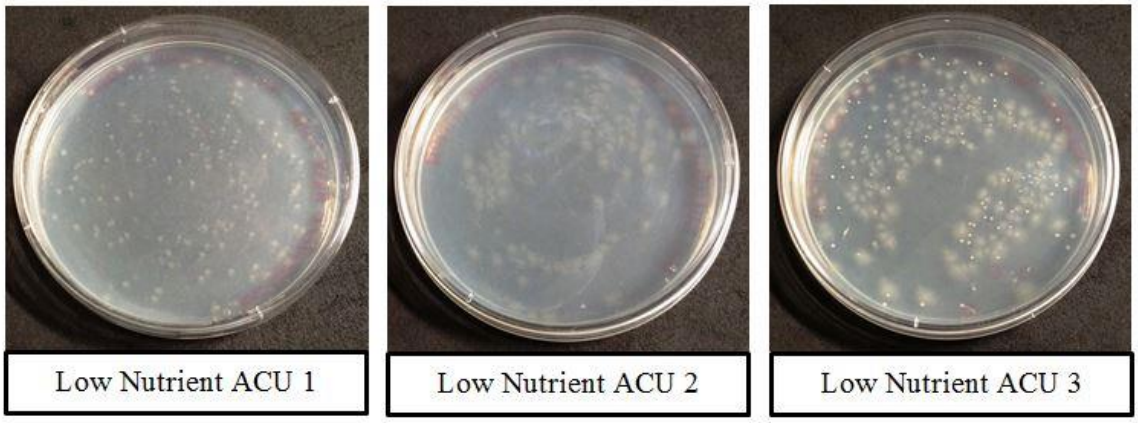


Figure 22: Microbial growth from unaffected A.clathrodes homogenate on Low Nutrient agar

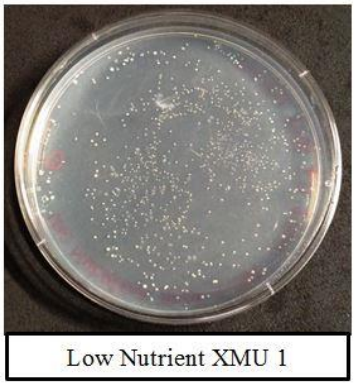


Figure 23: Microbial growth from unaffected X. muta homogenate on Low Nutrient agar

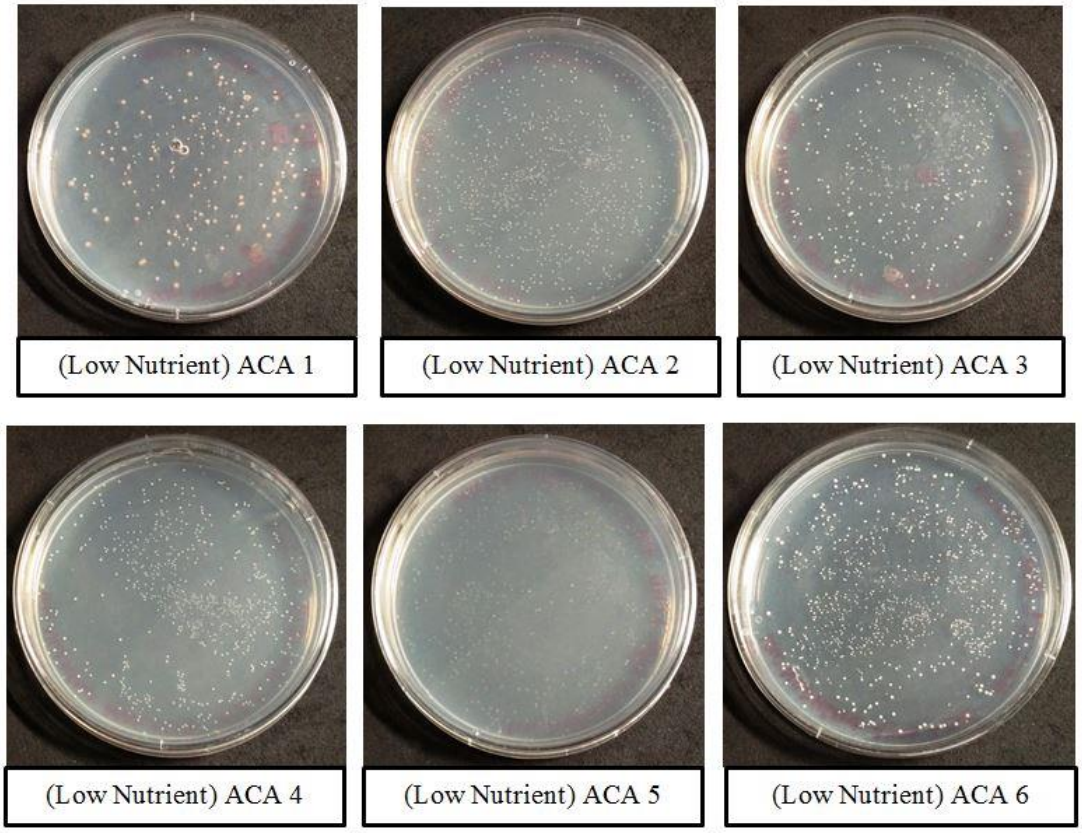


Figure 24: Microbial growth from affected A.clathrodes homogenate on Low Nutrient agar

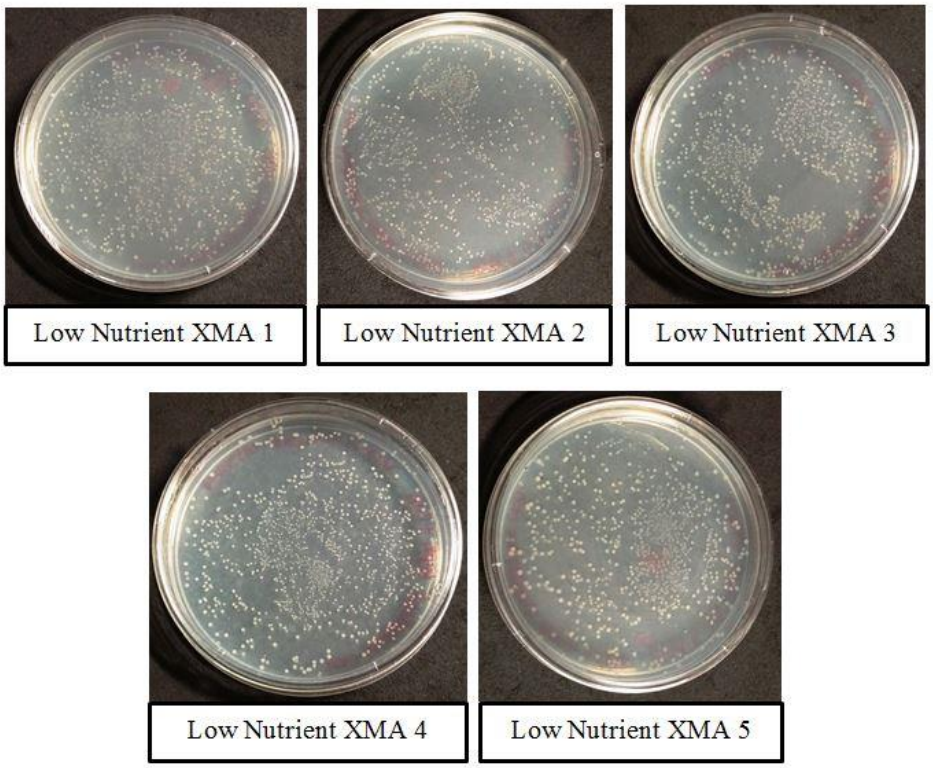
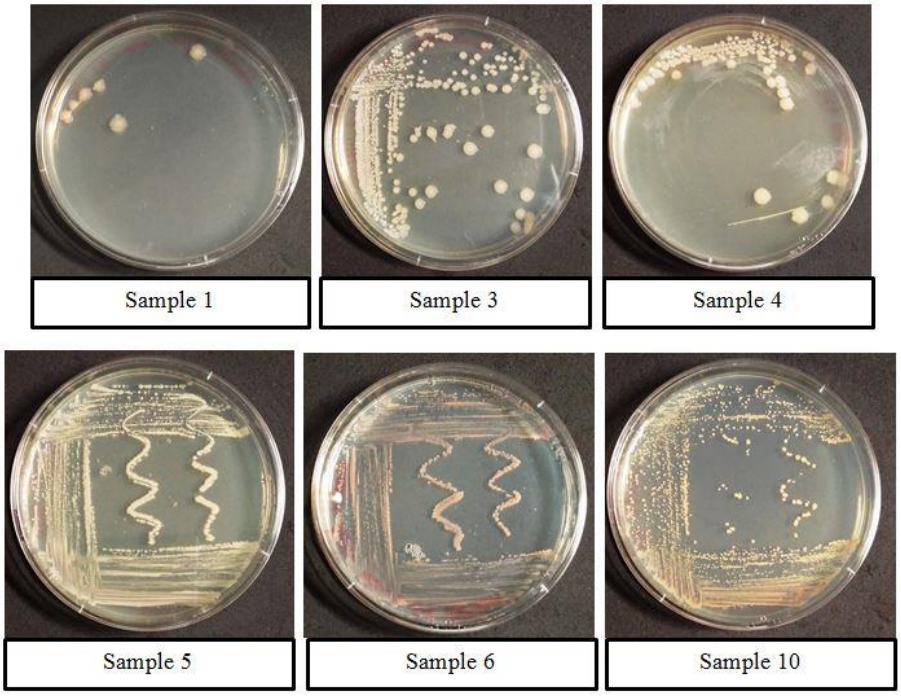


Figure 25: Microbial growth from affected X. muta homogenate on Low Nutrient agar



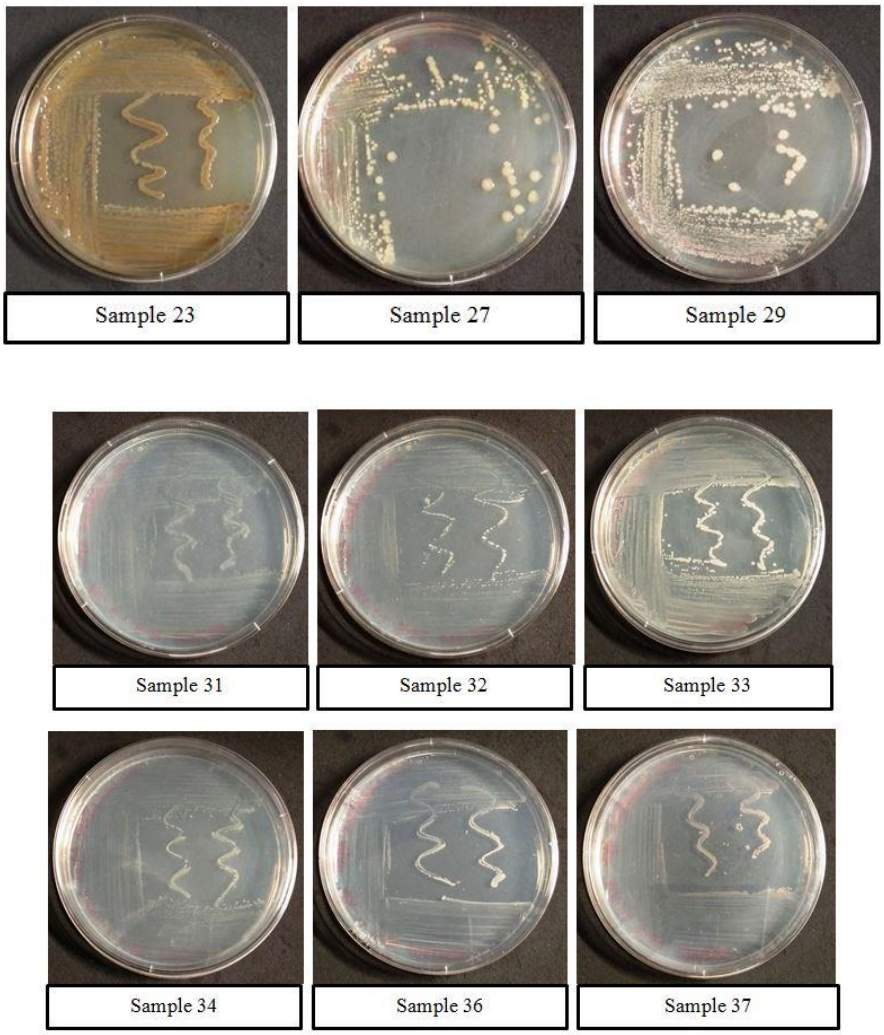


Figure 26: Single isolated colonies from Marine and Low Nutrient agar

As shown on Figures 18-26, a number of dominant bacterial communities were successfully revived on both Marine and Low Nutrient agar plates. All 22 sponge homogenates were spread on both media plates. Affected samples from both sponges showed growth on plates in 2-3 days on marine agar and showed growth on low nutrient agar in 4-5 days. Unaffected sponge samples showed growth on marine agar plates in 3-4 days while on low nutrient agar plates in 5-7 days.

MALDI – TOF – MS

Sr. No.	Analyte Name	Sponge Samples	Organism (Best Match)	Score Value
1	S - 1 (+) B	*X. muta* affected (XMA)	*Vibrio parahaemolyticus*	1.986
2	S - 1 (+) B		*Vibrio harveyi*	1.979
3	S - 2 (+) B	*X. muta* affected (XMA)	*Vibrio mytili*	1.858
4	S - 2 (+) B		*Vibrio harveyi*	1.992
5	S - 3 (+) B	*X. muta* affected (XMA)	*Vibrio harveyi*	1.978
6	S - 3 (+) B		*Vibrio mytili*	1.987
7	S - 4 (++) B	*X. muta* affected (XMA)	*Vibrio harveyi*	2.247
8	S - 4 (++) B		*Vibrio alginolyticus*	2.189
9	S - 5 (+) B	*A. clathrodes* affected (ACA)	*Bacillus firmus*	1.968
10	S - 6 (++) A	*A. clathrodes* affected (ACA)	*Bacillus vietnamensis*	2.135
11	S - 7 (-) C	*A. clathrodes* affected (ACA)	no peaks found	< 0
12	S - 8 (+) B	*A. clathrodes* affected (ACA)	*Bacillus firmus*	1.784
13	S - 9 (+) B	*A. clathrodes* affected (ACA)	*Bacillus firmus*	1.852
14	S - 10 (++) A	*A. clathrodes* affected (ACA)	*Bacillus megaterium*	2.241
15	S - 11 (+) A	*A. clathrodes* affected (ACA)	*Bacillus koreensis*	1.905
16	S - 12 (++) A	*A. clathrodes* affected (ACA)	*Bacillus marisflavi*	2.184
17	S - 13 (-) C	*A. clathrodes* affected (ACA)	no peaks found	< 0
18	S - 14 (-) C	*A. clathrodes* affected (ACA)	no peaks found	< 0
19	S - 15 (+) B	*A. clathrodes* unaffected (ACU)	*Bacillus firmus*	1.839
20	S - 17 (-) C	*A. clathrodes* unaffected (ACU)	no peaks found	< 0
21	S - 19 (-) C	*A. clathrodes* unaffected (ACU)	not reliable identification	1.336
22	S - 20 (+) B	*A. clathrodes* unaffected (ACU)	*Bacillus firmus*	1.782
23	S - 21 (+) B	*A. clathrodes* unaffected (ACU)	*Bacillus firmus*	1.803

Sr. No.	Analyte Name	Samples	Organism (Best Match)	Score Value
24	S - 22 (-) C	*A. clathrodes* affected (ACA)	no peaks found	< 0
25	S - 24 (+) B	*A. clathrodes* unaffected (ACU)	*Bacillus firmus*	1.81
26	S - 25 (-) C	*X. muta* unaffected (XMU)	no peaks found	< 0
27	S - 26 (-) C	*X. muta* unaffected (XMU)	no peaks found	< 0
28	S - 27 (++) A	*X. muta* unaffected (XMU)	*Vibrio neptunius*	2.002
29	S - 27 (+) B		*Vibrio alginolyticus*	1.869
30	S - 28 (+) B	*X. muta* unaffected (XMU)	*Bacillus firmus*	1.876
31	S - 29 (+) B	*A. clathrodes* unaffected (ACU)	*Bacillus firmus*	1.9
32	S - 30 (+) B	*A. clathrodes* unaffected (ACU)	*Bacillus firmus*	1.944
33	S - 31 (+) B	*A. clathrodes* unaffected (ACU)	*Bacillus firmus*	1.876
34	S - 32 (-) C	*A. clathrodes* unaffected (ACU)	not reliable identification	1.266
35	S - 33 (+) B	*X. muta* affected (XMA)	*Vibrio harveyi*	1.994
36	S - 33 (+) B		*Vibrio campbellii*	1.934
37	S - 34 (++) A	*X. muta* affected (XMA)	*Vibrio parahaemolyticus*	2.019
38	S - 35 (+) B	*X. muta* affected (XMA)	*Bacillus firmus*	1.926
39	S - 36 (+) B	*A. clathrodes* affected (ACA)	*Bacillus firmus*	1.804
40	S - 37 (-) C	*A. clathrodes* affected (ACA)	not reliable identification	1.545
41	S - 38 (-) C	*A. clathrodes* affected (ACA)	not reliable identification	1.514
42	S - 39 (-) C	*A. clathrodes* unaffected (ACU)	no peaks found	< 0

Table 7: MALDI – TOF – MS data

Overall MALDI – TOF – MS analysis showed presence of either *Vibrio* spp. or *Bacillus* spp. in all samples. *Vibrio* spp. or *Bacillus* spp. were detected in the affected samples that were isolated from marine agar (S – 1 to 14) while unaffected samples

isolated from marine agar (S – 15 to 26) had presence of mostly *Bacillus* spp. Affected samples isolated from low nutrient agar showed presence of *Vibrio* spp. only for samples 33 and 34, *Bacillus* spp. for 35 and 36 and non-reliable identification for rest of affected samples. Two of the unaffected samples from low nutrient agar showed presence of nonpathogenic *Vibrio* spp. and rest of unaffected samples from low nutrient agar showed presence of *Bacillus* spp. Samples having score values above 2.000 had high probable species identification while samples having score values between 1.700 and 1.999 had probable genus identification. Score values for samples ranging from 0.000 to 1.699 did not have reliable identification. Samples 7, 13, 14, 17, 22, 25, 26 and 39 did not show peaks and thus their score value was 0.

Range	Description	Symbols	Color
2.300 ... 3.000	Highly probable species identification	(+++)	Green
2.000 ... 2.299	Secure genus identification, probable species identification	(++)	Green
1.700 ... 1.999	Probable genus identification	(+)	Yellow
0.000 ... 1.699	Not reliable identification	(-)	Red

Table 8: Meaning of Score Value

Category	Description
A	Species Consistency: The best match was classified as 'green' (see above). Further 'green' matches are of the same species as the first one. Further 'yellow' matches are at least of the same genus as the first one.
B	Genus Consistency: The best match was classified as 'green' or 'yellow' (see above). Further 'green' or 'yellow' matches have at least the same genus as the first one. The conditions of species consistency are not fulfilled.
C	No Consistency: Neither species nor genus consistency (Please check for synonyms of names or microbial mixture).

Table 9: Meaning of Consistency Categories (A – C)

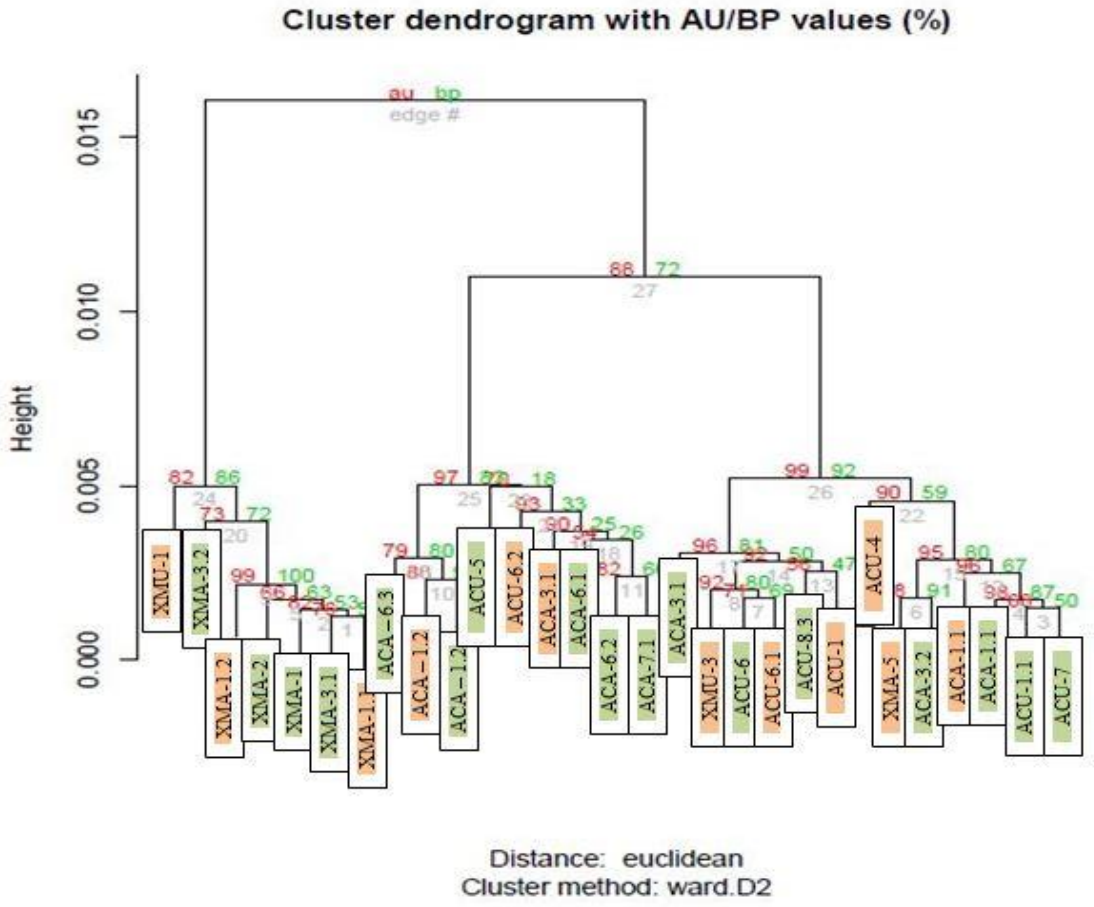


Figure 27: Phylogenetic tree from MALDI – TOF – MS analysis. Green boxes are isolates from Marine agar while orange boxes are isolates from Low Nutrient agar.

Cluster analysis of identified showed few bacteria were associated exclusively with a particular sponge species or corresponded to healthy or affected hosts (Fig. 28). Above phylogenetic tree from MALDI – TOF – MS analysis shows that samples are clustered according to the bacteria identified in them during this analysis. Most of the

affected *X. muta*, some affected *A. clathrodes* and one unaffected *X. muta* were clustered together due to identification of *Vibrio* spp. in them. Most of the affected *A. clathrodes*, all unaffected *A. clathrodes* and one of each affected and unaffected *X. muta* were clustered together as they were detected with *Bacillus* spp. Samples did not cluster together according to the media they were isolated from.

16S rDNA Sequencing

(A) NanoDrop Readings

Sr.		DNA Isolation		PCR Reaction		Purified PCR Products	
No.	Samples	ng/µl	260/280	ng/µl	260/280	ng/µl	260/280
1	XMA – 1	12.9	2.22	891.6	1.99	24.1	1.60
2	XMA – 2	11.1	2.27	776.0	2.04	15.4	1.66
3	XMA – 3.1	18.4	2.02	753.4	2.04	30.7	1.69
4	XMA – 3.2	10.8	2.02	857.1	2.04	23.4	1.82
5	ACA – 1.1	14.2	2.37	887.3	2.01	74.0	1.74
6	ACA – 1.2	10.1	2.47	936.8	2.01	73.6	1.78
7	ACA – 2	90.2	1.83	905.5	2.02	68.4	1.78
8	ACA – 3.1	13.6	2.19	858.2	2.00	110.5	1.82
9	ACA – 3.2	12.1	2.16	829.8	2.01	19.2	1.42
10	ACA – 6.1	9.6	1.98	865.2	2.02	75.7	1.75
11	ACA – 6.2	9.3	1.67	827.3	2.02	72.6	1.72
12	ACA – 6.3	20.0	1.86	783.0	2.01	78.2	1.74
13	ACA – 7.1	322.0	1.89	895.6	1.98	105.2	1.81
14	ACA – 7.2	65.5	1.80	904.9	2.00	72.6	1.81
15	ACU – 1.1	15.0	2.11	887.2	2.00	74.8	1.84
16	ACU – 1.2	14.7	1.63	866.3	2.01	102.0	1.86
17	ACU – 2.1	18.2	1.7	870.6	2.00	68.4	1.80
18	ACU – 2.2	13.0	1.85	871.5	2.00	109.0	1.83
19	ACU – 5	19.0	1.96	901.6	2.00	77.8	1.76
20	ACU – 6	13.6	1.92	900.7	1.99	88.6	1.79
21	ACU – 7	17.5	1.87	922.4	2.00	81.4	1.77
22	ACU – 8.1	15.0	2.00	822.5	2.00	62.5	1.83
23	ACU – 8.2	8.4	2.21	841.1	2.00	90.9	1.82
24	ACU – 8.3	15.1	2.07	885.6	2.00	83.9	1.82

Sr.		DNA Isolation		PCR Reaction		Purified PCR Products	
No.	Samples	ng/µl	260/280	ng/µl	260/280	ng/µl	260/280
25	XMU – 3.1	11.2	1.95	788.7	2.00	88.4	1.77
26	XMU – 3.2	9.4	2.28	854.1	1.99	72.3	1.79
27	XMU – 1	10.7	1.85	759.3	2.02	21.9	1.75
28	XMU – 3	12.2	1.91	822.5	2.01	34.9	1.66
29	ACU – 1	17.4	2.01	835.3	2.00	83.7	1.84
30	ACU – 4	14.9	1.63	851.5	2.02	18.8	1.78
31	ACU – 6.1	9.6	2.24	763.2	2.03	20.0	1.67
32	ACU – 6.2	14.4	1.72	727.3	2.02	21.3	1.83
33	XMA – 1.1	7.9	1.99	1093.3	2.02	42.0	1.73
34	XMA – 1.2	9.8	2.21	286.7	1.58	45.8	1.70
35	XMA – 5	9.2	2.06	958.6	2.01	62.8	1.78
36	ACA – 1.1	15.3	2.01	861.1	2.01	96.3	1.80
37	ACA – 1.2	21.1	1.96	926.0	2.01	90.4	1.81
38	ACA – 3.1	11.2	1.73	958.1	2.00	119.7	1.81
39	ACA – 3.2	9.9	1.84	890.9	1.99	100.8	1.76

Table 10: NanoDrop Readings

As seen in above table, concentration of isolated DNA ranges between 8.4 ng/µl and 322 ng/µl and purity also was from 1.7 to 2.47. The concentration of the PCR products suggests successful amplification of isolated DNA by 1055F and 1392R primers. It can be concluded that concentration of purified PCR products lied in the range that was required for 16S rDNA sequencing.

(B) Agarose Gel Electrophoresis (1st PCR Reaction)

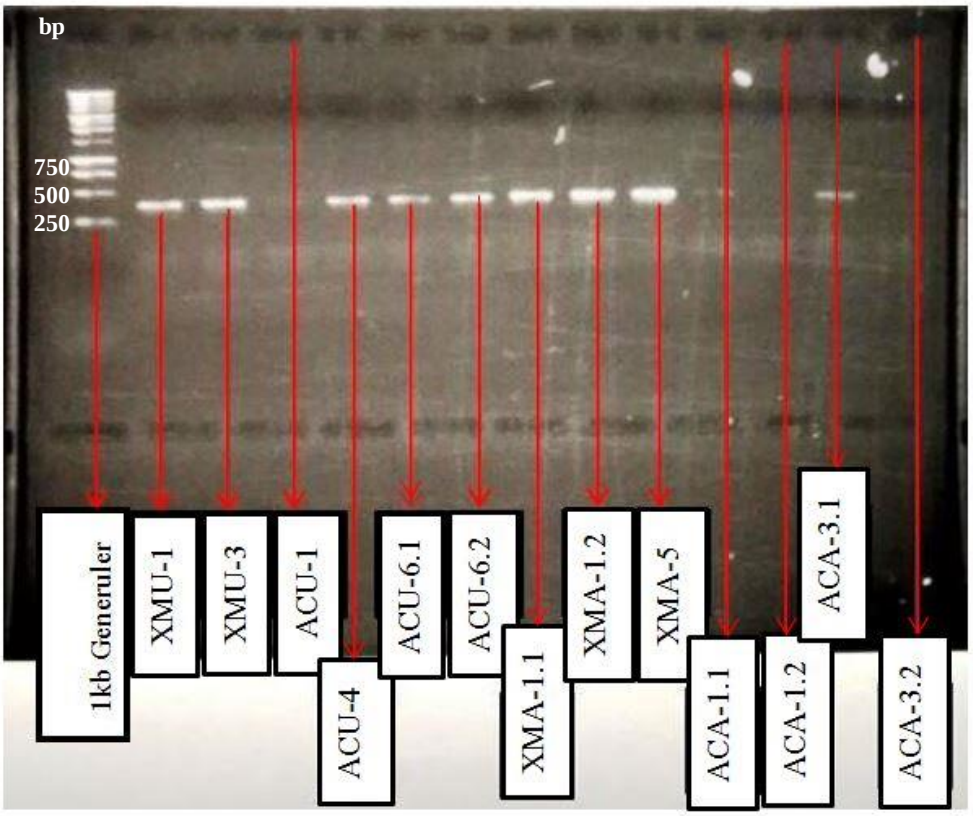


Figure 28: Agarose Gel from 1st PCR Reaction

Samples 27, 28, 30, 31, 32, 33, 34, 35 and 38 were successful amplified in the initial round of PCR of the V8 region of the 16S rDNA gene by 1055F and 1392R primers. Successful amplicons showed a strong band at ~350 bp. The samples that did not amplify on the first round were submitted to a second round of 16S rDNA PCR. The results of this second reaction are shown on the next section and on Figure 30.

(C) Agarose Gel Electrophoresis (2nd PCR Reaction)

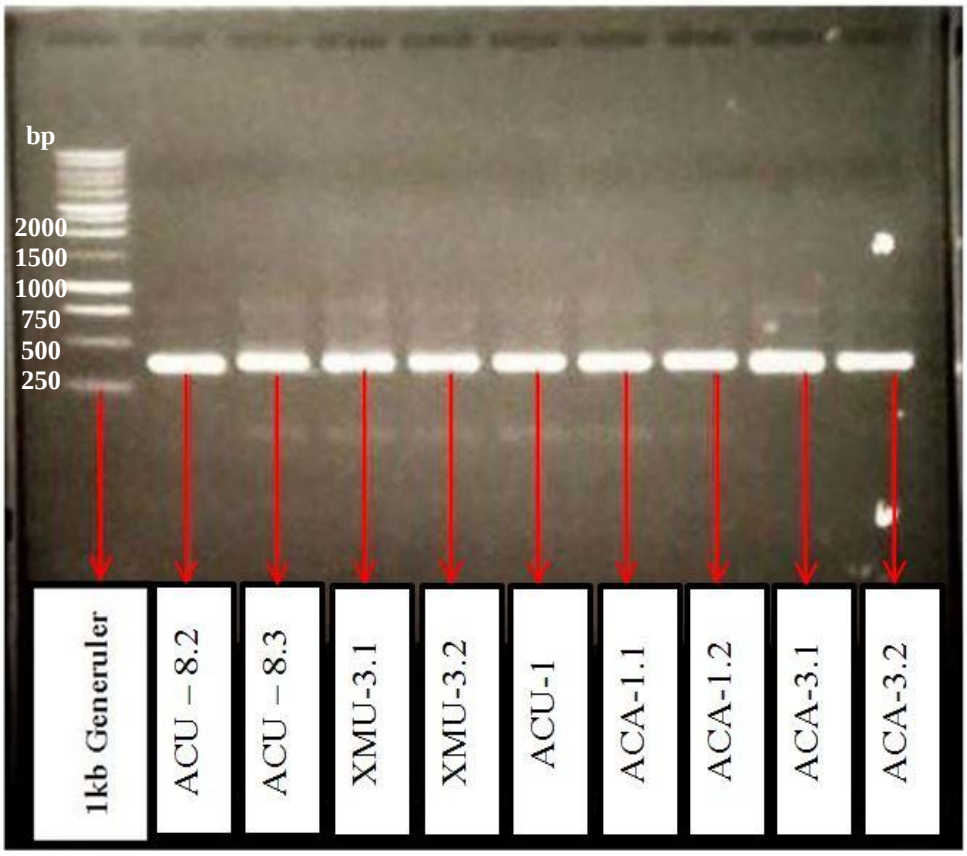


Figure 29: Agarose Gel from 2nd PCR Reaction

Samples 23, 24, 25, 26, 29, 36, 37, 38 and 39 were successful amplified in the second round of PCR (i.e. re-PCR) of the V8 region of the 16S rDNA gene by 1055F and 1392R primers. Successful amplicons showed a strong band at ~350 bp.

(D) 16S rDNA Sequencing Data

Sample	Description	E-value	ID	Accession
XMA – 1	*Vibrio harveyi* strain RCVH001 16S ribosomal RNA gene, partial sequence	0	100%	MF164179.1
XMA – 2	*Vibrio rumoiensis* strain NIOMR17 16S ribosomal RNA gene, partial sequence	2e-157	100%	KY673003.1
XMA – 3.1	*Vibrio harveyi* strain RCVH001 16S ribosomal RNA gene, partial sequence	0	98%	MF164179.1
XMA – 3.2	*Vibrio harveyi* strain SDMN-Y7 16S ribosomal RNA gene, partial sequence	0	100%	KY003120.1
ACA – 1.1	*Halanaerobium sehlinense* strain 2Sehl 16S ribosomal RNA gene, partial sequence	2e-131	95%	JN381501.1
ACA – 1.2	Halanaerobium sehlinense strain 2Sehl 16S ribosomal RNA gene, partial sequence	7e-177	99%	JN381501.1
ACA – 2	*Halanaerobium sehlinense* strain 2Sehl 16S ribosomal RNA gene, partial sequence	1e-179	99%	JN381501.1
ACA – 3.1	Not reliable identification	-----	------	------
ACA – 3.2	Not reliable identification	-----	------	------
ACA – 6.1	*Halanaerobium sehlinense* strain 2Sehl 16S ribosomal RNA gene, partial sequence	2e-166	99%	JN381501.1
ACA – 6.2	*Halanaerobium sehlinense* strain 2Sehl 16S ribosomal RNA gene, partial sequence	1e-178	99%	JN381501.1
ACA – 6.3	*Halanaerobium sehlinense* strain 2Sehl 16S ribosomal RNA gene, partial sequence	5e-173	99%	JN381501.1
ACA – 7.1	Not reliable identification	-----	------	------
ACA – 7.2	*Halanaerobium sehlinense* strain 2Sehl 16S ribosomal RNA gene, partial sequence	1e-178	99%	JN381501.1
ACU – 1.1	Not reliable identification	-----	-----	-----
ACU – 1.2	Not reliable identification	-----	-----	-----
ACU – 2.1	Not reliable identification	-----	-----	-----

Sample	Description	E-value	ID	Accession
ACU – 2.2	*Pseudovibrio denitrificans* strain Ab134 16S ribosomal RNA gene, partial sequence	2e-132	98%	KX990273.1
ACU – 5	*Halanaerobium sehlinense* strain 2Sehl 16S ribosomal RNA gene, partial sequence	6e-152	98%	JN381501.1
ACU – 6	Not reliable identification	-----	-----	-----
ACU – 7	Not reliable identification	-----	-----	-----
ACU – 8.1	Not reliable identification	-----	-----	-----
ACU – 8.2	*Pseudovibrio* sp. strain Bu15_13 16S ribosomal RNA gene, partial sequence	3e-149	99%	KY671138.1
ACU – 8.3	Not reliable identification	-----	-----	-----
XMU – 3.1	Not reliable identification	-----	-----	-----
XMU – 3.2	Not reliable identification	-----	-----	-----
XMU – 1	*Vibrio coralliilyticus* strain Bu15_04 16S ribosomal RNA gene, partial sequence	0	100%	KY671164.1
XMU – 3	*Vibrio coralliilyticus* strain Bu15_04 16S ribosomal RNA gene, partial sequence	1e-169	99%	KY671164.1
ACU – 1	Not reliable identification	-----	-----	-----
ACU – 4	*Microbulbifer variabilis* strain Mcap_H38 16S ribosomal RNA gene, partial sequence *Alteromonadaceae bacterium* GUDS1341 16S ribosomal RNA gene, partial sequence	0 0	100% 100%	KP640585.1 KF282396.1
ACU – 6.1	*Microbulbifer variabilis* gene for 16S rRNA, partial sequence	0	100%	AB266055.1
ACU – 6.2	*Pseudovibrio denitrificans* strain UST4-50 16S ribosomal RNA gene, partial sequence	1e-179	99%	KM196102.1
XMA – 1.1	*Vibrio harveyi* strain RCVH001 16S ribosomal RNA gene, partial sequence	0	99%	MF164179.1
XMA – 1.2	*Vibrio harveyi* strain RCVH001 16S ribosomal RNA gene, partial sequence	3e-140	96%	MF164179.1
XMA – 5	*Vibrio owensii* strain MC009 16S ribosomal RNA gene, partial sequence	1e-144	99%	MG016023.1

Sample	Description	E-value	ID	Accession
ACA – 1.1	Not reliable identification	-----	-----	-----
ACA – 1.2	Not reliable identification	-----	-----	-----
ACA – 3.1	Not reliable identification	-----	-----	-----
ACA – 3.2	Not reliable identification	-----	-----	-----

Table 11: 16S rDNA Sequence Analysis Data

Isolates cultured on marine from affected *X. muta* and *A. clathrodes* included *Vibrio* spp. and *Halanaerobium sehlinense* respectively (Table 12). Isolates cultured from healthy *A. clathrodes* were identified by sequencing as *Pseudovibrio* spp. and *Halanaerobium sehlinense*. Bacteria cultured on low nutrient agar from unaffected *X. muta* and *A. clathrodes* included *Vibrio coralliilyticus* and *Microbulbifer variabilis* respectively. Most of the unaffected *A. clathrodes* from marine agar and affected *A. clathrodes* samples from low nutrient agar did not have reliable identification due to either mixed signals or bad signals.

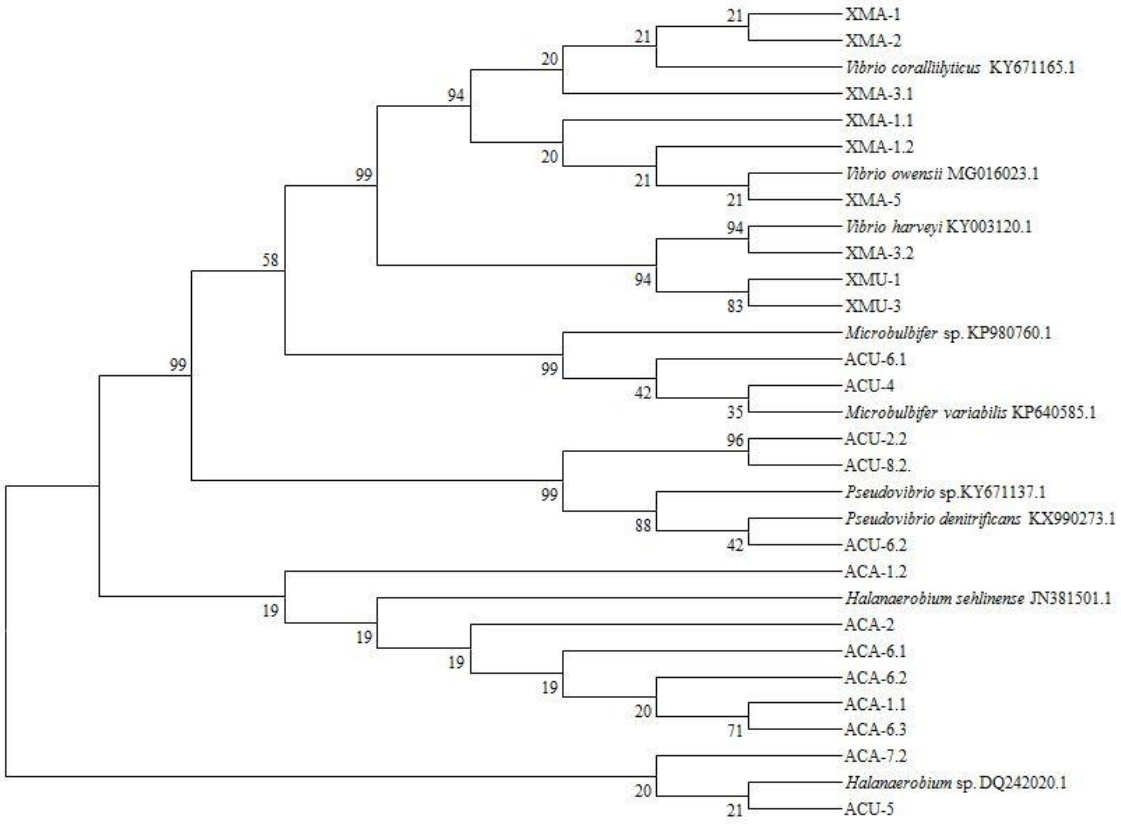


Figure 30: Maximum Parsimony analysis of taxa

The evolutionary history was inferred using the Maximum Parsimony method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed [1]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [1]. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (pg. 126 in ref. [2]) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). The analysis involved 31 nucleotide sequences. All positions containing gaps and missing

data were eliminated. There were a total of 167 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [3].

Gram Staining

Sr. No.	Samples	Shape of Bacteria	Gram +ve or -ve
1	XMA – 1	Rod	Gram -ve
2	XMA – 2	Rod	Gram -ve
3	XMA – 3.1	Rod	Gram -ve
4	XMA – 3.2	Rod	Gram -ve
5	ACA – 1.1	Rod	Gram +ve
6	ACA – 1.2	Rod	Gram +ve
7	ACA – 2	Rod	Gram +ve
8	ACA – 3.1	Rod	Gram +ve
9	ACA – 3.2	Rod	Gram +ve
10	ACA – 6.1	Rod	Gram +ve
11	ACA – 6.2	Rod	Gram +ve
12	ACA – 6.3	Rod	Gram +ve
13	ACA – 7.1	Rod	Gram -ve
14	ACA – 7.2	Rod	Gram -ve
15	ACU – 1.1	Rod	Gram +ve
16	ACU – 1.2	Rod	Gram +ve
17	ACU – 2.1	Rod	Gram +ve
18	ACU – 2.2	Rod	Gram +ve
19	ACU – 5	Rod	Gram +ve
20	ACU – 6	Rod	Gram +ve
21	ACU – 7	Rod	Gram +ve
22	ACU – 8.1	Rod	Gram +ve
23	ACU – 8.2	Rod	Gram +ve
24	ACU – 8.3	Rod	Gram +ve
25	XMU – 3.1	Rod	Gram +ve
26	XMU – 3.2	Rod	Gram +ve
27	XMU – 1	Rod	Gram -ve
28	XMU – 3	Rod	Gram -ve
29	ACU – 1	Rod	Gram -ve
30	ACU – 4	Rod	Gram +ve
31	ACU – 6.1	Rod	Gram +ve
32	ACU – 6.2	Rod	Gram -ve
33	XMA – 1.1	Rod	Gram -ve
34	XMA – 1.2	Rod	Gram -ve

Sr. No.	Samples	Shape of Bacteria	Gram +ve or -ve
35	XMA – 5	Rod	Gram -ve
36	ACA – 1.1	Rod	Gram -ve
37	ACA – 1.2	Rod	Gram -ve
38	ACA – 3.1	Rod	Gram -ve
39	ACA – 3.2	Rod	Gram -ve

Table 12: Results of Gram Staining

Above table represents Gram staining data. Affected *X. muta* samples from marine agar were detected by *Vibrio* spp. and thus gram staining results for theses samples was negative (i.e. pink). Whereas affected *A. clathrodes* showed presence of *Halanaerobium sehlinense*/ *Bacillus firmus*. Both of these bacteria are gram positive which can be seen from above table. Unaffected *A. clathrodes* samples from marine agar too were detected by *Halanaerobium sehlinense*/ *Bacillus firmus* and showed gram positive results. Unaffected *X. muta* samples from marine agar were not identified from either of the techniques. These samples showed gram positive results. While affected unaffected *X. muta* samples from low nutrient agar were identified as Vibrio spp. and their gram staining their gram results were negative. Affected *A. clathrodes* samples from low nutrient agar were not identified from either of the techniques. These samples showed gram negative results. For unaffected *A. clathrodes* samples from low nutrient agar, gram results were positive and they matched results from MALDI-TOF-MS analysis.

Data Comparison

Sr. No.	Samples	16S rDNA Sequencing	MALDI-TOF-MS	Gram Staining
1	**XMA – 1**	***Vibrio harveyi***	***Vibrio harveyi***	**Gram -ve**
2	**XMA – 2**	***Vibrio harveyi***	***Vibrio harveyi***	**Gram -ve**
3	**XMA – 3.1**	***Vibrio harveyi***	***Vibrio harveyi***	**Gram -ve**
4	**XMA – 3.2**	***Vibrio harveyi***	***Vibrio harveyi***	**Gram -ve**
5	ACA – 1.1	*Halanaerobium sehlinense*	*Bacillus firmus*	Gram +ve
6	ACA – 1.2	*Halanaerobium sehlinense*	*Bacillus vietnamensis*	Gram +ve
7	ACA – 2	*Halanaerobium sehlinense*	no peaks found	Gram +ve
8	ACA – 3.1	Not reliable identification	*Bacillus firmus*	Gram +ve
9	ACA – 3.2	Not reliable identification	*Bacillus firmus*	Gram +ve
10	ACA – 6.1	*Halanaerobium sehlinense*	*Bacillus megaterium*	Gram +ve
11	ACA – 6.2	*Halanaerobium sehlinense*	*Bacillus koreensis*	Gram +ve
12	ACA – 6.3	*Halanaerobium sehlinense*	*Bacillus marisflavi*	Gram +ve
13	ACA – 7.1	Not reliable identification	no peaks found	Gram -ve
14	ACA – 7.2	*Halanaerobium sehlinense*	no peaks found	Gram -ve
15	ACU – 1.1	Not reliable identification	*Bacillus firmus*	Gram +ve
16	ACU – 1.2	Not reliable identification	-	Gram +ve
17	ACU – 2.1	Not reliable identification	no peaks found	Gram +ve
18	ACU – 2.2	*Pseudovibrio denitrificans*	-	Gram +ve
19	ACU – 5	*Halanaerobium sehlinense*	not reliable identification	Gram +ve
20	ACU – 6	Not reliable identification	*Bacillus firmus*	Gram +ve
21	ACU – 7	Not reliable identification	*Bacillus firmus*	Gram +ve
22	ACU – 8.1	Not reliable identification	no peaks found	Gram +ve
23	ACU – 8.2	*Pseudovibrio spp.*	-	Gram +ve
24	ACU – 8.3	Not reliable identification	*Bacillus firmus*	Gram +ve
25	XMU – 3.1	Not reliable identification	no peaks found	Gram +ve
26	XMU – 3.2	Not reliable identification	no peaks found	Gram +ve
27	**XMU – 1**	***Vibrio coralliilyticus***	***Vibrio neptunius***	**Gram -ve**
28	XMU – 3	*Vibrio coralliilyticus*	*Bacillus firmus*	Gram -ve
29	ACU – 1	Not reliable identification	*Bacillus firmus*	Gram -ve
30	ACU – 4	*Microbulbifer variabilis*	*Bacillus firmus*	Gram +ve
31	ACU – 6.1	*Microbulbifer variabilis*	*Bacillus firmus*	Gram +ve
32	ACU – 6.2	*Pseudovibrio denitrificans*	not reliable identification	Gram -ve
33	**XMA – 1.1**	***Vibrio harveyi***	***Vibrio harveyi***	**Gram -ve**
34	**XMA – 1.2**	***Vibrio harveyi***	***Vibrio parahaemolyticus***	**Gram -ve**
35	XMA – 5	*Vibrio owensii*	*Bacillus firmus*	Gram -ve

Sr. No.	Samples	16S rDNA Sequencing	MALDI-TOF-MS	Gram Staining
36	ACA – 1.1	Not reliable identification	*Bacillus firmus*	Gram -ve
37	ACA – 1.2	Not reliable identification	not reliable identification	Gram -ve
38	ACA – 3.1	Not reliable identification	not reliable identification	Gram -ve
39	ACA – 3.2	Not reliable identification	no peaks found	Gram -ve

Table 13: Data comparison

Upon comparison, only 18% of the data was correlated between MALDI-TOF-MS analysis and 16S rDNA sequencing. Results of XMA-1, XMA-2, XMA-3.1 and XMA-3.2 from techniques correlated. These samples were isolated from marine agar. Data of isolates XMU-1, XMA-1.1 and XMA-1.2 from low nutrient agar correlated when compared. Data of unaffected and affected *A. clathrodes* isolated from both media plates did not correlate with each other.

CHAPTER IV

Discussion

The main aim of this project was to identify the endophytic bacterial communities of sponges involved in the massive die – off that occurred in August 2016 at the East Bank of the Flower Garden Banks National Marine Sanctuary (FGBNMS). For this purpose a culturomics approach was used with the sponges *Agelas clathrodes* and *Xestospongia muta* collected from affected and unaffected regions of East bank. Isolated bacteria were cultured in the lab using Marine agar/broth and Low Nutrient agar/broth. Isolated colonies were sub – cultured and subjected to three different identification methods: molecular techniques: MALDI – TOF – MS, 16S rDNA sequencing and morphological/microscopic identification.

Homogenate Preparation

After collection, samples were kept on ice, transported to the lab, and were stored at -80° C for 8 months without any cryoprotective agents, like dimethyl sulfoxide (DMSO) or glycerol since the main goal for these samples was the culture-independent identification of these microbial communities. Because the sample preparation and storage was not optimal, a fair amount of cellular damage during cryopreservation was expected [38]. Therefore, the total culturable endophytic microbial community present in either sponge might not be preserved was not expected to be cultured. Due to extreme temperature storage conditions, i.e. -80° C, and starving conditions, the bacteria present in sponge samples could have formed spores [39]. Thus to revive the remaining and

dominant bacterial population present in both sponges, small pieces of both organisms were used to inoculate Marine broth which was then incubated for 24 hours at room temperature [40]. These samples were chopped into smaller pieces to increase surface area thus allowing maximum penetration of the broth into sponge.

Inoculation of Homogenate on Marine and Low Nutrient Agar plates

Marine agar plates of full strength were used to plate the homogenates described above. This media was used to allow the growth of maximum generalist microbial communities [40]. Marine agar plates were prepared in DI water as marine broth already has a salt concentration similar to that of seawater. Low nutrient agar plates were diluted to 10% of the strength of marine broth and were used to obtain communities that thrive low nutrient concentrations or oligotrophic conditions, similar to those found in coral reef. Low nutrient agar plates were prepared in artificial sea water (ASW) to maintain salt concentration similar to natural sea water [40]. Cycloheximide was added as antifungal while preparing both agar plates to avoid any fungal contamination on plates [41].

A total of 30 µl of sponge homogenate was inoculated into 1 ml of marine broth and low nutrient broth and incubated at room temperature before platting on their corresponding agar plates. Extended incubation helped in reviving of bacterial populations on both media plates [42]. Visible turbidity was measured in *A. clathrodes* unaffected (ACU) 48 hours post inoculation suggesting that bacterial population in ACU was slow growing. In contrast, visible turbidity was measured in *A. clathrodes* affected (ACA), *X. muta* unaffected (XMU) and *X. muta* affected (XMA) after 24 hours indicating presence of fast growing microbial communities present in them. The Beijerinck strains

of bacteria were revived using method of extended incubation. These strains were the oldest bacterial cultures to be revived. Thus extended incubation does helped in reviving bacterial communities from sponge homogenates [42].

MALDI – TOF – MS

One of the hypothesis was that few of the isolated colonies will be identified using MALDI – TOF – MS. 24 out of 39 colonies were identified by this techniques. From other hypothesis, presence of endophytic bacteria belonging to phylum *Cyanobacteria*, *Proteobacteria*, *Chloroflexi*, *Firmicutes*, *Actinobacteria*, *Acidobacteria*, *Planctomycetes*, *Bacteroidetes* or *Gemmatimonadet*es were expected into healthy *A. clathrodes*. MALDI – TOF – MS analysis confirmed the hypothesis. Most of the unaffected samples showed presence of *Bacillus* spp. These species belongs to *Firmicutes* phylum.

For *X. muta* sponge, expected endophytic bacteria found in these sponges belonged to phylum *Chloroflexi*, *Actinobacteria*, *Acidobacteria*, and *Cyanobacteria*. MALDI – TOF – MS analysis detected presence of nonpathogenic *Vibrio* spp. and *Bacillus* spp. in them. *Vibrio* spp. belongs to *Proteobacteria* phylum and *Bacillus* spp. belongs to *Firmicutes* phylum. Expected results were not seen in these samples.

Bacillus firmus is a gram positive, nonpathogenic, spore forming alkaliphilic facultative aerobic bacterium. Production of strong acidic polysaccharide by *B. firmus* helps is bioabsorption of Pb, Cu and Zn [46]. This bacterium is also involved in oxidation, precipitation, bioaccumulation and manganese – oxidizing activity [47].

Presence of Bacillus spp. was seen in many samples as these species might have formed spores when sponge samples were stored in -80° C due to unfavorable conditions. Nonpathogenic *Vibrio* spp. produces nitrogenase that plays important role in nitrogen fixation [48]. All these properties might make *Bacillus firmus,* other *Bacillus* spp. and nonpathogenic *Vibrio* spp. a good endophyte of sponges.

Affected samples were expected to show presence of bacteria belonging to *Proteobacteria* and *Bacteroidetes* phylum. MALDI – TOF – MS analysis showed presence of both *Vibrio* spp. and *Bacillus* spp. in *A. clathrodes* and *X. muta*. *Vibrio* spp. belongs to *Proteobacteria* phylum. Thus expected results were obtained for affected samples.

Vibrio spp. occurring in sea water is dormant during cold temperatures and flourishes in warm water. Last year during July (2016), temperature of water in Gulf of Mexico had increased considerably thus leading to favorable conditions for *Vibrio* spp. During such favorable conditions, growth and metabolic rates of *Vibrio* spp. increases resulting in their higher concentrations [21]. Pathogenicity of these species is caused because of proteases, phospholipase, haemolysins and other exotoxins released by them. Major proteases released include cysteine protease and alkaline metal–chelator-sensitive proteases [45]. Thus *Vibrio* spp. occurring in sea water as opportunistic pathogen might have contributed in some way for the die off.

The limited coverage of environmental microbes in the MALDI-TOF database could account for the failure to identification of other bacterial communities.

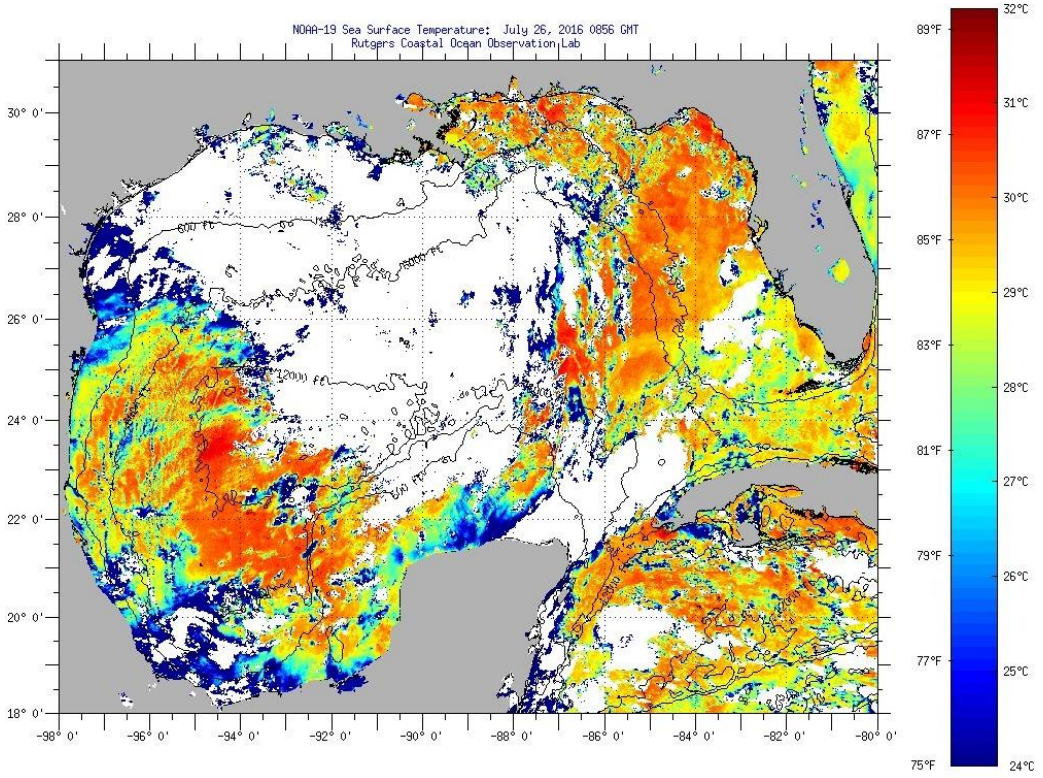


Figure 31: Temperature in Gulf of Mexico in July 2016

(https://marine.rutgers.edu/cool/sat_data/show/?file=../../regions/gulfmexico/sst/noaa/2016/img/160726.208.0856.n19.jpg)

16S rDNA Sequencing

Hypothesis for 16S rDNA sequencing analysis was that most of the cultured isolates will be identified using this molecular tool. Only 22 colonies out of 39 were identified. According to hypothesis, affected samples were expected to show presence of bacteria belonging to *Proteobacteria* and *Bacteroidetes* phylum. 16S rDNA sequencing confirmed this hypothesis. All affected *A. clathrodes* and *X. muta* sponge samples showed presence of *Vibrio harveyi* and *Halanaerobium sehlinense*. *Vibrio harveyi*

belongs to *Proteobacteria* while *Halanaerobium sehlinense* belongs to *Firmicutes* phylum. As described above in MALDI – TOF – MS, opportunist *Vibrio* spp. became active because of increase in temperature leading to pathogenicity in sponges. *Halanaerobium sehlinense* are extremely halophilic fermentative bacteria [49]. Metabolic fermentative pathways carried out by this species include lactate, acetate, ethanol, hydrogen, and carbon dioxide. Fermentation products by this species include ethanol, hydrogen, and acetate [50]. Relationship of *Halanaerobium sehlinense* with sponges is not clear yet and thus they cannot be categorized into either potential pathogenic community or endophytic community.

Healthy *A. clathrodes* were expected to show presence of *Cyanobacteria*, *Proteobacteria*, *Chloroflexi*, *Firmicutes*, *Actinobacteria*, *Acidobacteria*, *Planctomycetes*, *Bacteroidetes* or *Gemmatimonadet*es while healthy X. muta were expected to show presence of *Cyanobacteria*, *Chloroflexi*, *Actinobacteria* or *Acidobacteria*. From 16S rDNA analysis, unaffected *A. clathrodes* samples have presence of *Halanaerobium sehlinense*, *Pseudovibrio* spp. and *Microbulbifer variabilis* while *X. muta* samples were detected with *Vibrio coralliilyticus*. As discussed above, relationship of *Halanaerobium sehlinense* with sponges is not clear yet and thus they cannot be categorized into either potential pathogenic community or endophytic community. *Pseudovibrio* spp. are generally irregular rod-shaped and are capable of denitrification. They too are capable of fermenting glucose, mannose, sucrose and trehalose into acid [51]. *Microbulbifer variabilis* is gram-negative, strictly aerobic and non-motile bacteria. They perform nitrate

reduction and are catalase and oxidase positive [52]. These all features of *Pseudovibrio* spp. and *Microbulbifer variabilis* make it a good symbiont for sponges.

Vibrio coralliilyticus is gram negative temperature dependent pathogenic bacteria. They flourish in temperature above 25° C and upon infectious they cause tissue damage to sponges [53]. For these bacteria confluence of genetic mobility, temperature-dependent virulence and increased antimicrobial resistance gets higher in warm sea waters [54]. Presence of *Vibrio coralliilyticus* in unaffected samples concludes that *Vibrio* spp. is present in healthy sponges as nonpathogenic bacteria and turns into pathogen under favorable conditions. Also, there might be a possibility of cross contamination while performing sub – culturing.

Data comparison between MALDI – TOF – MS and 16S rDNA sequencing

Only 18% of data correlated between both the techniques. Data of affected *X. muta* samples isolated from marine correlated completely. Both the techniques, showed presence of *Vibrio* spp. in affected *X. muta* samples. Affected *A. clathrodes* isolates from marine agar were detected with *Halanaerobium sehlinense* by 16S rDNA and *Bacillus* spp. by MALDI – TOF – MS. Unaffected *A. clathrodes* isolates from marine agar were detected with *Halanaerobium sehlinense, Pseudovibrio* spp. by 16S rDNA and *Bacillus* spp. by MALDI – TOF – MS. Unaffected *X. muta* isolates from marine agar did not show reliable identification in either of the techniques. Affected *A. clathrodes* isolates from low nutrient were not identified by either of the techniques. Whereas affected *X. muta* isolates from low nutrient correlated completely and showed presence of *Vibrio harveyi*.

Unaffected *X. muta* isolates from low nutrient agar also correlated completely and showed presence of *Vibrio* spp. Unaffected *A. clathrodes* isolates from low nutrient showed presence of *Microbulbifer variabilis* and *Pseudovibrio denitrificans* by 16S rDNA sequencing and *Bacillus* spp. by MALDI – TOF – MS.

MALDI – TOF – MS identification of microbial community is done on bases of protein fingerprints. Unknown protein fingerprints from unidentified bacterial colony is matched with protein fingerprints present in MALDI – TOF – MS database. Upon matching of protein fingerprints, organism is identified. 2 organisms can have same proteins or peptides or biomolecules and thus during analysis, instead of showing 2 different organisms, only of the organism is detected. Also database of MALDI – TOF – MS is not fully developed. Thus MALDI – TOF – MS analysis showed presence of mainly 2 bacteria. In case of 16S rDNA sequencing, identification of microbial community is done using 16S gene present in microbe. As mentioned earlier, 16S gene is unique to each and every microbe and thus data analysis from 16S rDNA sequencing showed so much variation. Also, database of 16S rDNA sequencing is well developed.

CONCLUSION AND FUTURE DIRECTIONS

All the 4 factors i.e. environmental, physical, chemical and bacterial factors together might have contributed in massive die-off at East Bank of FGBNMS. Change salinity of Gulf water, rise in water temperature and presence if Vibrio spp. in affected samples leads to conclusion that rise in temperature of Gulf water lead to favorable conditions for *Vibrio* species to flourish and this might have caused pathogenicity by *Vibrio* species.

Future directions for this project includes re-sequencing by 16S rDNA and again performing MALDI – TOF – MS on the samples that were not identified, culturing of bacteria from sponge samples collected in 2017 to compare bacterial profile from 2 different years, samples can be subjected to FISH technique to find sequence complementarity in isolated samples, screening of isolates from unaffected samples for secondary metabolites that can be beneficial to humans and using *Microbulbifer variabilis* and nonpathogenic *Vibrio* spp. as potential nitrogen fixing agents for farming.

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