Bioprospecting Sediments from Red Sea Coastal Lagoons for Microorganisms and Their Antimicrobial Potential

Dissertation by

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In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy in Science

King Abdullah University of Science and Technology

Thuwal, Kingdom of Saudi Arabia

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ABSTRACT

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Since the soils nutrient composition along with the associated biotic and abiotic factors direct the diversity of the contained microbiome and its potential to produce bioactive compounds, many studies have been focused on sediment types with unique features characteristic of extreme environments. Red Sea lagoon ecosystems are environments with such unique features as they are highly saline. However, not much is known about the potential of their microbiomes to produce bioactive compounds. Here, we explored sediment types such as mangrove mud, microbial mat, and barren soil collected from Rabigh harbor lagoon (RHL) and Al-Kharrar lagoon (AKL) as sources for antibiotic bioprospecting. Our antibiotic bioprospecting process started with a metagenomic study that provides a more precise view of the microbial community inhabiting these sites and serves as a preliminary screen for potential antibiotics. Taking the outcomes of the metagenomic screening into account, the next step we established a library of culturable strains from the analyzed samples. We screened each strain from that library for antibiotic activity against four target strains (*Staphylococcus aureus* ATCC 25923, *Escherichia coli* dh5 α, *Pseudomonas syringae* pv. tomato dc3000 and *Salmonella typhimurium* dt2) and for the presence of polyketide synthase (PKS) and nonribosomal peptide synthetase (NRPS) genes known to support synthesis of secondary metabolites that act like antimicrobial agents. The metagenomic study showed a shift in dominant phyla consistent with a historical exposure to hydrocarbon contamination and that AKL unexpectedly displayed more contamination than RHL. This may be due to dominant phyla in AKL being consistent with early hydrocarbon exposure (when contamination levels are still high) and the dominant phyla in RHL being consistent with late

hydrocarbon exposure (when contamination levels are lower as a result of an extended period of hydrocarbon degradation). Additionally, RHL samples showed a higher percentage of enzymes associated with antibiotic synthesis, PKS and NRPS. When considering sediment type, mangrove mud samples showed a higher percentage of enzymes associated with antibiotic synthesis than microbial mat samples. Taken together, RHL was shown to be the better location with an increased probability of successful antibiotic bioprospecting, while the best sediment type in RHL for this purpose is microbial mat. Moreover, the phylum Actinobacteria tends to be the common target for research when it comes to antibiotic bioprospecting. However this culture-independent metagenomic study suggests the tremendous potential of Proteobacteria, Bacteroidetes, Cyanobacteria and Firmicutes for this purpose. Taking into account that the metagenomic screen suggests other phyla beyond Actinobacteria for antibiotic bioprospecting, the culture-dependent experiments were not designed to target actinobacteria alone. A total of 251 bacterial strains were isolated from the three collected sediments. Phylogenetic characterization of 251 bacterial isolates, based on 16S rRNA gene sequencing, supported their assignment to five different phyla: Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, and Planctomycetes. Fifteen putative novel species were identified based on a 16S rRNA gene sequence similarity of ≤ 98 % to other strain sequences in the NCBI database. We demonstrate that 52 of the 251 isolates exhibit the potential to produce an antimicrobial effect. Additionally, at least one type of biosynthetic gene sequence, responsible for the synthesis of secondary metabolites, was recovered from 25 of the 52 isolates. Moreover, 10 of the isolates had a growth inhibition effect towards all target strains. In conclusion, this study demonstrated the significant microbial diversity associated with Red Sea harbor/lagoon systems and their potential to produce antimicrobial compounds and novel secondary metabolites. To the best of our knowledge, this is the first study that has analyzed the microbiomes in Red Sea lagoons for antibiotic bioprospecting.

PUBLICATIONS

Publications generated as part of this PhD research:

- **Soha Al-Amoudi,** Magbubah Essack, Marta F. Simões, Salim Bougouffa, Irina Soloviev, John A. C. Archer, Feras F. Lafi and Vladimir B. Bajic, Bioprospecting Red Sea Coastal Ecosystems for Culturable Microorganisms and Their Antimicrobial Potential. *Marine Drugs*, 2016 Sep 10;14(9). pii: E165. doi: 10.3390/md14090165.

- **Soha Al-Amoudi**, Rozaimi Razali, Magbubah Essack, Mohammad Shoaib Amini, Salim Bougouffa, John A.C. Archer Feras F. Lafi, Vladimir B. Bajic, Metagenomics as a Preliminary Screen for Antimicrobial Bioprospecting. *Gene,* 2016 Dec 15;594(2):248-258. doi: 10.1016/j.gene.2016.09.021.

Other publications:

- Adil Salhi, Magbubah Essack, Aleksandar Radovanovic, Benoit Marchand, Salim Bougouffa, Andre Antunes, Marta Filipa Simoes, Feras F. Lafi, Olaa A. Motwalli, Ameerah Bokhari, Tariq Malas, **Soha Al Amoudi**, Ghofran Othum, Intikhab Allam, Katsuhiko Mineta, Xin Gao, Robert Hoehndorf, John A. C. Archer, Takashi Gojobori and Vladimir B. Bajic, DESM: portal for microbial knowledge exploration systems. *Nucleic Acids Research*, 2016 Jan 4;44(D1):D624-33. doi: 10.1093/nar/gkv1147..

ACKNOWLEDGEMENTS

My first and sincere appreciation goes to Prof. Vladimir Bajic, my supervisor, for everything I learnt from him and for his continuous help and support during all stages of my PhD research. I would also like to thank him for being a supportive and encouraging in helping me to shape my interests and ideas.

I also would like to express my deep gratitude and respect to Dr. Magbubah Essack and Dr. Feras Lafi whose advice and support were valuable to me and who were always available and helpful to all my queries and concerns. Special thank for Dr. Rozaimi Razali who helped me analyze the metagenomic data. Also, the support of Dr. Salim Bougouffa, Dr. Marta Simoes, Dr. Andre Antunes, Mohammad Shoaib, Dr. John Archer and Irina Soloviev are highly appreciated for their numerous discussions and help in many aspects of my PhD research.

Many thanks to Prof. Christoph Gehring and his group for their help with the use of instruments and equipment. Also, I want to thank the staff of the Analytic Core Laboratory and Bioscience Core Laboratory of KAUST for their great help in teaching me how to use some of the machines and instruments.

My greatest appreciation goes to my husband and soul mate Khaled Al-Hattami who was always a great supporter in all my struggles and frustrations in my life and studies. Thanks to him for questioning me about my ideas, helping me think rationally and for hearing my problems.

I would like to thank my family, especially my mother and father for always believing in me, for their continuous love and support in my decisions. Without them I could not have reached my goals.

Lastly but not least, I would like to thank all my colleagues and the administrative assistants specially Sarah Salhi and Hanouf Al-Hattan who were always available to provide support to me during my research.

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LIST OF ABBREVIATIONS

ACP	Acyl Carrier Protein
AIA	Actinomycete Isolation Agar
AKL	Al-Kharrar Lagoon
Anti-MA	Difco Marine Agar with Antibiotic
BTEX	Benzene, Toluene, Ethylbenzene and Xylene
BLAST	Basic Local Alignment Search Tool
BS	Barren Soil
C	Carbon
CTAB	Hexadecyltrimethyl Ammonium Bromide
ddH_2O	Double distilled water
EtBr	Ethidium Bromide
EtOH	Ethanol
KS	Ketosynthase Domain
MA	Difco Marine Agar
MB	Difco Marine Broth
MB-GM	Difco Marine Broth Gellan Gum
MM	Microbial Mat
MN	Mangrove Mud
N	Nitrogen
NRPS	Nonribosomal Peptide Synthetases
P	Phosphorus
PCR	Polymerase Chain Reaction
PKSs	Polyketide Synthases
Pro-K	Proteinase k
RHL	Rabigh Harbor Lagoon
SDS	Sodium Dodecyl Sulfate
SRSW	Sterilized Red Sea Water
TAE Buffer	Tris Base, Acetic Acid and EDTA Buffer
TE Buffer	Tris- EDTA Buffer

KEYWORDS

16S rRNA
Al-Kharrar Lagoon
Antimicrobial (Antibiotic)
Barren Soil
Bioactivity
Biodiversity
Bioinformatic
Bioprospecting
Culturable Bacteria
Firmicute
Mangrove Mud
Metagenomics
Microbial Mat (Salt Marches)
Non Ribosomal Peptide Synthesis Gene
Phylogenetic Diversity
Polyketide Synthase Gene
Rabigh Harbor Lagoon
Red Sea Lagoon

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DECLARATION

I hereby declare that this dissearation is my original work and it has been written by me in its entirety.

I have duly acknowledged all the sources of information, which have been used in the disseration.

Soha Al-Amoudi

Chapter 1

1 Introduction

1.1 Antibiotics: The Victory and Failure of ‘Miracle Drugs’

Antibiotics are chemical compounds produced by various organisms, e.g. bacteria or fungi, which are capable of killing or inhibiting the growth of undesirable pathogens [1]. Penicillin has been the first antibiotic and was discovered in 1928 by Alexander Fleming [2]. Since then, a plethora of new antibiotics have been discovered [3]. However, in the past two decades, the rate of antibiotic discovery has slowed down and although we have many new antibiotics on the market today, most are simply a chemical modification of the discovered naturally produces variants. In fact, very few antibiotic families have been in recent research [4]. Moreover, with the indiscriminate use of antibiotics in the health, agriculture and food industries, mankind has initiated a natural process called ‘antibiotic resistance’, whereby pathogens evolve and became unaffected by antibiotics. This phenomena has now became widespread, causing pathogens to gain resistance to more than one type of antibiotics, achieving the so-called multi-drug resistance. These multi-drug resistant pathogens are slowly taking us back to a time where pneumonia and wound infections killed patients by the masses. Consequently, researchers have intensified their search for new antibiotics.

1.2 The Search for New Antibiotics: What and Where?

Approximately, half a million natural products are derived from living organisms, and of these, approximately 80 000 are derived from microbes [5]. Thousands of known secondary metabolites are capable of inducing biological effects such as antimicrobial [6, 7], anti-tumor [8], anti-inflammatory [9, 10], anti-viral [11], antiparasitic [12], or can act as pesticides [12]. Approximately, 47% of the secondary metabolites of bacterial origin induce at least one of these activities, whereas much less plant-derived (7%) and animal-derived (3%) compounds induce such biological effects [5]. This may be due to bacterial survival within ecosystems depending on several shared and competitive mechanisms such as those of proteases, bacteriocins, antibiotic production, lysosomes, siderophores and production of organic acids to alter pH [13] that forces the production of specific types of beneficial natural products. Here, it should be noted that natural products with a wide range of biological activities that tend to be relevant pharmaceuticals are usually alkaloids, terpenoids, small peptides and polyketides [14]. Thus, bacteria seem to be the more promising source from which to acquire antibiotics.

Consequently, more research is currently focused on bioprospecting bacterial strains that produce novel antibiotic compounds potentially capable of combating multi-drug resistant pathogens. Since the 1940s, Actinomycetes has been the phylum targeted for bioprospecting of antibiotic compounds. However, sourcing diffusible broad-spectrum antibiotic agents from Actinomycetes in recent years has been met with diminishing returns. Since the 1960s, only daptomycin [15] and linezolid [16], have been successfully introduced in the medical treatments [17]. Nonetheless, several bioprospecting studies

have recently focused on a broader range of bacterial habitats including hydrothermal vents [18], sediments [19], plants [20], seawater [21], and eukaryotic marine organisms [22]. There are several factors that affect the species diversity in these various microbiomes including temperature, salinity, organic matter content, pH, hydrocarbon contamination, agricultural practices, and fertilizers [23]. This broader screening approach has successfully identified promising new antibiotics [24-26]. To increase success of such bioprospecting ventures there is now a particular focus on bacterial habitats with extreme characteristics. Particularly since [27] analyzed metagenomic data from 111 sites and pinpointed salinity as the major environmental determinant of microbiome composition, as opposed to extremes of other physical and chemical characteristics. They additionally reported that sediments and hypersaline mats exhibited more species diversity than other habitats. More recent studies reported that 71% of marine-derived natural products were not also terrestrial-derived natural products [28], and that marine-derived natural products are more broadly distributed, covering several drug-relevant areas [29]. These findings that marine ecosystems harbor more biological diversity than terrestrial ecosystems are to a large degree not unexpected, as 70% of the Earths' surface is covered by ocean harboring billions of distinct microbes with huge metabolic production diversity owing to 3.5 billion years of genetic evolution that had facilitated organisms' survival in the various ecosystems. Another important factor to consider when specifically selecting a site for bioprospecting antibiotic agents, is the selection of sites exposed to hydrocarbon contamination, as [30] reported an increase in microbial antibiotic resistance enzymes owing to selective pressure at sites exposed to

this type of contamination. Thus, an attractive antibiotic agent bioprospecting site would be one with hydrocarbon-contamination exposure and extreme salinity.

1.3 Functional Screening

Microbes are an important component of essentially all ecosystems, as they are key contributors to energy flow and organic matter mineralization [31]. For centuries microbes and their by-products have been used in several industrial applications [32], healthcare [33, 34], agriculture [35] and food industries [36, 37]. Moreover, the microbes' recognized potential as sources of novel antibiotics and enzymatic biocatalysts has been considerably reinforced by the advent of metagenomics. This comes as a result of capability of metagenomics to reveal dramatically borader spectrum of microorganisms present in the environmental samples as compared to those microorganisms that are culturable [38, 39]. Additionally, several metagenomic studies of microbial communities have provided a more accurate understanding of microbial diversity [40, 41], classification and enumeration of different taxa identified, without need to culture individual microbial species [42]. Moreover, metagenomic data allows for associating functional information to specific taxonomic groups, as well as provides insights into bacterial organisms that should be targeted in culture-based approaches [43]. This realization has brought about a surge in research focused on marine-derived natural product bioprospecting. The classical function driven approach for bioprospecting of antimicrobial naturally produced compounds is to use whole cells, supernatant or cell pellet extract to screen for a desired biological activity by testing for growth inhibition against a test microbe in top agar overlay assays from both cultured and metagenomic

samples. Also, key components associated with production of antimicrobial secondary metabolites, are gene clusters that contrian genes encoding for enzymes non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS). These can be easily screened for as primer sets for the PKS (ketosynthase domain) [44] and NRPS (adenylation domain) [45, 46] are available. Identifying these genes in the microorganisms helps in narrowing down specific classes of bioactive compounds producing microbes. This approach led to characterization of several antibiotics [47, 48]. Several bioactive compounds or activities have also been identified through function-based screening directly from metagenomic samples. As an example, since key components associated with secondary metabolite gene clusters encoding for non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) enzymes are activated by PPTases [49, 50], the phosphopantetheinyl transferase (PPTase)-targeting functional screen efficiently retrieved natural product gene clusters from metagenomic libraries [50]. For sequence/homology based screening, prior knowledge of the gene sequence associated with the targeted activity is required, which is not the case when using function-driven screening strategies [51-53]. Nonetheless, there are many challenges associated with these functional metagenomics screens: 1/ some PKS and NRPS pathways require enzymatic megacomplexes that are encoded by many genes that are distributed between multiple polycistronic transcriptional units [49, 54], making the expression of such biosynthetic pathway in heterologous host difficult; 2/ activity is restricted by the need for the entire cluster to be recovered on a single clone [55]; 3/ activities associated with a library clone extract are sometimes lost before chemical structure determination [48]; and 4/ microbial-

derived compounds often have multiple biological activities, one of which may be toxic to the heterologous host.

There are generally two competing strategies for natural products discovery, that is to 'isolate/sequence and then test' vs. 'test and then isolate/sequence' [56], both having their advantages and disadvantages. However, to identify pharmaceutically relevant compounds, integration and improvements of strategies are key to successful bioprospecting.

1.4 Red Sea Lagoon Systems as Sites for Antimicrobial Bioprospecting

We investigated the microbial community in sediments from two Red Sea lagoon systems for their unique physical-chemical environment as compared to Red Sea open waters. This difference is due to climatic factors such as temperature, evaporation rates, rainfall, winds, main sea currents and the lagoons' degree of isolation. Most of the studies carried out on Red Sea lagoons focused on the sediments chemical and textural composition [57-60] with one study discussing the foraminifera distribution in two Red Sea lagoons [61]. To the best of our knowledge there has been no systematic investigation of the microbial component of sediments in Red Sea lagoons. This is crucially important since the different physical and chemical component of sediment within lagoons will dictate a different and perhaps unique microbial community structure. One marked difference that distinguishes lagoon sediment from the other Red Sea sediments is that lagoons are considered rich in nutrients when compared to the low level of nutrient in Red Sea waters [62-66]. This affects competition between microbial species and opens possibility to find those species that show antimicrobial effects caused by novel antibiotics.

We used two lagoons for our sediment sampling efforts, Al-Kharrar lagoon (AKL) and Rabigh harbor lagoon (RHL). AKL, located north of Jeddah, is a shallow lagoon that lies on the coastal plain northwest of Rabigh city, on the western coast of Saudi Arabia; GPS (22º45′ 23º00′ N 38º45′ 39º00′ E). The lagoon depth ranges from 2-14 meters and is connected to the Red Sea through a narrow channel on its north western side, and the lagoon extends for about 17 km long and about 2-3 km wide [61]. Both, the southern and eastern part of the lagoon. are submerged in seawater on a regular basis (twice a day), with a tidal range of 20-30 cm; this creates salt marsh micro-environments (Sabkha), and contributes to the development of microbial mats. Also, a heavy mangrove system (*Avicennia marina*) coexists closely to salt marsh systems and surrounds the lagoon at certain places. Thid site contains diverse marine communities with an extensive sea grass bed of *Halophila stipulacea* in the middle of the lagoon. Several ephemeral streams have been reported to supply the lagoon with fresh water; however, this is unlikely to affect the high salinity of the AKL. RHL is similar to AKL in its general structure and sampling sites, however, a big part of the lagoon has been converted into a harbor serving the Petro Rabigh petrochemical and refining complex. Due to the massive opening of the harbor we assume that RHL would be closer in genetic and microbial profile to Red Sea open sea sites.

This research is focused on bioprospecting for antimicrobial potential of microbes that inhabit soils of two unstudied Red Sea lagoon systems. Most of the research focusing on natural products has been carried out on locations other than the Red Sea regions [67], while most Red Sea lagoon studies have focused on the chemical and textural structure of sediments rather than the microbial, genetic or bioactivity aspects. For these reasons the

RHL and AKL in the Red Sea were selected. Unique chemical and physical characteristics of these environments are likely suitable for unique or even completely novel microbes to inhabit such environments. The main difference is in the level of nutrition since a lagoon has a higher nutrient content and organic matter compared to the poor nutrient level in the open sea. The two lagoon locations can be inhabited by microbial species that may possibly produce novel antimicrobial compounds.

My PhD dissertation is composed of six chapters, beginning with an overview of the literature in Chapter 1. In Chapter 2, we investigated the metagenomic profiles of sediments from RHL and AKL, as well as a preliminary screen to pinpoint the location and sediment type of mangrove mud (MN) and microbial mat (MM), which show the most promise for successful antimicrobial bioprospecting. Chapter 3 reports on our study of isolates and bacterial strains generated from MN, MM and barren soil (BS) from AKL and RHL. Five different media were used: Difco Marine Agar (MA), Difco Marine Agar with Antibiotic (Anti MA), 10% Difco Marine Agar (10%MA), Actinomycete Isolation Agar (AIA), and Difco Marine Broth Gellan Gum (MB-GM). The purpose of this isolation step was to achieve axenic cultures: pure cultures of bacterial strains originating from one single original cell.

In Chapter 4 we present and discuss the phylogenetic analysis for bacterial identification based on 16S rRNA gene sequencing. In Chapter 5, we show our screening assay for antimicrobial effects, using both the antimicrobial activity screen and a molecular screening for PKS and NRPS genes on all isolates. In the last chapter, a summary of the results are presented. A general overview of this study is summarized in Figure 1.1

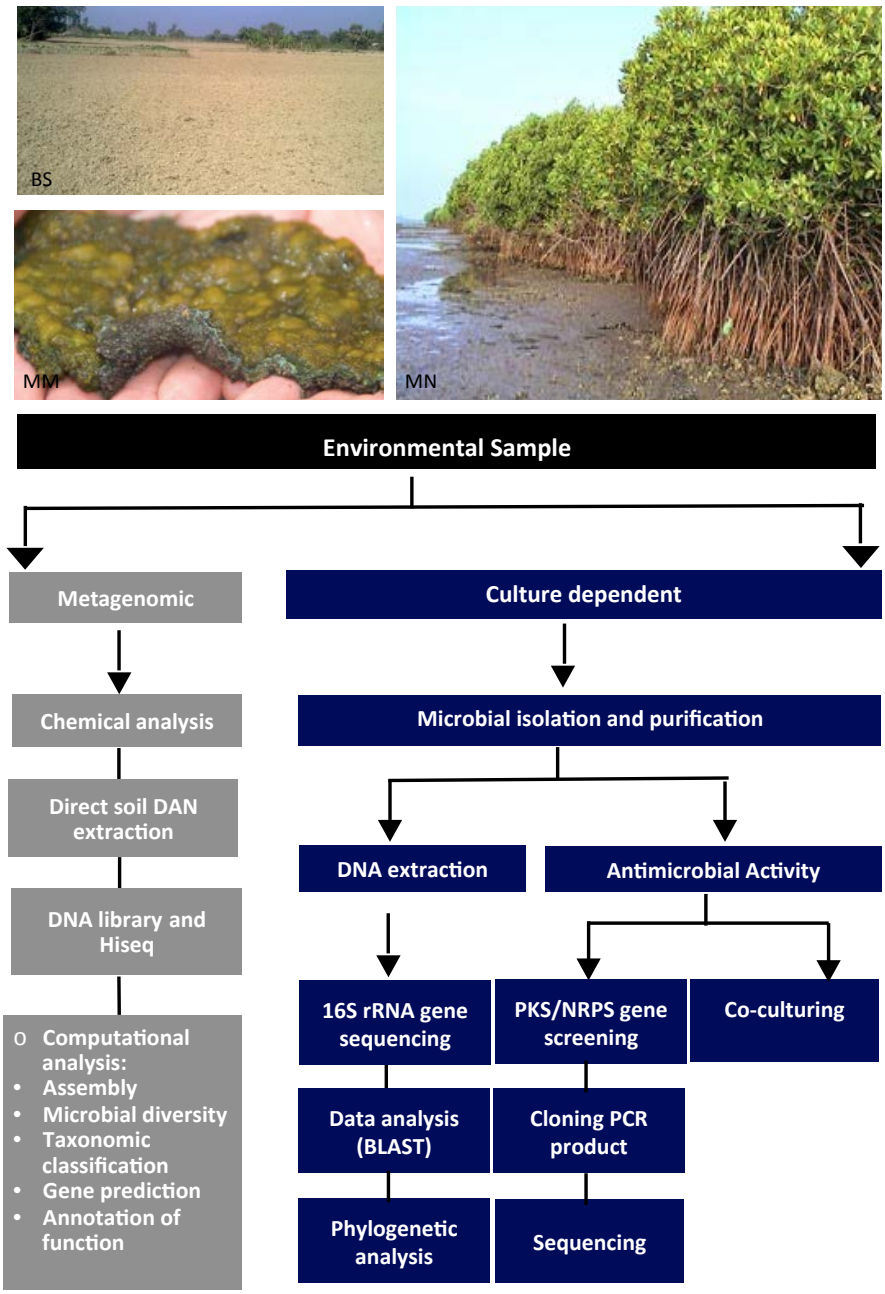

Figure 1.1. Project outline, both metagenomic- and culture-dependent methods were used

Chapter 2

2 Determining the suitability of the locations of interest for bioprospecting antimicrobial effect via metagenomic analysis

2.1 Introduction

Mangroves and coastal microbial mat ecosystems are recognized as high biodiversity hotspots [68, 69]. The microbial communities that inhabit these sediments have been shown to play fundamental roles in the functioning and maintenance of the food web in the ecosystem, and its biogeochemical and nutrient cycling [70-73]. In the same context, the structure of sediment microbial communities is strongly influenced by the availability of nutrients, anthropogenic and ecological properties [74, 75]. The microbial inhabitants of both MN and MM ecosystems have been assessed through several cultivation approaches. However, metagenomics combined with phylogenetic studies reveals that less than 1 % of bacterial diversity has been successfully cultured to date [76]. Metagenomic approaches have facilitated a more comprehensive and culture-independent description of the microbial populations present in mangroves [77-81] and coastal MM ecosystems [82-84]. Heterologous cloning and expression of metagenomic samples have been used for functional-based or sequence-based screening [85-89]. Thus, microbial diversity and activity are not only fundamental for the productivity and conservation of

mangroves, but may also serve as a reservoir of compounds with biotechnological interest.

Nonetheless, because of their exposure to environmental pollutants, these sediment types (MN and coastal MM) are often contaminated. Thus, dos Santos *et al*. [90] used mangrove metagenomics to identify bacterial genera that could be possible signals for the biomonitoring of oil pollution of mangroves. Abed *et al*. [91] categorized the bacterial communities in the anoxic layer of a heavily polluted MM and demonstrated the growth of specific strains on hydrocarbon carbon-energy sources under sulfate-reducing conditions. These results suggest that pollutants cause a change in the composition of the microbial community. In soil microbiota, selection pressure associated with hydrocarbon-contamination has been associated with increased antibiotic microbial resistance and an increased capacity to produce antibiotics [30]. Thus, it has become common practice to use cultures from contaminated sites for antimicrobial bioprospecting [92-94]. However, [27] investigated the environmental distribution of bacteria based on a comprehensive analysis that included 111 studies of diverse physical environments. They reported that sediments and hypersaline mats are more phylogenetically diverse than other environmental types, and that salinity is the major environmental determinant of microbiome composition, rather than extremes of other physical and chemical characteristics of the samples. Thus, contaminated sites with extreme salinity may hold a lot of promise for antimicrobial bioprospecting. Nonetheless, microbiological screening of natural products is time consuming and expensive. Metagenomics-based approaches are however far more attractive. Because it is culture independent, metagenomics provides an attractive and increasingly cost-effective preliminary screen to identify the

location and sediment type, with the highest possibility for successful antimicrobial bioprospecting.

In this study, we carried out a metagenomic screen of sediments from two highly saline and hot Red Sea coastal sites. The first site is the heavily industrialized RHL. The second site, AKL, not industrialized, is located 16 nm north of Rabigh harbor lagoon. For each site, mangrove-associated and microbial mat-associated sediment samples were collected and metagenomic DNA samples were sequenced, and annotated with function. From these experiments, we present a comparison of biodiversity, taxonomical abundance and an enrichment of antibiotic biosynthesis and hydrocarbon degradation enzymes. This is the first metagenomic study focused on the microbiomes in RHL and AKL, as well as the first to use metagenomics as a preliminary screen to pinpoint the location, sediment type and microbial phyla that shows the most promise for successful antimicrobial bioprospecting.

2.2 Materials and Methods

2.2.1 Site Description and Sample Processing

Sediment samples were collected from RHL (39° 0' 35.762" E, 22° 45' 5.582" N) and AKL (38°54' 39.638" E, 22°54' 50.251" N), locations separated by approximately 11 km of Rabigh coast in Saudi Arabia; in April 2012 (Figure 2.1). For each location, samples were collected from two mangrove- and microbial mat-associated sediments. At MN sites, sediment was collected from a 5-30 cm depth, approximately 15-20 cm away from mangrove (*Avicennia marina*) root. At MM sites, samples were collected from a 5-30 cm

depth beneath the salt layer of the salt marshes. The dimensions of each core sample were 20-25 cm long and 5 cm in diameter. All samples were collected in triplicate, adjacent to each other to minimize microhabitat difference. Samples were transported on ice to the laboratory and processed the same day. Samples were collected by the Coastal & Marine Resources Core Lab (CMOR) of King Abdullah University of Science and Technology (KAUST).

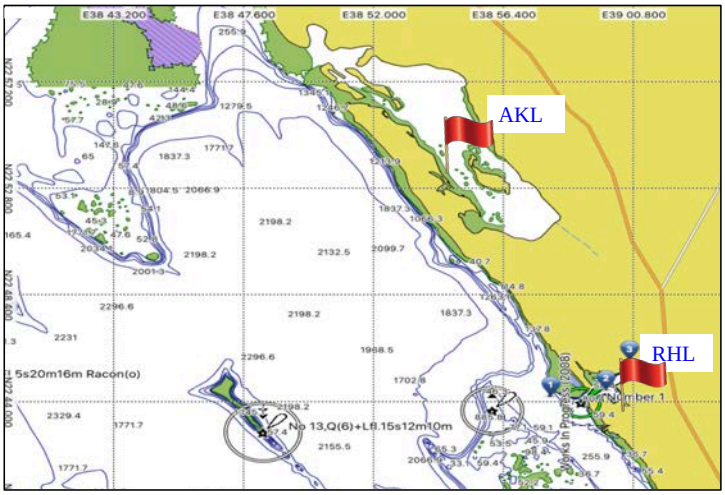

Figure 2.1. Maritime map showing location of two sample sites depicted by the red flags.

2.2.2 Chemical Analysis

Environmental parameters were measured for all sites (MNR, MMR, MNK and MMK) using four different analyzers. A 1:10 soil to water ratio was used to homogenize samples before measuring the pH, temperature and conductivity using the multi-parameter sensor assembly (YSI 6600 Sonde). The Flash 2000 Organic Elemental Analyzer (Thermo Scientific, USA) was used to measure the element flows of carbon (C), hydrogen (H),

nitrogen (N) and sulphur (S) according to EPA methods 440.0 [95]. These elements were measured by placing 3 mg of dried and homogenized sediment into a tin capsule that was inserted into the burning reactor via the MAS 200R auto sampler, together with a suitable amount of oxygen according to EPA methods 3052 for digestion. Then, the Inductively Coupled Plasma Optical Emission Spectrometry (Varian 720-ES ICP OES, Australia) was used to measure the concentration of trace elements such as iron (Fe), phosphorous (P), potassium (K) and magnesium (Mg), according to EPA methods 200.7, Varian [96]. These elements were measured by dissolving approximately 90 mg of soil sample in 5 ml of 70% of HNO_3 (trace metal grade, Fischer Scientific) and 1ml of 30% of H_2O_2 (trace metal grade, Fischer Scientific). Samples were fully digested and analyzed using the ICP/OES analyzer.

Gas Chromatography/mass spectroscopy – Selective ion monitoring (GC/MSD) 7890A (Agilent technology, USA) - was used to determine concentrations for BTEX (benzene, toluene, ethylbenzene and *m*-xylene, *o*-xylene and *p*-xylene). Here, 2g of each sediment sample were added to a glass vial containing 3 ml of NaCl/water liquid (to keep compounds in sediments unchanged and not evaporating). Total run time to analyze samples via GC was 22 min, with a split ratio of 20:1 using the J&W 29505-USLB-IL60-SUPELCO column (250 °C: 30 m x 250 µm x 0.2 µm). In GC acquisition parameters: temperature program was set at 165 °C for 0 min, run time: 7.1212 min. In the front detector FID, the heater was set at 280 °C, H_2 flow at 35ml/min, airflow at 400ml/min, and makeup flow at 15ml/min. In mass spectroscopy (MS) acquisition parameters: the solvent delay was 4 min. GC/MSD was connected to MSD 5975C from Agilent

technology with afull scan from 35 to 150 amu in SIM mode. The SIM parameters are listed in Table 2.1.

Table 2.1. Parameters used for BTEX analyses

Group	**Benzene**	**Toluene**	**Ethylbenzene**	**m,p-Xylene**	**o-Xylene**
Group start time	0 min	5 min	5.70 min	5.85 min	6.30 min
Ion	77, 78	91, 92	77, 91, 106	77, 91, 105, 106	91

2.2.3 DNA Extraction and Metagenomic Library Construction

Metagenomic DNA was extracted from 12.5 g of each sediment sample using the PowerSoil ® DNA Isolation Kit (Mo Bio Laboratories, USA), in accordance with the manufacturers protocol. DNA quality and quantity were assessed using the NanoDrop (Thermo Fischer Scientific, USA) spectrophotometer and the Qubit (Thermo Fischer Scientific, USA) fluorometer. For the DNA sequencing library construction, we used Illumina TruSeq® DNA sample Prep LS protocol. Libraries were sequenced using the Illumina HiSeq 2 × 100-bp paired-end technology. Each library sample was run in a single lane. This approach generated 85.41 GB of sequence data for all 12 samples, consisting of 3 replicates for each of MNR, MNK, MMR, and MMK.

2.2.4 Data Trimming and Quality Control

Pre-assembly quality control (QC) was performed on the Illumina HiSeq raw reads using the Trimmomatic ver 0.33 [97]. QC includes read trimming, removal of ambiguous bases,

removal of short reads, removal of duplicates, removal of foreign vectors, filtering out low quality reads and removal of sequencing adapters. In total, QC performed on 12-paired end HiSeq raw reads with the minimum acceptable Phred score of 20. Fastqc [98] was also used to visualize the data quality and assurance, pre and post trimming.

2.2.5 Metagenome Assembly and Post Assembly Quality Control

Metagenome paired end read data was assembled using a MegaHit de-novo assembler [99]. In total, 12 metagenome samples were assembled. We opted to assemble each of the replicates separately, because by pooling the replicates together we would lose all information on variability and hence will be of little use for statistical purposes [100]. The assemblies were performed using the meta-sensitive option - k-mers of 21, 41, 61, 81 and 99. In the post assembly stage, we removed all contigs with lengths of <= 1000bp in order to remove fragmented sequences which can introduce false positives during downstream analyses. Additionally, we used the UCHIME module in MOTHUR [101] to identify and subsequently remove possible chimeric sequences from the assembled metagenomes. Finally, we performed Principal Component Analysis (PCA) of the contigs against the M5 non-redundant protein database (M5NR) [102] using the Burrows-Wheeler Alignment tool (BWA) [103] in *METAGENassist* [104].

2.2.6 Feature Prediction, Annotation of Function and Taxonomic Assignment of Metagenomic Sequences

To annotate the four metagenomes, we used the Automatic Annotation of Microbial Genomes (AAMG) module in DMAP [105]. The AAMG includes annotation from various databases such as InterProScan [106], Gene Ontology [107], KEGG [108], MetaCyc [109] and Pfam [110]. In addition, it contains a database of common bioactive compounds. The two-sided Fisher's Exact Test was used for the comparison of datasets in terms of their taxonomical abundance and enrichment of functions. Correction for multiple tests was done using Storey's FDR [111]. For both functions and taxa, an FDR value of ≤ 0.05 was deemed significant. We also analyzed enzymes associated with antibiotic production/resistance such as PKS, NRPS, antibiotic synthesis and antibiotic resistance. For every predicted ORFs, we use AAMG to align ORF to proteins from the UniProt database, and if there was a hit, we extracted the UniProtKB controlled vocabulary information of the target. We limited the consideration of bioactivity to the four groups mentioned above.

For annotation, we merged metagenomic assemblies from replicates into a single fasta file for each of the MMK, MMR, MNK and MNR samples. The merged fasta files were then given as input into DMAP. Then, we normalized the data by obtaining the average abundance value. Previous studies have showed that combining different assemblies produces better results [112, 113]. There are two common methods in merging assembled contigs together. The first method, Dedupe (BBMap), merges different assembled contig files and removes redundant contigs in order to obtain a single unified assembled contig

file (https://sourceforge.net/projects/bbmap/). The other method is by ‘joining’ different overlapping contigs together in order to produce a longer contig [114]. However, neither method deals directly with taxonomical abundance because their main objective is to produce the best contig in order to predict as many ORFs as possible, which will then be aligned to the raw reads in order to calculate the abundance. Therefore, for our study, we used FragGeneScan [115] and Meta_RNA [116] to predict the ORFs and ribosomal RNAs respectively. We took a different approach by aligning the predicted ORFs and rRNA from each of the replicates against the Best Global Taxonomies [105]. We opted to predict the abundance by aligning the predicted ORFs and rRNA against GBT because of it’s ability to obtain insights into complex gene structure, which would not be possible to obtain from short read data. Furthermore, performing taxonomic classification using raw sequence reads has been shown to result in lower statistical confidence [117, 118]. We performed binning of the predicted genes in order to get the taxonomic assignment. This method produces a similar taxonomic pattern as when the 16s rRNA genes are used. A comparison of the two methods can be found in Figure S2.1.

2.2.7 Diversity Calculation

To compare the diversity between the AKL and RHL environments, we used the Whittaker concept of diversity [119]. There are three terms proposed by Whittaker: gamma diversity (γ-diversity), alpha diversity (α-diversity) and beta diversity (β-diversity). The γ-diversity refers to the total species diversity in a landscape [119]. Two different parameters determine γ-diversity: the mean species diversity in habitats at a local level (α-diversity) and the differentiation among those β-diversity habitats [119]. To

determine the β-diversity and γ-diversity, we used the pre-calculated MG-RAST alpha-diversity values, which were calculated from the Shannon diversity index [120]. We calculated both the location and sediment specific diversity values. We also calculated the α-diversity for each of the 12 assembled metagenomes, and we averaged the α-diversity per tri-replicates.

2.3 Results and Discussion

2.3.1 Sample Description and Environmental Parameters

Two different locations (RHL and AKL) with distinct features were chosen. As denoted by its name, “Rabigh harbor” is a lagoon that has been converted into a commercial harbor for the shipping of industrial products from Rabigh industrial city. This lagoon/harbor extends only 1.3 km in length and 0.24 km in width, with a lagoon depth reaching approximately 28m in depth and has a daily tide cycle of approximately 0.34 m. The sediment structure in this harbor/lagoon consists mainly of dark brown clay in the mangrove, and Greenish brown multi-layered sheets in the microbial mat. The AKL is increasingly being threatened by urban development and is connected to the Red Sea via a tight channel. AKL extends for 17 km in length and 2-3km in width, with a lagoon depth ranging from 2-3 meters. Both the southern and eastern parts of the lagoon are submerged in seawater, with the daily range of the tide cycle being 20-30 cm. The sediment structure in the lagoon consists mainly of white sandy mud in the northern part and dark brown mud in the southern part.

Although RHL and AKL differ in terms of their preservation state and anthropic action, they are similar in their environmental parameters (Table 2.2). They both have an average

temperature of 27°C, average salinity of 39 ppt and neutral pH of 7.8. Thus trace elements such as Fe, K, Mg, P, S, N, C, H were also measured in the four sample sites (MNR, MNK, MMR and MMK).

Table 2.2: Environmental Factors measurements of RHL and AKL sediments

Factors	**MNR**	**MNK**
Specific Conductivity	58.120 micro Siemens/cm	60.980 micro Siemens/cm
Conductivity	60.270 micro Siemens/cm	63.900 micro Siemens/cm
Total Dissolved Solids (TDS)	37.780 g/L	39.640 g/L
Temperature	26.93°C	27.51°C
Depth	10-15 cm	10-15 cm
Salinity	38.73 ppt	40.88 ppt
pH	7.94	7.77
Oxygen Reduction Potential	103 mV	125 mV
Dissolved Oxygen	6.58 mg/L	6.07 mg/L

MNK displayed the highest concentrations of trace elements such as K (0.6 %), S (1.25%), N (2.09%), C (24.35%), and H (3.76%) compared with other sample sites (MNR, MMK and MMR). While, the highest values for Mg and Fe were found in MMR and MMK sample sites (Figure 2.2). Jeyabal and Ramasamy (2014) showed that mangrove sediments have a great organic matter content and a high capacity to accumulate many organic and inorganic pollutants [121]. This suggests that microbes accumulate in MN sites that have more organic matter more than in MM sites.

Taking into account that MNK is not a pristine mangrove but is one that has suffered relatively less impact compared to MNR, we further assessed sediment contamination with petroleum hydrocarbons because aromatic hydrocarbons such as benzene, ethylbenzene, xylene, toluene, phenanthrene and naphthalene are phytotoxic [122] and have been demonstrated to affect microbial diversity [90]. A BTEX assessment at MN sites has shown both MNK and MNR sites displayed Toluene, Ethylbenzene and Xylene contamination at very low concentrations (Figure 2.3 and 2.4).

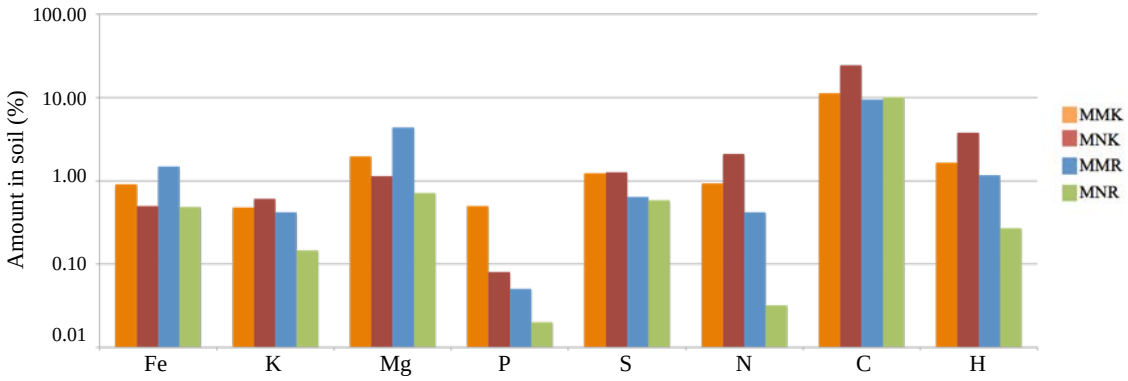

Figure 2.2. Trace elements comparison of different sediment at AKL and RHL, the data correspond to average from 3 different values MM and MN.

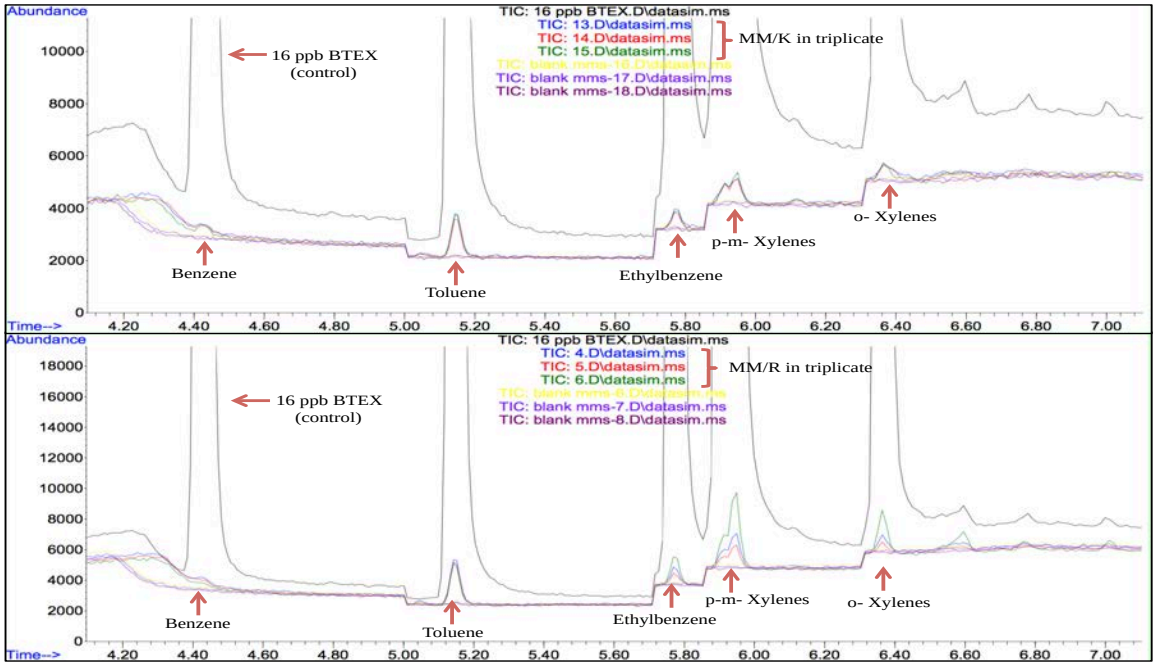

Figure 2.3. GC-Mas spectrometry results, for BTEX concentration found in MMK and MMR.

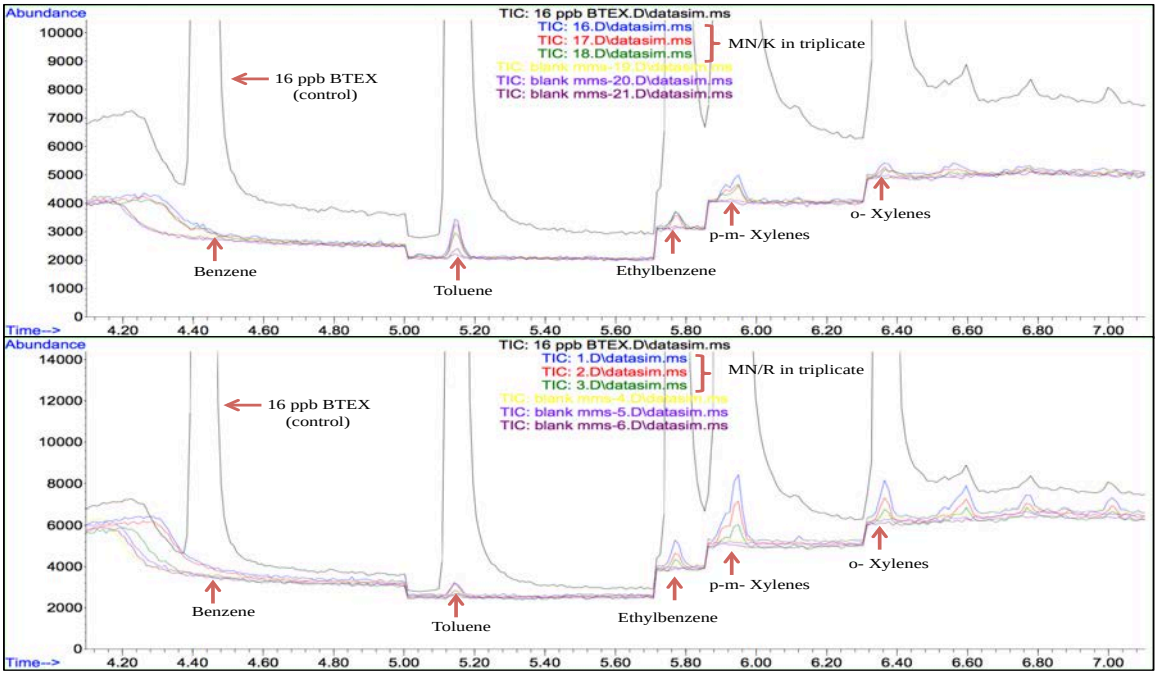

Figure 2.4. GC-Mas spectrometry results, for BTEX concentration found in MNK and MNR.

2.3.2 Microbial Diversity

Illumina sequencing generated a total of 422.79 million raw read sequences from the four sediment samples (MNK, MMK, MNR and MMR), with an average length of 101bp. After trimming, a total of 419.33 million sequences were obtained: 67.22 million reads for MNK, 59.85 million reads for MNR, 234.07 million reads for MMK, and 58.20 million reads for MMR (Table 2.3). The metagenome assembly generated 12 individual assembled contigs datasets for the four samples – three assembled contigs per sample.

To confirm microbial diversity patterns observed across MNR, MNK, MMR and MMK samples, we performed a Principal Component Analysis (PCA) to verify if the three replicates are clustered near each other, that is, if replicates show low variability. For this analysis, microbial communities were assessed at both a phylum and genus level (eukaryotic reads were excluded). At both levels, microbial communities for MNR, MNK, MMR and MMK were distinct from each other, with replicates displaying lower variability at the genus level (Figure 2.5). The distinction between microbial communities of the four samples is further augmented at the species level as depicted by the bar chart (Figure 2.6), and sediment specific and location specific alpha, beta and gamma diversity calculations (Table 2.4).

Our results reveal that MNK has a significantly higher species diversity compared to MNR ($p<0.05$). Similarly, MMK has a significantly higher species diversity compared to MMR ($p<0.05$). Overall, we observed greater diversity in the mangrove environment compared to the microbial mat environment ($p<0.05$). The reason for the enrichment of species in the mangrove environment compared to the microbial mat of the salt marsh could be due to a sustainable nutrient cycle (organic material sourced from mangrove

plants) in the mangrove environment. In contrast, the microbial mat samples came from the low marshes. Additionally, we observed that the AKL samples are more species rich compared to the RHL samples

Table 2.3. The assembly statistics.

	Pre-trimming #Reads	**Post-trimming - #Reads**	**% #Reads Dropped**	**N50**	**Total size (Mb)**	**Min length (bp)**	**Max length (bp)**	**Average length (bp)**	**#contigs**
MMK1	19964711	19882619	0.41	1901	16785778	1000	43022	1892	8874
MMK2	25051699	24925293	0.50	2448	13844845	1000	63525	2340	5916
MMK3	190682693	189259143	0.75	2410	502912101	1000	107678	2236	224901
MMR1	22087831	21802193	1.29	3592	34092493	1000	50463	2815	12110
MMR2	14861974	14771142	0.61	3504	33831219	1000	173647	2774	12195
MMR3	21734064	21629985	0.48	2421	62646981	1000	60925	2225	28159
MNK1	22163310	21993504	0.77	2406	49170378	1000	79639	2257	21784
MNK2	24396180	24101284	1.21	2591	84012947	1000	127238	2389	35160
MNK3	21300172	21123037	0.83	1544	16962471	1000	44155	1587	10690
MNR1	22292713	22068422	1.01	3060	60115822	1000	162858	2551	23563
MNR2	17468092	17128022	1.95	2387	32813842	1000	81568	2264	14492
MNR3	20793784	20650379	0.69	2786	72564776	1000	104985	2443	29706

Table 2.4. Sediment specific and location specific alpha, beta and gamma diversity between all samples.

	Sediment specific diversity			**Location specific diversity**			
	α	β	γ		α	β	γ
MNR	319.47	301.31	96,259.51	MNK	620.78	69.20	42,957.98
MNK	620.78		187,047.22	MMK	551.508		38,163.80
MMK	551.58	333.76	184,095.34	MNR	319.47	101.65	32,474.13
MMR	217.82		72,699.60	MMR	217.82		22,141.40

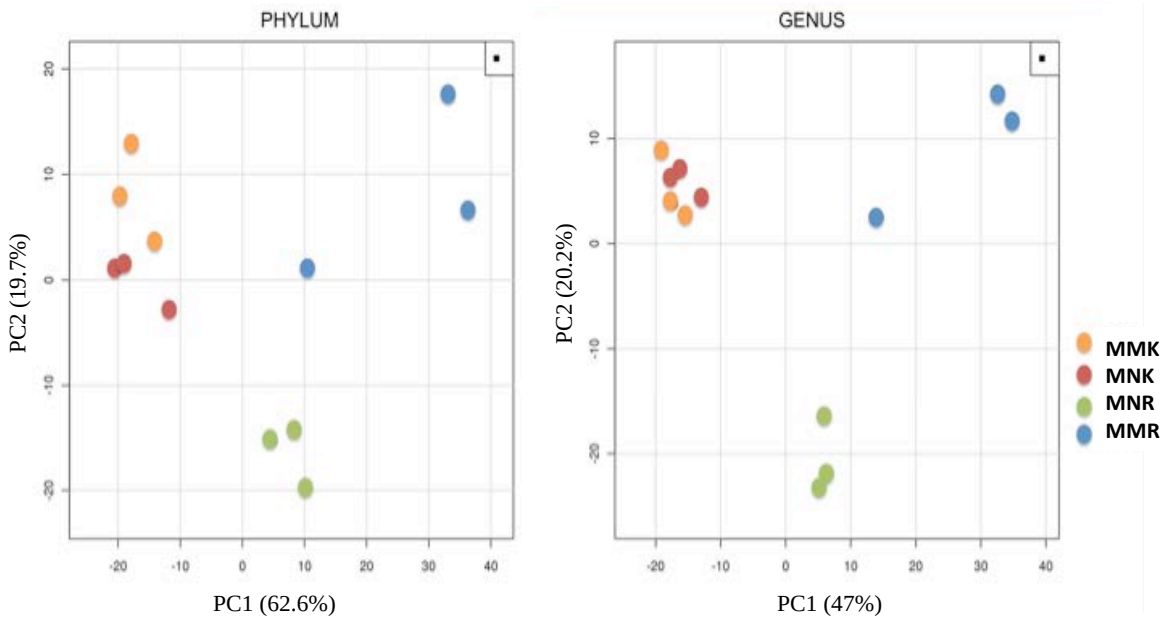

Figure 2.5. Principal Component Analysis showing the biological replicates of all four samples.

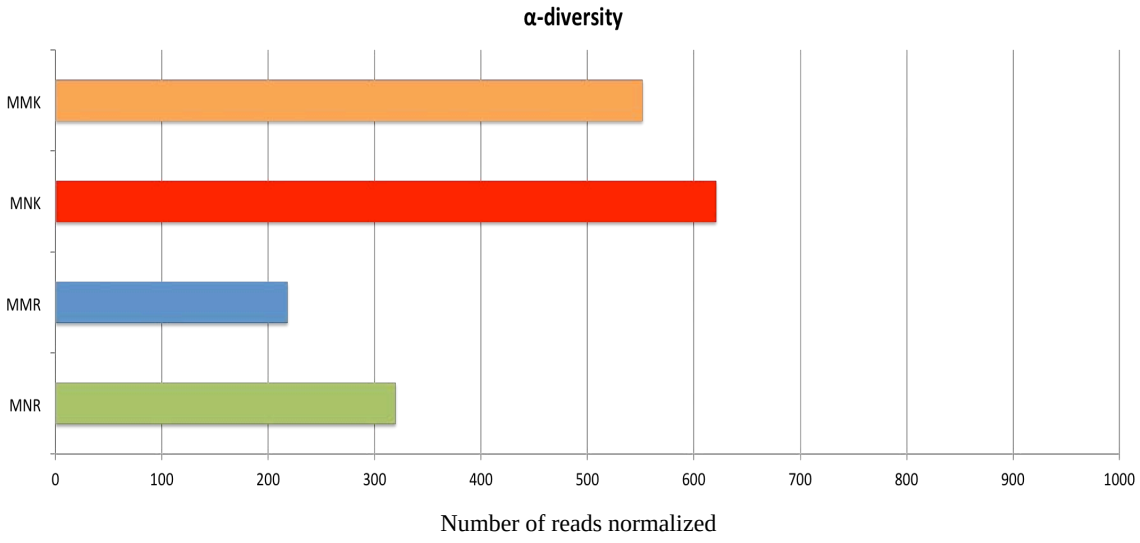

Figure 2.6. Bar chart showing species diversity over number of reads

Our observation supports a previous study [123], which suggests that the diversity in the salt marsh decreases as elevation decreases. This is because the low marsh is often submerged in salty or brackish water, which limits the species to those that can survive in an anoxic environment [124]. Additionally, the low marsh tends to be dominated and influenced by autotrophs that limit nutrient resources available to non-autotrophic species [125].

2.3.2 Microbial Abundance Analysis at The Phylum Level

For the assignment of taxonomic affiliations to the metagenomic libraries of each sample, we used DMAP. Metagenomic libraries show a predominance of prokaryotic taxa ranging from 84 % (MMK) - 85 % (MNK) of the sediment community in AKL, this is further magnified to 93 % (MNR) – 94 % (MMR) of the sediment community in RHL. RHL samples had less Archaeal and Eukaryotic sequences compared to AKL samples. DMAP showed Archaeal phylum Halobacteriales (Euryarchaeota) to be dominant in all the samples, except MNK where Bathyarchaeota comprised the majority. Additionally, Methanomicrobiales (Euryarchaeota) are enriched in all the samples, while Methanobacteriales (Euryarchaeota) and Thermococcales (Euryarchaeota) were only enriched in the AKL samples. Moreover, Cenarchaeales (Thaumarchaeota), Nitrosopumilales (Thaumarchaeota) and Thermoproteales (Crenarchaeota) were specifically enriched in MNK. For bacteria, the phyla dominant in RHL and AKL samples differed (Figure 2.7). Bacterial phylum Proteobacteria dominated in AKL samples, followed by Bacteroidetes, Chloroflexi, Firmicutes and Actinobacteria (unclassified sequences were not taken into account), while dominating bacterial phyla in

RHL samples differed not only from AKL samples, but also differed from each other. In MMR, bacterial phylum Cyanobacteria dominated, followed by Bacteroidetes, Proteobacteria, Planctomycetes and Firmicutes. Whereas in MNR, bacterial phylum Bacteroidetes dominated, followed by Proteobacteria, Cyanobacteria, Planctomycetes and Firmicutes (unclassified sequences were not taken into account) (Figure 2.7). The complete list of taxonomy for each of the four samples is given in Table S2.1.

A recent metagenomics study reported the microbiome that inhabits the gray mangroves rhizosphere from the Red Sea, are in close proximity (Thuwal, Saudi Arabia) to the mangroves researched in this study [77]. This mangrove located in Thuwal represents a pristine mangrove. Our results are not in general agreement with this study regarding the profile of the dominant microbial phyla, as the proportion of Firmicutes in the pristine mangrove is higher (6.5 %) than in MNK (5.4 %) and MNR (3.5 %). Also, the proportion of Cyanobacteria (2.3 %) and Bacteroidetes (9 %) in the pristine mangrove is lower than in both MNK (Cyanobacteria 3.5 %, Bacteroidetes 15 %) and MNR (Cyanobacteria 11.4 %, Bacteroidetes 46 %). However, it is quite clear that proportion of phyla in Thuwal correlates more with the proportion of phyla in MNK. Metagenomic data for MM in close in proximity to our locations is scarce, not allowing for in depth comparative studies. However, our results are in general agreement with previous studies for MM regarding dominant microbial groups [69, 83, 126]. Moreover, it has been reported that cyanobacteria occur in and dominate polluted sites by forming a bacterial filled cyanobacterial mat that plays a significant role in hydrocarbon degradation [127, 128]. Also, Acosta-Gonzalez *et al*. [129] characterized the bacterial populations after the

Prestige oil spill at two sampling times (2004 and 2007), and demonstrated that the community structure was initially dominated by Proteobacteria in 2004. However, three years later, in 2007, Acosta-Gonzalez *et al*. found that Bacteroidetes was identified as the dominant phylum. Thus, dominant phyla in RHL are congruent with phyla found to dominate MN that have been exposed to hydrocarbon contamination. Additionally, the analysis showed greater diversity in AKL samples compared to RHL ($p<0.05$), which could be due to the detrimental impact of pollution.

2.3.3 Microbial Abundance Analysis at The Genus Level

A more detailed analysis of the data based on DMAP at the genus level revealed that when comparing MNR and MNK, 12 genera were enriched in one of these samples compared to the other (Table 2.5). Also, when comparing MMR and MMK, 19 genera were enriched in one of these samples compared to the other (Table 2.6).

For the MNR sample we found an enrichment of the Cyanobacterial class Oscillatoriophycideae (genera *Dactylococcopsis* and *Halothece*), while for the MNK sample we found only an enrichment of the Cyanobacterial class Gloeobacteria (genus unclassified) (Table 2.5). MMK samples showed the same enrichment as MNK, while MMR samples also showed enrichment of Cyanobacteria genera from class Oscillatoriophycideae (genera *Microcoleus*, *Coleofasciculus* and *Lyngbya*) when compared to MMK samples.

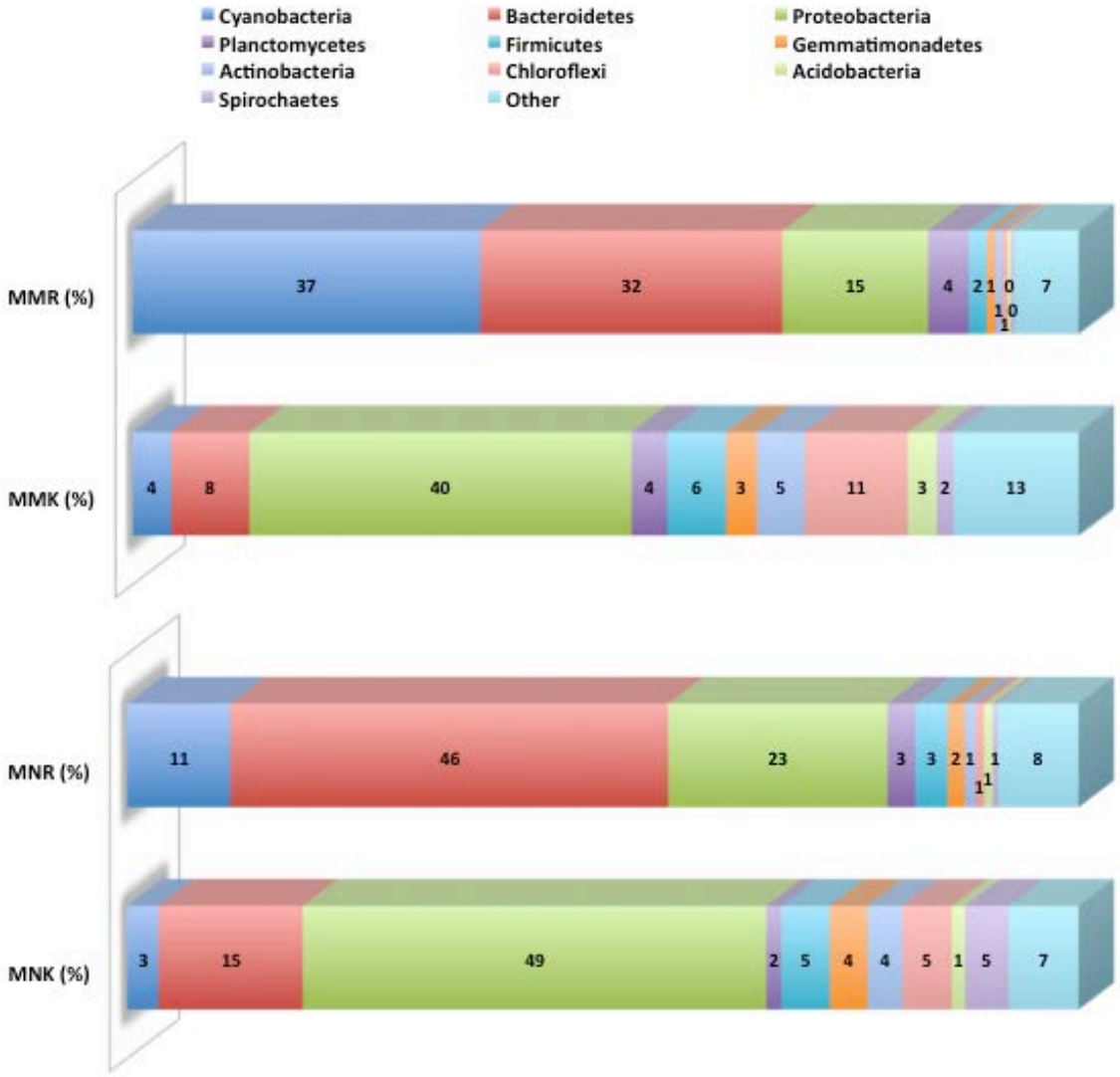

Figure 2.7. Taxonomic distribution of phyla across the 12 samples, averages into four groups: MMR, MMK, MNR and MNK

Studies have reported increased cyanobacterial counts and dramatic shifts in the composition of the cyanobacterial community, induced by exposure to hydrocarbon contamination [127, 128]. Moreover, studies have reported the degradation of aromatic compounds by cyanobacteria, such as *Anabaena cylindrica*, *Phormidium faveolarum* and *Oscillatoria* sp. strain JCM [130, 131]. Cyanobacteria *Microcoleus chthonoplastes* and

Phormidium corium, isolated from oil-contaminated sediments, were also shown to degrade *n*-alkanes [132], as well as *Aphanothece halophyletica, Dactyolococcopsis salina*, *Halothece* strain EPUS, *Oscillatoria* strain OSC, and *Synechocystis* strain UNIGA [133]. Cyanobateria also plays an indirect role in the overall success of the biodegradation process by supplying commensal oil-degrading bacteria, with oxygen produced by photosynthesis, and the fixed N needed for their activity in the degradation processes. The cyanobacteria found enriched in our samples: Gloeobacteria, *Coleofasciculus* and *Halothece* were reported to be abundant in hypersaline microbial mats, but have not been linked to hydrocarbon degradation [134, 135].

The MNR sample showed an enrichment of genera from the class Sphingobacteria (genera *Salinibacter* and *Rhodothermus*), Cytophagia (genera *Pontibacter* and *Fulvivirga*) and Flavobacteria (genus *Flavobacterium*), when compared to the MNK sample. Similarly, the MMR samples also showed an enrichment of several Bacteroidetes genera from the class Cytophagia (genera *Pontibacter, Fulvivirga Marivirga, Cesiribacter and Cyclobacterium*), Flavobacteria (*Nonlabens*), Sphingobacteria (genera *Salinibacter* and *Rhodothermus*) and Bacteroidia (genus unclassified) compared to the MMK sample (see Table 2.6). Both MNK and MMK showed no enrichment of Bacteroidetes genera compared to the corresponding RHL sample.

A recent study reported the core microbiome of two sites, with high hydrocarbon contamination, located along the coast of Italy at the Po River Prodelta (Northern Adriatic Sea) and the Mar Piccolo of Taranto (Ionian Sea) [136]. There, results revealed that the core microbiome include *Clostridia*, *Cytophagia*, *Flavobacteria*, Archaea (within the classes Methanobacteria and Methanomicrobia) and several classes within the phylum

Proteobacteria. Abed *et al*. [137] reported a similar microbiome (Beta-, Gamma- and Deltaproteobacteria, Cytophaga-Flavobacterium-Bacteroides group and Spirochetes) for a cyanobacterial mat, which degraded petroleum compounds at elevated salinity and temperature, sampled from the Saudi Arabian coastline. Abed *et al*. [138] also reported the significant Sphingobacteria counts in oil-contaminated cyanobacterial mat in a constructed wetland. Hemalatha and Veeramanikandan [139] reported the isolation of two *Flavobacterium* species from oil contaminated soil samples. They demonstrated that the optimum temperature for hydrocarbon degradation by these *Flavobacterium* strains were at 40°C. These finding suggest that Bacteroidetes classes, such as Cytophagia, Flavobacteria and Sphingobacteria, are key contributors in the hydrocarbon-degrading microbiome.

Table 2.5. Log Odds Ratio for mangrove (MNK versus MNR) enriches microbial classes and genera.

Phylum	Class	Genus	Log Odds Ratio	P-Value	Q-Value (FDR)
Bacteroidetes	Flavobacteriia	Flavobacterium	-1.11	0	0
Bacteroidetes	Sphingobacteriia	Rhodothermus	-3.074	0	0
Bacteroidetes	Sphingobacteriia	Salinibacter	-4.531	0	0
Bacteroidetes	Cytophagia	Pontibacter	-1.004	0	0
Bacteroidetes	Cytophagia	Fulvivirga	0.129	1.00E-04	1.00E-04
Chloroflexi	Caldilineae	Caldilinea	1.767	0	0
Cyanobacteria	Oscillatoriophycideae	Dactylococcopsis	-4.652	0	0
Cyanobacteria	Oscillatoriophycideae	Halothece	-5.386	0	0
Gemmatimonadetes	Gemmatimonadales	Gemmatimonas	0.654	0	0
Proteobacteria	Deltaproteobacteria	Desulfococcus	3.493	0	0
Proteobacteria	Deltaproteobacteria	Desulfobacterium	3.607	0	0
Spirochaetes	Spirochaetales	Spirochaeta	2.941	0	0

Table 2.6. Log Odds Ratio for microbial mat (MMK versus MMR) enriches microbial classes and genera.

Phylum	Class	Genus	Log Odds Ratio	P-Value	Q-Value (FDR)
Bacteroidetes	Sphingobacteriia	Rhodothermus	-0.832	0	0
Bacteroidetes	Sphingobacteriia	Salinibacter	-0.446	0	0
Bacteroidetes	Cytophagia	Cyclobacterium	-2.019	0	0
Bacteroidetes	Cytophagia	Pontibacter	-1.688	0	0
Bacteroidetes	Cytophagia	Fulvivirga	-2.553	0	0
Bacteroidetes	Cytophagia	Marivirga	-2.786	0	0
Bacteroidetes	Cytophagia	Cesiribacter	-2.551	0	0
Bacteroidetes	Flavobacteriia	Nonlabens	-4.637	0	0
Caldithrix	Caldithrix_unclassified	Caldithrix	1.581	0	0
Chloroflexi	Anaerolineae	Anaerolinea	4.012	0	0
Chloroflexi	Caldilineae	Caldilinea	3.075	0	0
Cyanobacteria	Lyngbya	Lyngbya	-4.411	0	0
Cyanobacteria	Oscillatoriophycideae	Microcoleus	-2.279	0	0
Cyanobacteria	Oscillatoriophycideae	Coleofasciculus	-5	0	0
Planctomycetes	Planctomycetia	Planctomyces	-1.305	0	0
Proteobacteria	Alphaproteobacteria	Azospirillum	-0.909	0	0
Proteobacteria	Alphaproteobacteria	Skermanella	-1.048	0	0
Proteobacteria	Deltaproteobacteria	Desulfococcus	2.955	0	0
Spirochaetes	Spirochaetales	Spirochaeta	2.236	0	0

The MNK showed an enrichment of Proteobacteria genera when compared to the MNR sample, specifically from the class Deltaproteobacteria (*Desulfococcus*, *Desulfobacterium*), followed by a genus from the class Zetaproteobacteria, Betaproteobacteria, Alphaproteobacteria and Gammaproteobacteria (genus unclassified), while the MNR sample showed no enrichment of Proteobacteria genera. MMR samples showed enrichment of Proteobacteria genera when compared to the MMK sample, specifically from the class Alphaproteobacteria (genera *Skermanella* and *Azospirillum*).

Likewise, MMK showed enrichment of Proteobacteria genera when compared to the MMR sample, specifically from the class Deltaproteobacteria (*Desulfococcus*) and followed by a genus from the class Zetaproteobacteria, Betaproteobacteria and Gammaproteobacteria (genus unclassified).

The Deepwater Horizon (DWH) oil spill (2010) in which ~4.1 million barrels of oil were released into the Gulf of Mexico resulted in a deep-sea hydrocarbon plume that caused a shift in the indigenous microbiome. Several studies reported that oil-degrading Gamma- and Deltaproteobacteria dominated the deep-sea plume [140-144].

Interestingly, despite observing a significantly decreased proportion of Firmicutes in RHL samples compared to AKL samples, genera of the class Bacilli (*Bacillus*) and Clostridia (*Clostridium*) are enriched in the MNR sample compared to the MNK sample. There are several reports demonstrating the hydrocarbon degrading ability of *Bacillus* strains [145, 146]. One study in particular, demonstrated that the *Bacillus* sp. strain DHT, isolated from oil contaminated soil, has the ability to grow when cultured in the presence of a variety of hydrocarbons, including crude oil, hexadecane, pyrene, dibenzothiophene, diesel oil, salicylate, naphthalene, catechol, and phenanthrene as the sole sources of carbon (in 0–10% salinity and at 30–45°C) and it produced biosurfactant [147]. In the same report, no growth was observed when culturing was done on toluene, phenol, 2-hydroxyquinoline and carbazole.

2.3.4 Functional Analysis of Mangrove and Microbial Mat Samples

For functional analysis, sample sites were combined for metagenome assembly using the de-novo assembler – MegaHit [99]. Functional Analysis was primarily focused on; 1/

"Metabolism of Aromatic Compounds" to provide more insight into the possibility that these sites were historically exposed to hydrocarbon contamination (BTEX assessment at MN sites showed that both MNK and MNR sites displayed Toluene, Ethylbenzene and Xylene contamination at very low concentrations), and 2/ enzymes associated with the "Production of bioactive secondary metabolites" to pinpoint the location and sediment type that shows the most promise for successful antimicrobial bioprospecting.

2.3.4.1 Metabolism of Aromatic Compounds

The commonly reported pollutants in the Red Sea [148] are oil spills from ships that use the Suez Canal, and from local oilfields. However, a caveat associated with our study is that the BTEX analysis was only performed after metagenomic sequencing revealed a shift in dominant microbial phyla that was consistent with hydrocarbon contamination. Thus, we used pathway enzyme hits associated with "Aromatic Compound Degradation" from MetaCyc and "Xenobiotics biodegradation" from KEGG, to find further support of the hydrocarbon contamination. The metagenomic data reveals that "Aromatic Compound Degradation" and "Xenobiotics biodegradation" are significantly enriched in AKL samples compared to RHL samples (see Table S2.2).

MMK was shown to be the most enriched sample followed by MNK, MNR and MMR. Moreover, the increase in AKL samples is primarily due to increased "anaerobic aromatic compound degradation (Thauera aromatica)" and Benzoate degradation (see Table S2.2). Also, "Styrene degradation" is significantly enriched in the MMK sample compared to MMR. Other aromatic compound degradations were also observed such as Toluene degradation, Ethylbenzene degradation and Xylene degradation. These findings make

sense as anaerobic hydrocarbon-degrading Deltaproteobacteria were shown to be enriched in AKL samples.

Moreover, the metagenomic data associated with “Aromatic Compound Degradation” are in general agreement with our BTEX results as it shows Toluene, Ethylbenzene and Xylene degradation, but with the exception of Benzene degradation.

2.3.4.2 Production of Bioactive Secondary Metabolites

Polyketide synthases (PKS) and non-ribosomal peptide synthetase (NRPS) are enzymes known to synthesize bioactive secondary metabolites with commercial importance such as antibiotics, siderophores, cytostatics, toxins and pigments [70, 149]. We assessed the percentage of sequences that matched: 1/ PKS and NRPS enzymes, 2/ enzymes involved in antibiotic synthesis and antibiotic resistance pathways. Table 2.7 show the percentage of enzymes associated with PKS, NRPS, antibiotic synthesis and antibiotic resistance, in all samples with amino acid sequence alignment score of > 45 %.

The MMR sample displayed a higher percentage of PKS and NRPS matches compared to MMK. Similarly, MNR showed higher PKS matches than MNK, but MNK showed higher NRPS matches than MNR. Also, for all four samples, the percentage of enzymes associated with antibiotic resistance pathways is significantly higher compared to the PKS, NRPS, and antibiotic synthesis enzymes. The high abundance of antibiotic resistance enzymes in all samples, suggests both locations are likely holding high quantities of antibiotic. Both MNR and MMR (RHL samples) showed a higher percentage of enzymes associated with antibiotic synthesis than both MNK and MMK

(AKL samples), respectively. However, when considering sediment type, MN samples showed a higher percentage of enzymes associated with antibiotic synthesis than the MM samples. The complete list of enzymes for each of the four samples is given in Table S2.3.

Table 2.7. Higher sequence identities (> 45%) enzymes associated with PKS, NRPS, antibiotic synthesis and antibiotic resistance.

	% Antibiotic synthesis	% Antibiotic resistance	% PKS	% NRPS
MMK (n=30)	3.33%	83.33%	10.00%	3.33%
MMR (n=140)	5.00%	70.71%	17.86%	7.86%
MNK (n=346)	9.25%	71.68%	12.72%	6.94%
MNR (n=209)	10.05%	67.94%	13.40%	9.09%

The results describe in Table 2.7, 2.8 and 2.9 show a high microbial species diversity. For example, in Table 2.8, we observed 243 sequences that were found to be associated with antibiotic synthesis enzymes derived from 45 species. Similarly, in Table 2.7, we observed ~13% or 28 of MMR enzymes from Cyanobacteria sequences for PKS being derived from 13 Cyanobacteria species (see Table S2.4 and S2.5).

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We observed that Bacteroidetes, Proteobacteria, Cyanobacteria and Firmicutes are commonly dominant bacteria in both MN and MM samples, and also the key antibiotic producers in our samples (see Table 2.8-2.9). This does not mean that Bacteroidetes, Proteobacteria, Cyanobacteria and Firmicutes are better target phyla for antimicrobial bioprospecting than the currently preferred phylum, Actinobacteria, in general. Thus, we further determined the odds of antibiotic biosynthesis activity occurring in the phyla of interest compared to the odds of antibiotic biosynthesis activity occurring in Actinobacteria, represented as the odds ratio in Table 2.10. The odds ratio was

determined for MNK, MNR, Thuwal – mangrove (MNT) [77], Lake Washington - Freshwater lake (LWF) [150] and Lake Tyrell - Hypersaline lake (LTHS) [151].

We observe that locations associated with exposure to hydrocarbon contamination (MNK, MNR and LWF) show a higher abundance of phyla that should be targeted for antimicrobial bioprospecting, while locations that are considered pristine (MNT and LTHS) only point to the Firmicutes and the commonly targeted Actinobacteria phyla. Interestingly, Firmicutes is appearing in all locations as a phylum that should be targeted and show consistently higher odds for successful antimicrobial bioprospecting than Antinobacteria. This data suggest that hydrocarbon contaminated environments yield more phyla (Bacteroidetes, Proteobacteria, Cyanobacteria and Firmicutes) that could be targeted for antibiotic bioprospecting and that Firmicutes is likely a phylum that should be targeted in all environments.

Interestingly, the sequences associated with antibiotic synthesis enzymes from Proteobacteria are primarily derived from Gamma-, Delta- and Alpha-proteobacteria, while those from Firmicutes are primarily derived from Bacilli and Clostridia (see Table 2.11). Here it should be noted that Mason *et al.* [152] reported that the sediment microbiomes' responses to the DWH oil spill showed surface sediment layers were enriched with uncultured Gamma-proteobacteria, similar to previous observations in the deep-sea hydrocarbon plume. Kimes *et al.* [153] also used metagenomics to evaluate deeper layers (1.5–3 cm below seafloor) of the same sediment and reported an enrichment of anaerobic hydrocarbon-degrading Delta-proteobacteria. Additionally, Acosta-Gonzalez *et al.* [129] reported that the Prestige oil spill was dominated by Proteobacteria in 2004, that primarily comprised of Gamma- and Delta-proteobacteria, before the

bacterial community shift to Bacteroidetes as the dominant phylum. Thus, our findings not only show an identical shift in phyla associated with the hydrocarbon contamination detected (that is, enrichment of Gamma- and Delta-proteobacteria), but also report that sequences associated with antibiotic synthesis enzymes for Proteobacteria are primarily derived from the same classes Gamma-, Delta- and Alpha-proteobacteria, suggesting that selection pressure associated with hydrocarbon-contamination enrich the bacterial classes with sequences associated with antibiotic synthesis enzymes.

Table 2.8. Metagenomic contigs associated with enzymes involved in antibiotic synthesis and resistance and the phyla from which they are derived.

	Number of sequences associated with antibiotic synthesis enzymes				Number of sequences associated with antibiotic resistance			
	MMK	MMR	MNK	MNR	MMK	MMR	MNK	MNR
Proteobacteria	11	13	243	37	27	40	928	130
Bacteroidetes	2	61	54	99	7	325	259	431
Acidobacteria	5	1	9	0	19	2	40	1
Firmicutes	4	3	39	6	10	11	92	30
Cyanobacteria	0	26	35	54	4	128	68	167
Chloroflexi	3	3	29	3	14	1	126	10
Gemmatimonadetes	0	0	20	5	3	3	129	24
Actinobacteria	2	0	18	1	1	0	43	10
Planctomycetes	0	51	5	2	3	134	23	9

Table 2.9. Metagenomic contigs associated with PKS and NRPS enzymes and the phyla from which they are derived.

Phyla	PKS				NRPS			
	MMK	MMR	MNK	MNR	MMK	MMR	MNK	MNR
Proteobacteria	9	14	498	58	6	12	194	41
Bacteriodetes	1	112	88	143	1	53	45	101
Acidobacteria	6	2	15	4	6	0	2	2

Firmicutes	1	2	54	16	2	0	20	4
Cyanobacteria	9	58	24	55	1	33	19	45
Chloroflexi	5	1	39	5	4	1	21	2
Gemmatimonadetes	0	0	20	3	1	2	13	9
Actinobacteria	4	1	38	5	1	1	18	1
Planctomycetes	0	75	11	6	2	32	5	2

Table 2.10. Odds ratio of different phyla of interest in different locations.

Location	Phyla	Type	Count	Odds ratio
MNK (Hydrocarbon contamination exposure)	Proteobacteria	#Enzyme	243	1.0
		#Contigs	24003	
	Bacteroidetes	#Enzyme	54	0.7
		#Contigs	8241	
	Firmicutes	#Enzyme	39	2.0
		#Contigs	1895	
	Cyanobacteria	#Enzyme	35	2.7
		#Contigs	1306	
	Actinobacteria	#Enzyme	18	1.0
		#Contigs	1787	
MNR (Hydrocarbon contamination exposure)	Proteobacteria	#Enzyme	37	1.2
		#Contigs	13382	
	Bacteroidetes	#Enzyme	99	1.7
		#Contigs	24794	
	Firmicutes	#Enzyme	6	1.9
		#Contigs	1300	
	Cyanobacteria	#Enzyme	54	6.7
		#Contigs	3383	
	Actinobacteria	#Enzyme	1	1.0
		#Contigs	420	
Thuwal (Considered pristine)	Proteobacteria	#Enzyme	73	0.8
		#Contigs	1756	
	Bacteroidetes	#Enzyme	3	0.8
		#Contigs	72	
	Firmicutes	#Enzyme	8	1.3
		#Contigs	128	
	Cyanobacteria	#Enzyme	1	0.5
		#Contigs	42	
	Actinobacteria	#Enzyme	2	1.0

		#Contigs	40	
Lake Washington - Freshwater lake (Hydrocarbon contamination exposure)	Proteobacteria	#Enzyme	3	0.5
		#Contigs	127	
	Bacteroidetes	#Enzyme	70	1.7
		#Contigs	804	
	Firmicutes	#Enzyme	9	2.3
		#Contigs	77	
	Cyanobacteria	#Enzyme	3	1.3
		#Contigs	46	
	Actinobacteria	#Enzyme	2	1.0
		#Contigs	40	
Lake Tyrell - Hypersaline lake (Considered pristine)	Proteobacteria	#Enzyme	2005	0.8
		#Contigs	24110	
	Bacteroidetes	#Enzyme	34	0.5
		#Contigs	649	
	Firmicutes	#Enzyme	57	1.0
		#Contigs	552	
	Cyanobacteria	#Enzyme	42	0.8
		#Contigs	474	
	Actinobacteria	#Enzyme	130	1.0
		#Contigs	1244	

Table 2.11. Metagenomic contigs associated with enzymes involved in antibiotic synthesis and the taxonomic class from which they are derived. The numbers in table that are not representing percentages, show the number of enzymes involved in antibiotic synthesis. The percentages give the proportions of enzymes per taxonomic class**.**

	MMK	MMR	MNK	MNR
Proteobacteria	**11**	**13**	**243**	**37**
• Gamma-proteobacteria	36%	69%	23%	59%
• Delta-proteobacteria	18%	23%	31%	27%
• Alpha-proteobacteria	27%	8%	39%	14%
Bacteriodetes	**2**	**61**	**54**	**99**
• Cythophagia	50%	34%	44%	13%
• Flavobacteria	0%	48%	24%	21%
Cyanobacteria	**0**	**26**	**35**	**5**
• Nostocales	0%	31%	26%	20%
• Oscillatoriophycideae	0%	62%	66%	80%
Firmicutes	**4**	**3**	**39**	**6**
• Bacilli	75%	67%	44%	50%

• Clostridia	25%	33%	51%	50%

2.4 Conclusion

Our study is the first to compare Red Sea lagoon microbiomes in terms of diversity, taxonomy and function. Overall, our results suggest that the mangrove environment is superior in terms of species diversity and taxonomical abundance compared to microbial mat from salt marsh. Furthermore, we have shown that similar sediments in the two Red Sea lagoons are not homogeneous; each particular locality exhibits its own unique taxonomical abundance and biological pathways. Specifically, we show a shift in dominant phyla consistent with a historical exposure to hydrocarbon contamination and that AKL unexpectedly displayed more contamination than RHL. This may be due to dominant phyla in AKL being consistent with early hydrocarbon exposure (when contamination levels are still high) and the dominant phyla in RHL being consistent with late hydrocarbon exposure (when contamination levels are lower as a result of an extended period of hydrocarbon degradation). Additionally, RHL samples showed higher counts for PKS and NRPS matches in comparison to AKL samples, even though differences were minimal. However, both MNR and MMR (RHL samples) showed a higher percentage of enzymes associated with antibiotic synthesis than both MNK and MMK (AKL samples), respectively. In addition, when considering sediment type alone, MN samples showed a higher percentage of enzymes associated with antibiotic synthesis than MM samples. Additionally, diversity results showed mangrove samples are more diverse compared to microbial mat samples. Thus, we conclude, RHL is the better location with an increased probability of successful antimicrobial bioprospecting, while the best sediment type in RHL for this purpose is MN. Additionally, even though

Actinobacteria tends to be the common target for antimicrobial bioprospecting, our study suggest that Firmicutes (Bacilli and Clostridia) should be a target plylum for antimicrobial bioprospecting in all locations, while in hydrocarbon contaminated sites Bacteroidetes, Proteobacteria, Cyanobacteria and Firmicutes frequently appear to be better targets than Actinobacteria. Moreover, phyla that should be targeted are increased at site exposed to hydrocarbon contamination. Thus, our study suggests that is will be beneficial to use metagenomics as a preliminary screen to comprehensively identify target phyla for this type of bioprospecting.

Next, we will use traditional culturing methods to establish a bacterial library that can be screened for the *in silico* detected antimicrobial effects, and hopefully provide further evidence that Bacteroidetes, Proteobacteria, Cyanobacteria and Firmicutes should be targeted for antimicrobial bioprospecting in addition to Actinobacteria.

Chapter 3

3 Establishing a bacterial library of targeted phyla.

3.1 Introduction

In the late 1800 Koch and coworkers innovatively pioneered the growth and maintenance of pure cultures isolated from environmental samples using flasks, petri dishes and solid agar. This culture technique is in principle still being used today. Nonetheless, it is now known that only a small proportion of bacteria are cultivated *in vitro,* while the majority of bacteria are yet to be grown on artificial media using standard methods. Signifying that key organisms are likely being overlooked. Consequently, there has been a move towards the development of molecular culture-independent techniques. Despite which culture-dependent analyses are not redundant as pure isolates in pure media are required for characterization of physiological properties and virulence potential.

There are several factors that makes culturing bacteria difficult including low prevalence, slow growth rates and fastidious growth requirements (such as specific nutrients, pH conditions, incubation temperatures or oxygen levels) [154]. Thus if these specific requirements are not met, competition for nutrients exist or bacteriocins or antimicribial agents are released, some bacteria may not grow [155].

To increase the recovery of “unculturables”, several methods are being used including extended incubation times [156, 157] filtration [158], density-gradient centrifugation or elutriation and extinction-dilution [157, 159, 160], cocultivation [161, 162], simulating their natural environment [162, 163], using water from the natural environment for

media preparation [157, 164]. The culture media used to date has also been scrutinized and dubbed nutrient-rich, a condition that favors faster-growing bacteria, as slow-growing bacteria was shown to thrive with the use of use of dilute nutrient media [164-166].

The metagenomic study (chapter 2) provided directive of the bacterial phyla (Bacteroidetes, Proteobacteria, Firmicutes and Actinobacteria) that should be cultivated as a target for antimicrobial bioprospecting. Cyanobacteria was excluded as its cultivation differs from the other bacteria, that is, cyanobacteria are sensitive to standard microbiological agar or don't grow well on agar that is too dry and take 14 days of incubation, which is long period comparing to other bacteria [167]. Based on the select phyla, identified 5 different types of media known to grow the phyla of interest. Media used include Difco Marine agar 2216 (MA) (BD Difco^{TM}, USA) for cultivating heterotrophic bacteria [168]. This media usually gives higher colony-forming unit (CFU) than other enriched agar media [169]. Other study shows that the greatest bacterial morphotypes diversity was seen on plates of MA [168]. We also used 10% MA for cultivating oligotrophic bacteria, Anti-MA for bacteria that are resistant to antibiotic, and MB-GM for bacteria that do not prefer agar. AIA for cultivating Actinobacteria,

Overall this chapter demonstrates the diversity and cultivability of microbes from from three different sediments types: MN, MM, and BS located in AKL and RHL. To date there are no microbiological studies of such environments, and data on nutrient cycling and eutrophication levels are limited.

3.2 Materials and Methods

3.2.1 Media Preparation

The isolation of microbes in the laboratory normally allows us to capture only 1% of the microbial community found in most environments [170]. This is because of the large number of strains that resist growth on solid medium, and the difficulties of replicating real life environments in the lab. The use of advanced methodologies in culturing will improve the number of cultured genera. For example, the use of unusual solidifying agents such as gellan gum instead of agar, liquid and dilution media, have established a new isolation and a variation in the collection of isolations, and may also isolate rare species [166, 171, 172]. Isolation experiments employ liquid and solid media (with agar and gellan gum). Also, samples were subjected to heating and drying, to inhibit the growth of Gram-negative bacteria and allow the growth of Gram-positive bacteria. The types of media that were used are described in the following sections.

3.2.2 Antibiotic Preparation

A 1g/L final concentration stock solution of streptomycin and a 0.2g/L stock solution of ampicillin were prepared in ddH_2O, sterilized by filtration and kept at 4°C until used. Anti- BD Difco™ Marine Broth 2216 was prepared with a 0.002g/L of streptomycin and 0.004g/L ampicillin [173] and kept at 4 °C.

3.2.3 Antifungal Agent

Cycloheximide (50 mg/L) was added to the media, the streptomycin (0.002g/L) and ampicillin (0.004g/L) were first mixed in a beaker and the cycloheximide powder was dissolved as completely as possible in this stock solution before incorporation to the rest of the broth [173].

3.2.4 Enrichment Broth

Cultivation in selective broths was initiated for two purposes, first as an intermediate step to introduce a limiting step (heat) to enrich the isolation of Gram-positive bacteria (Firmicutes and Actinobacteria) and second as a media to isolate planktonic microbes, the growth of which is inhibited in culture media with agar as a solidifying agent. Another approach was to use antibiotics to investigate the cultivability of members that are resistant to antibiotic such as archaeal domain, and other bacteria like Planctomycetes.

3.2.4.1 Actinomyces Broth Media (BD Difco™ Actinomycete Isolation)

Of the Actinomyces broth media [174], 57g of Actinomyces broth powder were dissolved in 1L of double distilled (dd) H_2O, and was mixed using a magnetic stirrer bar at 180 °C until the media became clear. The stir bar was removed with a sterile magnetic stir rod, the beaker containing the broth was autoclaved for 10 minutes at 121°C, 50mg/L cycloheximide was added to enrichment broth, and was then stored at 4°C.

3.2.4.2 Difco Marine Broth 2216 (MB)(BD Difco™ Marine Broth 2216)

Of the Difco Marine Broth 2216 [175], 37g of Difco Marine Broth 2216 powder was suspended in 1L of dd H_2O; mixed using a magnetic stir bar under a heat source (heating block) at 180°C until completely dissolved. Then autoclaved the media for 15 minutes at 121°C. Once cooled to 55°C, 50mg/L of cycloheximide was added and then stored at 4°C.

3.2.4.3 Difco Marine Broth 2216 Plus Antibiotics (Anti-MB)

Difco Marine Broth 2216 was supplemented with antibiotics. After making MB as in section (3.2.4.2), streptomycin (1g/L) and ampicillin (0.2g/L) stock solutions were added along with 50mg/L cycloheximide to enrichment broths. All broth solutions were stored at 4°C.

3.2.4.4 1/10 Difco Marine Broth 2216

Of the Difco Marine Broth 2216 [175], 1/10 was prepared by mixing 3.74g Difco Marine Broth 2216 powder with 500 ml of dd H_2O and 500ml of sterilized Red Sea water (SRSW) (was collected and autoclaved) to adjust for loss of salt concentration in the full strength Difco 2216 media. Using a magnetic stir bar and heating block, the solution was heated at 180°C until clear, then autoclaved for 15 minutes at 121°C and. Once cooled to 55°C, 50mg/L of cycloheximide was added and then stored at 4°C.

3.2.5 Solid Media

Solid media allows for the macroscopic visualization of microbes as colonies while permitting the separation and identification of single microbes from complex mixed cultures or environmental samples containing multiple types of microbes.

Solid media was prepared by adding 18g of agar or gellan gum. Agar media allows the selection of various microbes. On the other hand, some studies show that gellan gum is more successful with isolated microbes that are difficult to culture [156].

3.2.5.1 Actinomycete Isolation Agar (AIA)

Of the Actinomycete isolation agar (BD Difco™, USA) [174], 22g of the powder media was suspended in 1L of dd H_2O, with 18 g of granulated agar [176] added as a solidifying agent, and placed on heating blocks at 180°C until the media was completely dissolved. Then, 1ml of 99% glycerol was added, and autoclaved at 121 °C for 15 minutes. Once cooled to 55°C, 50mg/L of cycloheximide was added. The media was poured into 8cm Petri dishes, approximately 30ml of media per dish, and then stored at 4°C.

3.2.5.2 Difco Marine 2216 Agar (MA)

Of the Difco marine broth 2216 powder [175], 37g was suspended in 1L of dd H_2O and then mixed using a magnetic stir bar. Afterwards, 18g of granulated agar was added [176] as a solidifying agent. The media was heated on a heating block at 180°C until the agar was completely dissolved. It was then autoclaved at 121°C for 15 minutes. After cooling to 55-60°C, cycloheximide was added to a final concentration of 50mg/L. The media was

then poured into 8cm Petri dishes, approximately 30ml of media per dish, and then stored at 4°C.

3.2.5.3 Difco Marine 2216 Agar plus antibiotics (Anti-MA)

The procedure was the same as the one described for Difco marine agar 2216 (section 3.2.5.2), with the addition of streptomycin (1 g/L) and ampicillin (0.2g/L) along with cycloheximide added to the final concentration of 50 mg/L

3.2.5.4 1/10 Difco Marine 2216 Agar (10% MA)

Of the Difco Marine 2216 agar, 10% was prepared by mixing 3.74g of MA powder [175] with 500 ml of dd H_2O and 500 ml of SRSW [176]. Using a magnetic stir bar and heating block, the solution was heated at 180°C until clear. During the heating 18g of Agar was added gradually until completely dissolved. The stir bar was removed with a sterile magnetic stir rod, and the beaker containing the media was autoclaved for 15 minutes at 121°C. Plates were kept at 4°C.

3.2.5.5 Difco Marine Broth 2216 Gellan media (MB-GM)

Difco Marine Broth 2216 with gellan gum (MB-GM) plates were prepared as described for Difco Marine Broth 2216. A gellan gum based media was prepared by adding 6g/L of gellan gum and 1g/L of $CaCl_{2*}6H_2O$ (to assist in dissolving) in the prepared media.

After autoclave (121°C, 10 min), the prepared media cooled to 55°C, the cycloheximide was then added to the final concentration of 50 mg/L. The media was poured into 8 cm Petri dishes, approximately 30 ml of media per dish, and then stored it at 4°C.

3.2.6 Samples Processing

As soon as the samples arrived, MM was washed with seawater to remove or separate any mud from the microbial layer. Eight grams of wet weight sediment were homogenized at low speed with 10 ml of sterilised Red Sea water, The supernatant (100 µl) was diluted 5 and 25-fold and subsequently plated out on different media. Processing was the same for all samples.

3.2.6.1 Wet Processing- Direct

- Enrichment

From each of the three types of sediments samples – MM, MN, and BS – the process was done only in the primary (5^0) step. The mixture (sediment + SRSW) was incubated in a 56-60°C water bath for 20-30 min, then 1ml of the mixture was placed directly into a 100ml flask for enrichment in MB. Flasks were placed on shaking incubators at 28°C and 220 rpm for 3-7 days, until a biofilm could be seen surrounding the inside walls of the flasks.

- Plating

From each concentration (undiluted, $^1/_5$ dilution, and $^1/_{25}$ dilution) 100 µl were taken from all three sediments samples and spread across the following plates: AIA, anti-MA, 1/10

MA, MA and MB-GM. Samples were spread using L-shaped cell spreaders under the laminar flow hood. Plates were incubated upside down to prevent contamination through condensation, at 28 °C from 5-28 days.

3.2.6.2 Airdry Processing- Indirect

Samples were dried under the fume hood for several weeks. The same process was employed as the wet processing, as far as enrichment and plating for all three types of sediments. Two grams from each sample were added to 5ml of SRSW and mixed well. From the mixture, 1ml was placed directly into a 100ml flask for enrichment in MB. The process will be completed as described in the wet processing enrichment.

3.2.7 Isolation and Purification Procedures

Microbial growth in solid media was detected by the presence of different colonies on plates after several days (3-28days) of incubation. Single colonies were picked with sterilized inoculating loops and transferred to individual new plates of the same media. Re-streaked colonies were done using a four-quadrant streak and the above steps continued until the axenic colonies were achieved.

In cultivation experiments in liquid media, microbial growth was detected by the visible turbidity of the broth and biofilm formation in the flask's wall after 1-3 weeks incubation. These indicated that bacteria were growing and thus the enrichment broths were ready for culture and isolation into solid media. A hundred μl of broth with signs of bacterial growth was transferred with pipette into a new solid media of the same type of broth. The solution was spread using L-shaped cell spreaders. Also, biofilm was collected with a cotton swab

and mixed in with 1ml of SRSW. A hundred µl of the obtained biofilm mixture was spread onto new plates using L-shaped cell spreaders. After a few days of incubation at 28°C, colonies were observed and transferred to new plates for purification until all colonies have the same morphology in all plates.

3.2.8 Microbial Strains Storage

Colonies of purified microbial strains were picked using loops, placed with 0.5 ml of dd H_2O (for DNA extraction and amplification) and 1ml of glycerol solution (30%) into 1.5 ml of screw cap tubes (For long term storage). They were stored at -80°C until all isolates were ready for DNA extraction.

3.2.9 Macroscopic Morphology and Staining

The pure colonies were examined macroscopically, valuing their characteristics such as size, elevation, color, shape, surface and texture. Staining of bacteria strains, such as Methylene blue was done to get a general idea about the bacteria's cellular shape.

3.2.10 SPSS Analysis

This analysis was performed using SPSS software v.13.0 (SPSS Inc., Chicago, IL, USA). Data was processed using squared Euclidian distance and Ward method from SPSS.

3.3 Results and Discussion

3.3.1 Bacterial Isolation and Morphology

As mentioned above, five different types of media (AIA, MA, 10% MA, anti-MA and MB-GM) were used to isolate the phyla of interest [174, 175]. Since it is now commonly known that bacteria from environmental ecosystems are largely uncultured [177], a combination of several methods was implemented to increase recovery of so called “unculturables”. These methods include extended incubation times, heat shock, air-dry processing, using serial dilution, antibiotics, sterilized Red Sea water and both liquid and solid media. Here we also used diluted nutrient media (10% MA) in which slow-growing bacteria were shown to thrive [164-166], and gellan gum for bacteria that do not prefer agar.

Overall inoculum was taken directly from the soil sample, heat-shocked samples, air-dried sediment liquid media and biofilm (build-up on the wall of the flask) for streaking on the solid media. No significant morphological differences were observed for bacterial growth between these different methods used.

However, bacterial growth on plates that contained antibiotics (Anti-MA) was generally very low, with morphological differences compared with non-antibiotic plates. The colonies on the antibiotic plates were observed to be more convex and raised, glossy, homogeneous, and pigmented with colors including creamy, peach, black, or orange. It was most apparent that bacteria on plates without antibiotics grew significantly faster than those with antibiotic.

Overall, growth on both MA and 10% MA plates seemed very similar with high bacterial density, and exhibited a range of colony shapes and the colors. Colony counts and

morphology was less diverse on Anti-MA and AIA. While inoculum from the serial dilutions allowed for differences to be seen between colonies on 10% MA plates that were not observed on MA. Additionally a decrease in the number and size of colonies was observed with increased dilutions as expected. This may likely be as a consequence of the more oligotrophic media providing an opportunity for the slower-growing bacteria to compete, similar to the oligotrophic media improving the culturability of SAR11 strains [157, 164].

Moreover, the bacterial growth on MB-GM was faster than on any of the agar-based media. This finding is not surprising as several studies have shown MB-GM to be more successful in isolating bacterial species from marine environments [156] and may isolate specifically rare Actinomycetes [171, 172].

Isolates were selected for propagation based on morphological distinctiveness (Table S3.1). MB-GM produced the highest recovery with 72 isolates (29%), followed by Difco marine 2216 agar (61 isolates =24.3%), 10% MA (57 isolates- 23%), AIA (56 isolates = 22.3%), and Anti-MA (6 isolates =2.4%). Even though isolate counts are relatively similar from all media except Anti-MA, more morphological differences were observed for isolates acquired from the dilutes media (10% MA) and AIA.

3.3.2 SPSS Data Analysis

Consequently, isolate morphology was used for a preliminary analysis via SPSS software v.13.0 (SPSS Inc., Chicago, IL, USA) to determine if bacterial diversity is sufficient for evaluating phyla suitable of bioprospecting. Here, SPSS software v.13.0 was used for comparison of the 251 isolates morphology (color, shape, size, surface, elevation and

texture), media types used to isolate the bacteria, sediments types and locations of selected samples. Based on these features, dendogram in Figure 3.1 clear show that the isolated form five major clusters.

These five major clusters include cluster A with 100 strains, cluster B with 73 strains, cluster C with 13 strains, cluster D with 49 and cluster E with 17 (Figure 3.1). Cluster A (Figure 3.1), with the highest population number, morphology characteristics were primarily smooth, moist, small or micro size, 85% were circular, 63% were white in color and half of the population were convex while the other half were flat.

For colonies in population B (Figure 3.1), 88% creamy and beige (Figure 3.2, 1a,b) while the rest were orange (Figure 3.2, 2a,b) and light brown (Figure 3.2, 3a,b). Colonies were further mostly, circular, small to micro sized, convex, moist and smooth surfaced.

Population C (Figure 3.1) is minus population B, this population' colonies displayed a mixture of colors including peach, creamy, black (Figure 3.2, 4a,b), light pink (Figure 3.2, 5a,b), white (Figure 3.2, 6a,b), and yellow (Figure 3.2, 7a,b). Most of the sizes were large, smooth, moist, and between irregular to circular shaped. While in population D (Figure 3.1), colony colors were brighter including yellow, pink and orange. Most of this group had smooth surfaces and were circular with few being rhizoid and irregularly shaped. Here more colonies were dry textured instead of moist. Population E (Figure 3.1) was primarily dry textured, flat, creamy or pink and had a combination of shapes including rhizoid, irregular (Figure 3.2, 8a,b), filamentous and circle. None of the clusters were significantly higher in types of media, sediment types and locations. Random isolates were selected to view cells shape under the microscope (Figure 3.3).

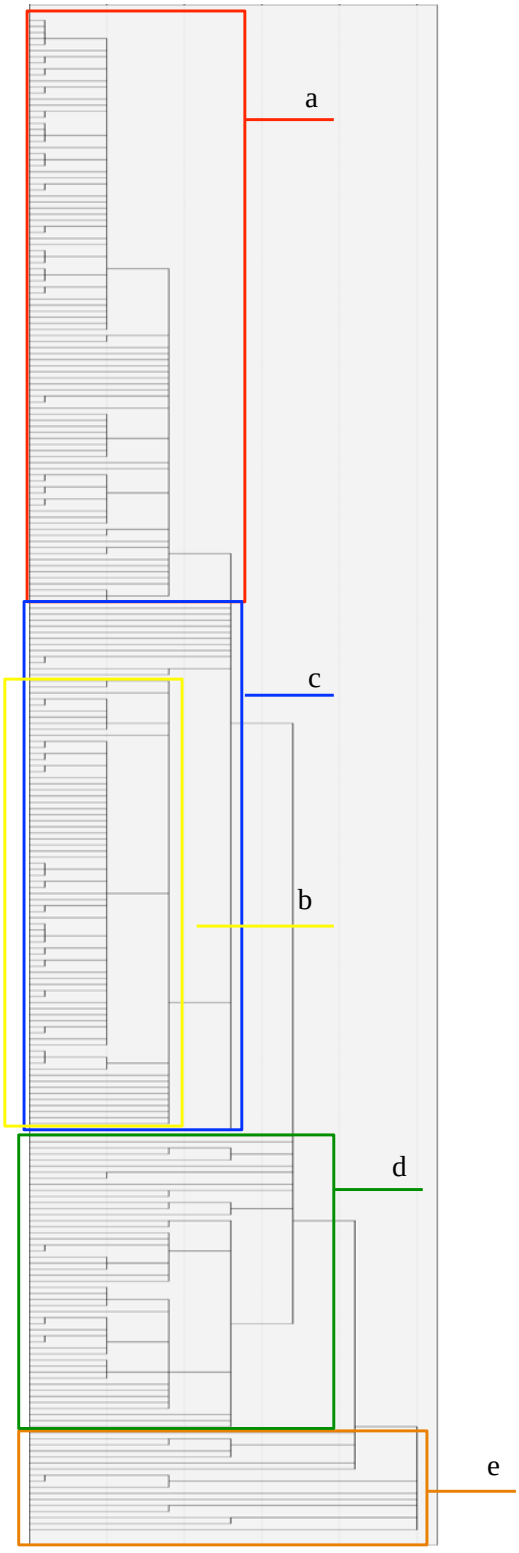

Figure 3.1. Dendogram of all data obtained for colonies morphology. Data was processed using squared Euclidian distance and Ward method from SPSS.

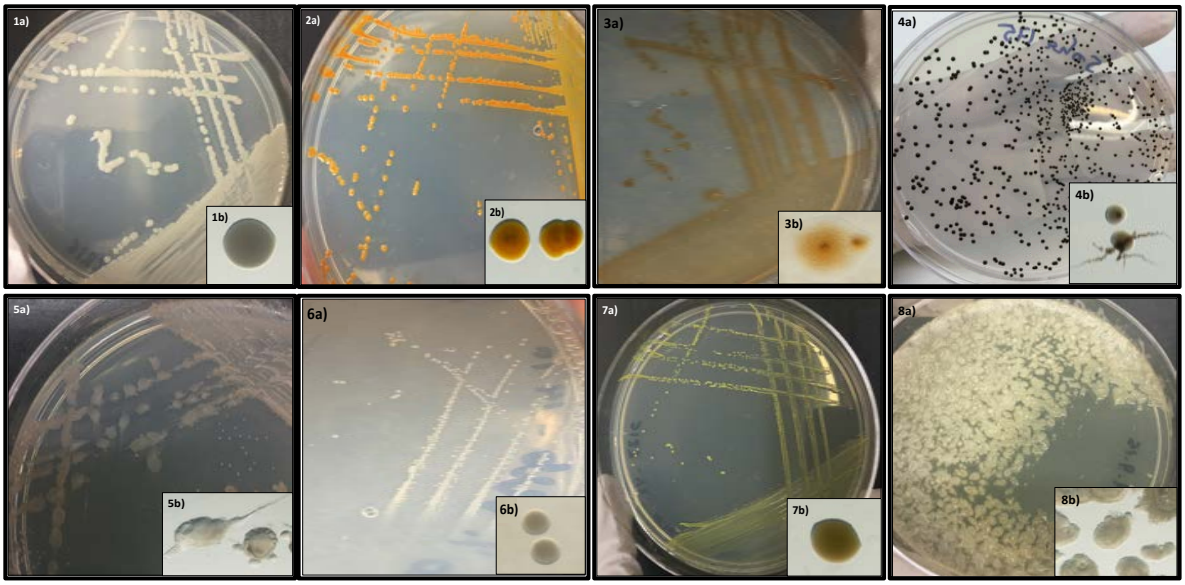

Figure 3.2. Representation of the different morphology of bacteria strain observed that grown in MA2216 that incubate at 30°C in the dark. They grown in 2 days except figure 4 and 8 they took 7 days. 1a. Bac 371, 2a. Bac 194, 3a. Bac 261, 4a. Bac 175, 5a. Bac 248, 6a. Bac 242, 7a. Bac 42, 8a) Bac 90.

b) Stereomicroscope image, magnification 1.6X.

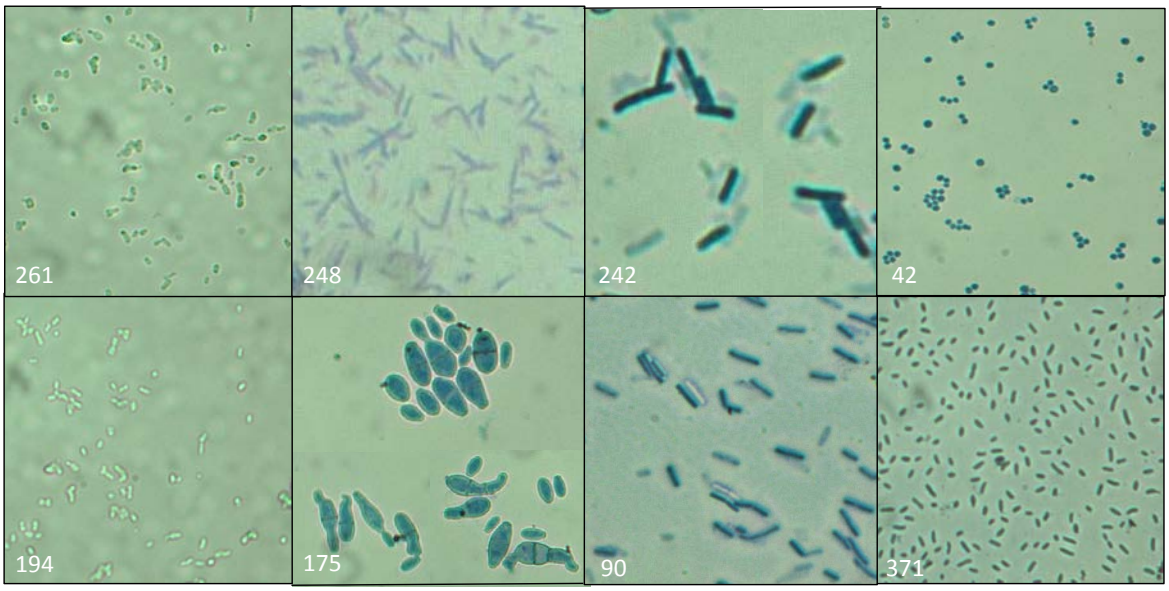

Figure 3.3. Random isolates was selected using Methylene blue stain to show cells shape under the microscope. Magnification of 1000x. The numbers in white represent the assigned bacterial ID.

3.4 Conclusion

Here, a bacterial library including 251 isolates has been successfully established. Cultured isolates display rich diversity based on colony morphology. Moreover, placing the colonies into five separate clusters via SPSS analysis may suggest the possibility of five different phyla. Additionally, according to the SPSS data, no significance has been associated sediment type and location.

Chapter 4

4 Bacterial Identification Based on 16S Ribosomal RNA Gene Sequencing Analysis

4.1 Introduction

Taxonomy is a part of biological science that includes classification, nomenclature and identification. This is applied to all-living things, to classify, name and identify organisms. For bacterial taxonomy and phylogeny studies, DNA-DNA hybridization is undeniably the "gold standard" for the taxonomic classification of species with ambiguous properties and identification of novel species. Based upon DNA-DNA hybridization, common species is defined as having ≥70% DNA-DNA relatedness. The shortcomings of this method are it being expensive, labor-intensive and time-consuming. Consequently these assays are rarely performed. Instead, studies describing novel species are solely based upon small subunit (SSU) sequences.

Johnson [178] (1984) demonstrated that species with <70% but >20% similarity in their total genomic DNA are considered to be different species within a genus. In line with this, Wayne *et al.* [179] (1987) also proposed that phenotypically related bacterial strains that show 70% or greater genomic DNA relatedness constitute a single bacterial species. While, Boone, Whitman [180] (1993) research showed that 16S ribosomal RNA (16S rRNA) gene sequence similarity of 98% or less is sufficient evidence for new species assignment in methanogens. Later, Stackebrandt and Goebel [181] (1994) reported a clear

correlation between total genomic DNA similarity and 16S rRNA gene sequence similarity. They showed that a 16S rRNA gene sequence similarity value below 97% corresponds to DNA reassociation value of not more than 60% in defined separate species. Thus, today the most common housekeeping genetic marker used for bacterial phylogeny and taxonomy studies are the 16S rRNA gene sequences. 16S rRNA gene sequences are attractive genetic marker as their 1/ function over time rarely changes, thus, their random sequence changes are recognized as a more accurate measure of evolution, 2/ presence in bacteria is common, and 3/ size is large enough for informatics-related studies.

Thus, it has been general practice [182-184] to describe strains with ≤ 98% 16S rRNA gene sequence similarity to other strains deposited in GenBank as 'putative novel species' with emphasis on putative as 16S rRNA gene sequence similarity alone is not sufficient to determine the novelty of a bacterial strain. Nonetheless, 16S rRNA gene sequence similarity is commonly used for phylogenetic analysis. For example, Gontang *et al*. (2007) performed phylogenetic analysis of 189 representative isolates based on 16S rRNA gene sequence data and reported that 65.6% of strains were under the class *Actinobacteria (*124 isolates), while the remaining 34.4% of strains belong to the class *Bacilli (*65 isolates) [185]. In another study, Ravenschlag *et al*. (1999) reported that 19.0% of strains were related to Desulfotalea sp., 13% were related to *Desulfuromonas palmitatis*, *Myxobacteria* spp. and *Bdellovibrio* spp. and 18.1% belonged to the class γ- *Proteobacteria* based on 16S rRNA gene sequence [186]. Many such studies have been reported and taken together, the most common genera found in sediments are: *Bacillus, Pseudomonas, Arthrobacter, Achromobacter, Micrococcus, Flavobacterium, Corynebacterium, Sarcina, Azosprillium, Clostridium,* and *Mycobacteria* [187-189].

In this chapter, the bacterial library established in Chapter 3 comprising 251 isolates are subjected to bacterial identification based on their 16S rRNA gene sequences for phylogenetic analysis.

4.2 Materials and Methods

4.2.1 DNA Extraction

Several procedures were investigated for find the best method of extracting DNA from different marine sediments (For more details please see supplementary for DNA extraction optimization text S4.1- S4.2). The combination of cell lysate overnight incubation in Sodium Dodecyl Sulphate (SDS) and Hexadecyltrimethyl ammonium bromide (CTAB) seemed to work best for extracting DNA from marine sediments. The DNA extraction process is divided into three major steps: cell lysis, phenol-chloroform extraction, and ethanol precipitation.

4.2.2 Cell lysis using CTAB

Cell lysis is used to lyse the cell membranes that are in contact with the sediment particles. CTAB was used to break down the cell. CTAB is efficient in removing humic acid with no DNA loss [190]. We took 1-3 loops of bacterial culture and added it to a 1.5ml tube, 500µl of TE Buffer was added to the bacterial culture, vortexes for 30sec., and then 25µl of lysozyme stock solution (20mg/ml) was added, which was then further mixed by overturning. It was incubated at 37°C for 30min with shaking. Then 1% of SDS (25µl) and 1% CTAB (50µl) were added, placed it on spinning wheel at 4°C for 1h. After, 12.5µl

proteinase-K stock solution 20mg/ml was added and then mixed thoroughly. We also Incubated at 65°C for 15min, briefly vortexed every 5min, then cooled to 37°C. Of the RNase stock solution (5µg/µl), 1µl was mixed thoroughly, and incubated at 37°C for 30min. Samples were placed on ice for 3-5 minutes before continuing with phenol extraction.

4.2.3 Phenol-Chloroform Extraction and Ethanol Precipitation

Phenol-chloroform extractions were done to purify DNA from a solution that also had proteins. The DNA was dissolved in the aqueous layer, and the rest disseminated into the non-aqueous layer. Phenol should be pH 7.5-8. The following are the steps of Phenol-Chloroform extraction; First, One equal volume of phenol: chloroform:IAA, was added to a DNA solution (100–700 µL). It was gently mixed and spun for 5 minutes at max speed (16,000x g). The aqueous layer (top layer) was removed to a new 1.5ml tube. The bottom layer was discarded. The first steps were repeated one more time and the aqueous layer removed to another new 1.5m tube. Second, an equal volume of chloroform:IAA was added. This was mixed and spun for 3min at max speed (16,000x g). The aqueous layer (top layer) was removed to a new tube. The bottom layer was discarded into a waste container. The second steps were repeated one more time. For the ethanol precipitation steps, $^{1}/_{10}$ sodium acetate was added to the sample. Two volumes of 100% ethanol (-20°C) were added to them and mixed gently. The sample was placed at -80°C for 1hr or at -20°C for overnight. It was spun for 30 min at 4°C and centrifuged at 12,000 rpm, then the supernatant was removed carefully. The tube was filled halfway (~2volume of the original sample) with 70% ethanol (RT), and the mixture was incubated at RT for 5-10min and

spun for 5 min (wash step). The supernatant was then carefully pipetted out or decanted. It showed a clear pellet on the bottom which may be difficult to see. The pellet was dried by placing the tube upside down on a rack or in a speedvac. It takes no longer than 30min to dry– just until all residual ethanol has evaporated. The pellet was dissolved in an appropriate amount of TE buffer (100- 200µl), or desired buffer. At this point the samples were ready for PCR

4.2.4 Polymerase Chain Reaction (QIAGEN® Fast Cycling PCR Kit)

Polymerase chain reaction (PCR) was used to amplify [191] the 16s rRNA gene for microbial identification and molecular classifications. The following are the primers that we used (Table 4.1).

Table 4.1. Primers targeting 16rRNA for this study.

Primer	Sequence	Target	Ref.
27F	AGAGTTTGATCMTGGCTCAG	16SrRNA gene	[192]
1492R	GGTTACCTTGTTACGACTT	16SrRNA gene	[192]

In PCR tube we prepared the following: 25µl of PCR Master Mix (ready made), 2.5µl of forward primer 27F, 2.5µl of reverse primer 1492R, 17.5µl of RNase- free water, 2.5µl of DNA template. Table 4.2 describes the experimental set up of each PCR reaction.

Table 4.2. Experimental set up of the PCR reaction.

Reagents	1X

Ready-to-use PCR master mix	25µl
27 forward primer (2µM)	2.5µl
1492 reverse primer (2µM)	2.5µl
RNase- free water	17.5µl
DNA template (~25-30ng)	2.5µl
Total volume	50µl

with some parameters modified to the thermocycler (Bio-Rad, Hercules, California, United State) used. Initial denaturation occurred at 95 °C for 7 min, followed by 35 cycles of: denaturation at 95 °C for 1 min, annealing at 54 °C for 45 s, and an extension at 72 °C for 90 sec, with a final extension at 72 °C for 5 min

4.2.5 Agrose Gel Electrophoresis

Amplified DNA was electrophoresed on 1% agarose gel. The amplified PCR products of bacterial gene fragments were purified according to the manufacturers recommendations: Nucleospin recommended protocol (Macherey-Nagel GmbH, Düren, North Rhine-Westphalia, Germany). One gram of agarose mixed into 100ml 1XTAE with EtBr. The mixture was heated to dissolve the solution until it becomes clear. The mixture was cooled down for 3-5min, and the gel was then poured into the casting tray containing a sample comb. The comb was removed and the gel inserted into the electrophoresis chamber (make sure that the TE buffer covers the gel surface).

The Running buffer (2L of 1XTAE with EtBr) was prepared by adding 40ml of 50XTAE stock into 1960ml of dH_2O with 32µl Ethidium Bromide solution- EtBr. (10mg/ml).

The 50XTAE stock was prepared by adding 242.28g Trizma base (Tris), 100ml (0.5M) EDTA (PH 8), 57.1ml Acitic Acid, up to 1000ml ddh2O was added and then Autoclaved at 121°C for 20min.

For every 5µl of DNA solution, 1µl of 6X gel-loading dye was added and mixed well. Per well, 5-12µl of DNA-dye sample was loaded. The gel was run at 80-100 volts until the markers had migrated to the end of the gel. The gel was transferred to be viewed under a short waved UV light to detect the amplified bands. Once the band size was corrected and confirmed, then PCR can performed to remove any unspecific binding nucleotides.

The amplified PCR products of bacterial gene fragments were purified according to the manufacturer's recommendations. Nucleospin recommended protocol (Macherey-Nagel GmbH, Düren, North Rhine-Westphalia, Germany) as the following: The volume of the reaction mixture was adjusted to 100µL using a binding buffer NT or water. One volume of sample was mixed with two volumes of NT Buffer, for example 100µL of PCR reaction with 200µL of NT buffer. The Nucleospin column was placed into a 2ml collection tube and the sample was loaded. The sample was centrifuged for 1 min at 11,000-x *g*. The flow-through was discarded and the nucleospin column was placed back into the collection tube. Next, 700µL of washing buffer NT3 was added to the Nucleospin column. Samples were centrifuged for 1 min at 11,000-x *g*. Flow-through was discard and the Nucleospin column was placed back into the collection tube. To remove the NT3 Buffer completely, centrifugation was used for 2 min at 11,000-x *g*. For Elute DNA: the NucleoSpin column was placed into a new 1.5 mL microcentrifuge tube and 15-50µL of Elution buffer NE was added and incubated at room temperature for 1 min to increase the yield of the elute DNA. Centrifugation was used for 1 min at 11,000 x*g*.

4.2.6 Sequence and Phylogenetic Analysis

Purified products were submitted for Sanger sequencing of the 16S rRNA gene at the King Abdullah University of Science and Technology (KAUST) Bioscience Core Laboratory. The 16S rRNA gene sequences were compared to sequences within the NCBI database using the Basic Local Alignment Search Tool (BLAST). The phylogenetic tree was built in the ARB package [193], using the SILVA SSU Ref NR 99 (version 119) as the reference tree [194]. Alignment of the 16s rRNA genes (also referred to as species in the text) was performed using SILVA's SINA on-line aligner [195], with the variability profile set to "Bacteria", and all other parameters set to default. The alignment was inspected and edited in the ARB package. Initially, we added our species to the reference tree using the parsimony method. Close relatives of the reference tree were selected and a new tree was built using MrBayes [196] in ARB (filter: bacteria, number of substitution type 4, number of cycles for Markov Chain Monte Carlo = 100,000, number of chains = 6, temperature parameter for heating the chains = 0.5, Markov chain sample frequency = 500). *Thermogymnomonas acidicola* (archaea) was added to the tree as an outgroup.

4.2.7 Accession Numbers of Nucleotide Sequence

The 16S rRNA gene sequences of isolates were submitted in the GenBank database with the following accession numbers KP795796-KP795924 and KP980708- KP980808.

4.3 Results And Discussion

4.3.1 16S rRNA Sequencing and Phylogenetic Analysis

Sanger sequencing of microbial 16s rRNA genes was used for microbial identification [197]. However, several studies published in the 1990s established that sequencing of the 16s rRNA gene could be suitable for identification [198]. Previous studies of microbial phylogeny provided the basis for sequencing methodologies, based on sequencing the 16S rRNA genes [199]. Sanger sequencing [200] enabled us to sequence the 16s rRNA gene and allowed for the identification of many different genera and species that could not be identified using biochemical testing alone [201]. According to the partial sequencing of the 16S rRNA gene, 251 isolated strains were sequenced as a taxonomic marker. Our analysis revealed that 16S rRNA gene sequences for all the strains showed similarity with 16S rRNA gene sequences deposited in the NCBI GenBank database in the range of 91-100 %. The taxonomic classification placed the 251 isolates into five phyla (Figure 4.1) comprising 32 genera: Firmicutes (135 strains; 12 genera), Proteobacteria (99 strains; 11 genera), Planctomycetes (6 strains; 2 genera), Actinobacteria (6 strains; 4 genera) and Bacteroidetes (5 strains; 3 genera). Interestingly, the number of cultured Proteobacteria decreased in the three sediment types, in accordance with the reduction in exposure of the sediments to Seawater. In contrast, the number of cultured Firmicutes increased in the three sediment types, in accordance with reduced sediments exposure to seawater. Additionally, the contrasting counts, of Proteobacteria and Firmicutes, in these different sediments, may be due to the spore-producing Firmicutes ability to survive in more adverse oligotrophic environments such as BS compared to MN [202]. Additionally, Proteobacteria has the known ability to utilize chemical elements in soil more than other

microorganisms making the nutrient rich MN sediment more favorable for this phylum [203, 204]. Bacteria from the Actinobacteria phylum were isolated from BS at Rabigh and from MN collected from RHL and AKL, while Bacteroidetes were only isolated from BS collected from both locations. Planctomycetes were cultured from various sites such as MM and BS at RHL and MN at AKL. The Firmicutes genus *Bacillus* has been dominantly isolated from all samples, especially from BS. We found that the most abundant and widely distributed species in RHL and AKL were: *Bacillus subtilis, Bacillus sonorensis* and *Bacillus licheniformis*. Also, *Virgibacillus pantothenticus*, *Microbulbifer maritimus*, *Bacillus foraminis, Vibrio alginolyticus* and *Vibrio furnissii* strains were isolated three times or more from MN only. *Bacillus*, recognized as important sources of natural bioactive products [205], are commonly found in nutrient-poor soils [206].

From the 251 isolates, 15 likely represent new species as they have ≤ 98% similarity to 16S rRNA gene sequences deposited in GenBank [207] (Table 4.3). Interestingly, 11 of the 15 putative novel species were isolated from the 10% MA medium. Thus, low nutrient media may be more suitable for targeting the isolation of those bacteria that are more difficult to culture. The rationale being that it provides an environment that does not facilitate the growth of easily culturable bacteria that thrive in high nutrient media and usually overshadow or outcompete the “so-called” unculturable bacteria. Moreover, from the putative novel species, 56.0% have a genetic affiliation with the phylum Proteobacteria, 12.5% have a genetic affiliation with the phylum Firmicutes, and 75.0% were isolated from Rabigh

.

Table 4.3. 16SrRNA gene taxonomic affiliation of the novel cultivated isolate

Strain ID	16S rRNA sequences: BLAST hit	Phylum	Location	Sediment type	Media
Bac34	98% Vibrio alginolyticus	Proteobacteria	RHL	MN	10% MA
Bac56	98% Microbulbifer maritimus	Proteobacteria	RHL	MN	10% MA
Bac77	98% Pseudoalteromonas flavipulchra	Proteobacteria	RHL	MM	10% MA
Bac85	95% Microbulbifer gwangyangensis	Proteobacteria	RHL	MM	10% MA
Bac92	97% Planctomyces brasiliensis	Planctomycete	RHL	MM	Anti- MA
Bac94	98% Bacillus simplex	Firmicutes	RHL	MM	10% MA
Bac120	98% Oceanicaulis sp.	Proteobacteria	RHL	BS	10% MA
Bac131	98% Planctomycete sp.	Planctomycete	RHL	BS	Anti- MA
Bac140	97% Flavobacteriaceae bacterium	Bacteroidetes	RHL	BS	10% MA
Bac175	98% Blastopirellula cremea	Planctomycete	AKL	MN	Anti- MA
Bac181	91% Brevibacterium avium	Actinobacteria	AKL	MN	AIA
Bac216	97% Marinobacter xestospongiae	Proteobacteria	AKL	MM	10% MA
Bac319	98% Pseudoalteromonas espejiana	Proteobacteria	RHL	MM	10% MA
Bac320	98% Pseudoalteromonas atlantica	Proteobacteria	RHL	MM	MA-GM
Bac387	96% Exiguobacterium profundum	Firmicutes	RHL	MN	10% MA

Twelve different genus were identified in Firmicutes, with 27 different species belonging to the genus *Bacillus;* 5 species belonging to the genus *Virgibacillus*; 2 different species form each genus *Brevibacillus, Exiguobacterium* and *Paenibacillus* and one for each genus *Enterococcus, Oceanobacillus, Ornithinibacillus, Sporosarcina, Staphylococcus, Terribacillus, Aneurinibacillus.* Eleven different genus were found in Proteobacteria, with 8 different species belonging to the genus *Pseudoaltramonas,* 7 belonging to *Vibrio*; 2 form each genus *Marinobacter, Microbulbifer* and *Ruegeria*; and one for each genus *Labrenzia, Pseudomonas, Psychrobacter, Saccharospirillum, salinivibrio, Stappia* and *Stenotrophomonas.* In Planctomycetes, there are 3 different species that belong to the genus *Planctomycete*, while in Bacteroidetes, 3 different genus belong to

Flavobacteriaceae, *Fulvivirga* and *Pontibacter*. Finally in Actinobacteria, 4 different genuses belong to *Brevibacterium*, *Gordonia*, *Isoptericola* and *Micrococcus* (Figure 4.2.).

Bacillus sonorensis and *B. licheniformis* were widely distributed in RHL and AKL. Analysis of *B. sonorensis* isolates revealed very different morphologies such as pink rhizoid, clear filamentous, white, orange, and creamy smooth colony, with either convex or flat colonies.

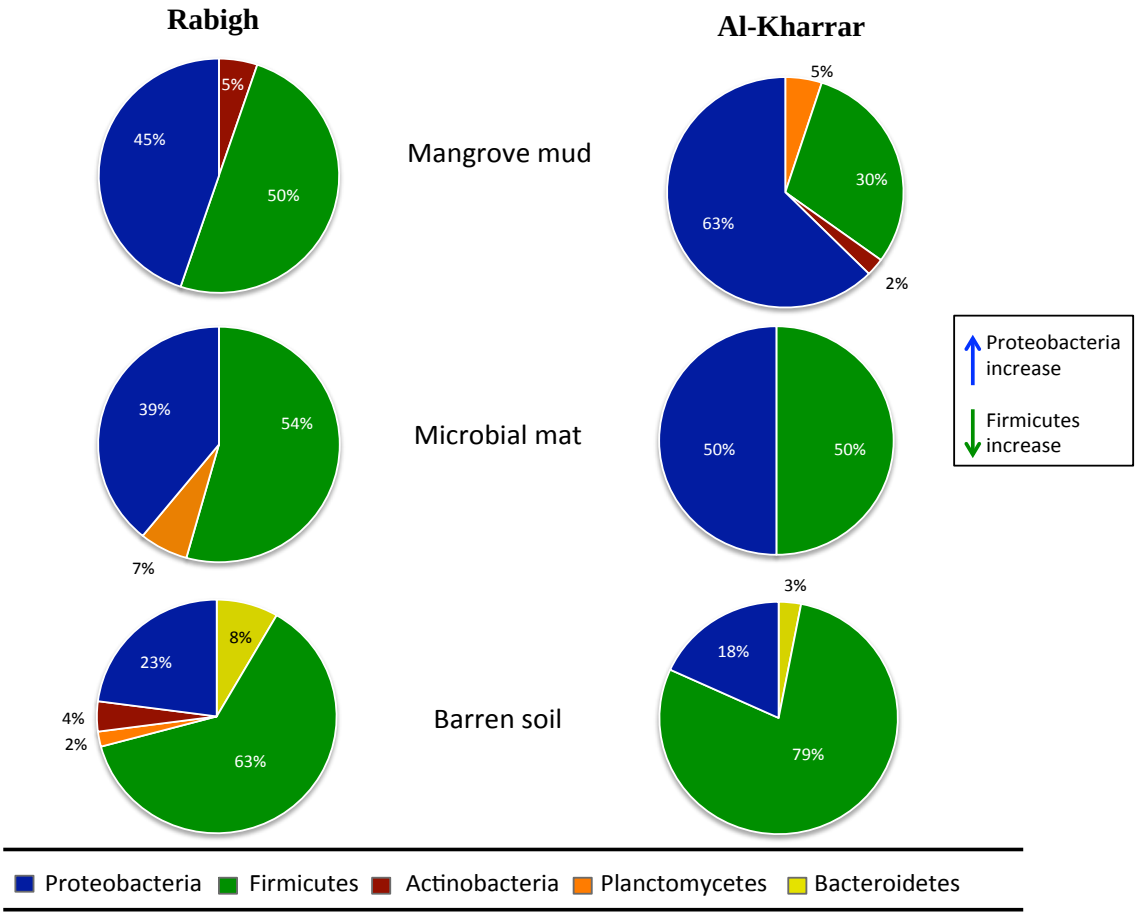

Figure 4.1. Overview of culturable microbial diversity on RHL and AKL (phylum level).

The phylogenetic analyses showed that 15 isolates might represent new species, since they showed ≤ 98% similarity to the sequence in the database. The isolates NO. 34, 56, 77, 94, 120, 131, 175, 319, and 320 shared a similarity of 98% with *Vibrio*, *Microbulbifer*, *Pseudoalteromonas*, *Bacillus*, *Oceanicaulis*, *Planctomycetes* and *Blastopirellua*. The isolate No. 92, 140, 216 shared a similarity of 97% with Planctomycetes, *Flavobacteraceae* and *Marinobacter*, respectively. Isolates No. 85, 181, and 387 shared a similarity ≤96% with *Microbulbifer*, *Brevibacterium*, *Exiguobacterium*, respectively (Table 4.3 and Figure 4.3).

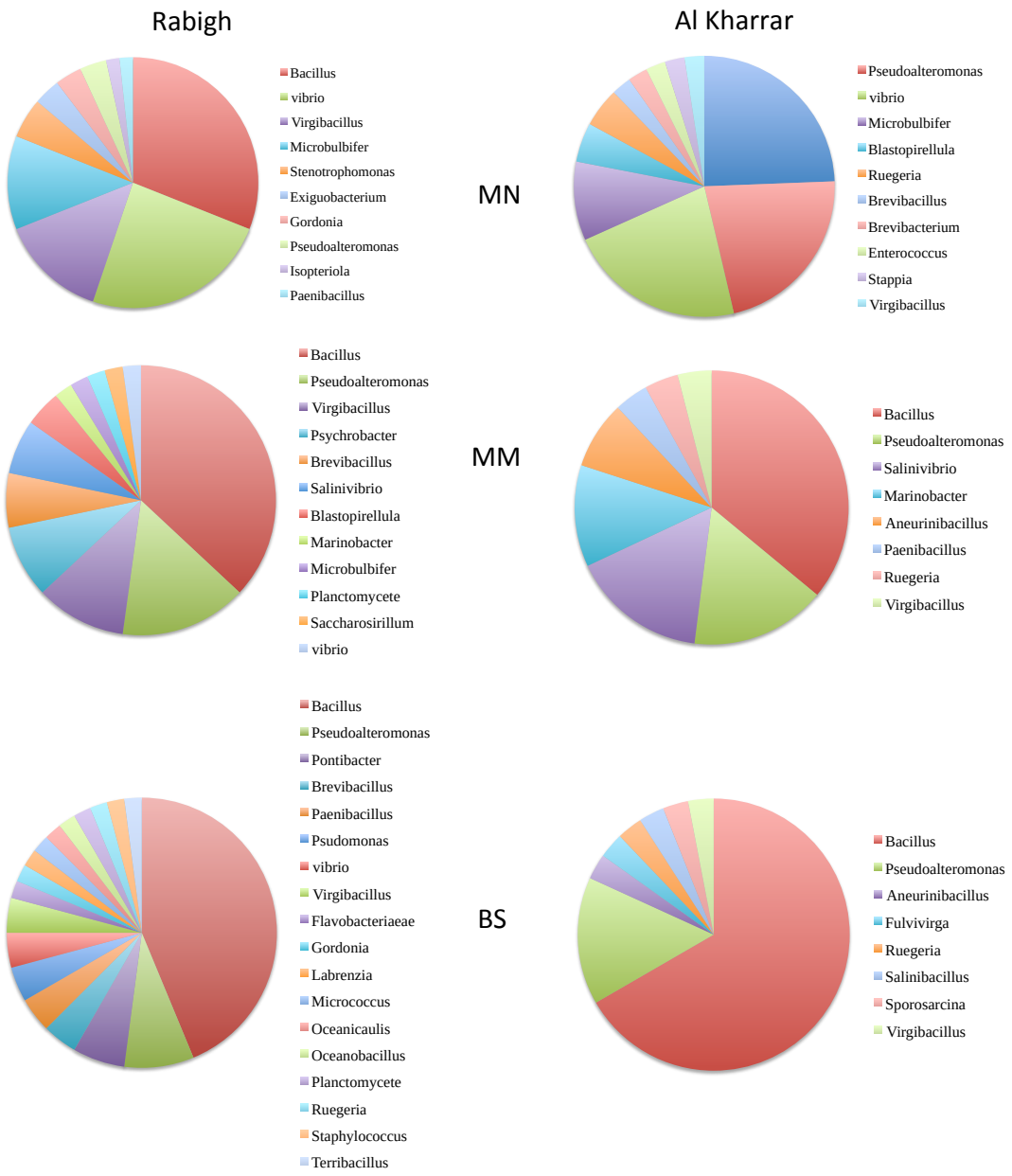

Figure 4.2. Overview of cultured microorganisms, at genus level, isolated from RHL and AKL sediments.

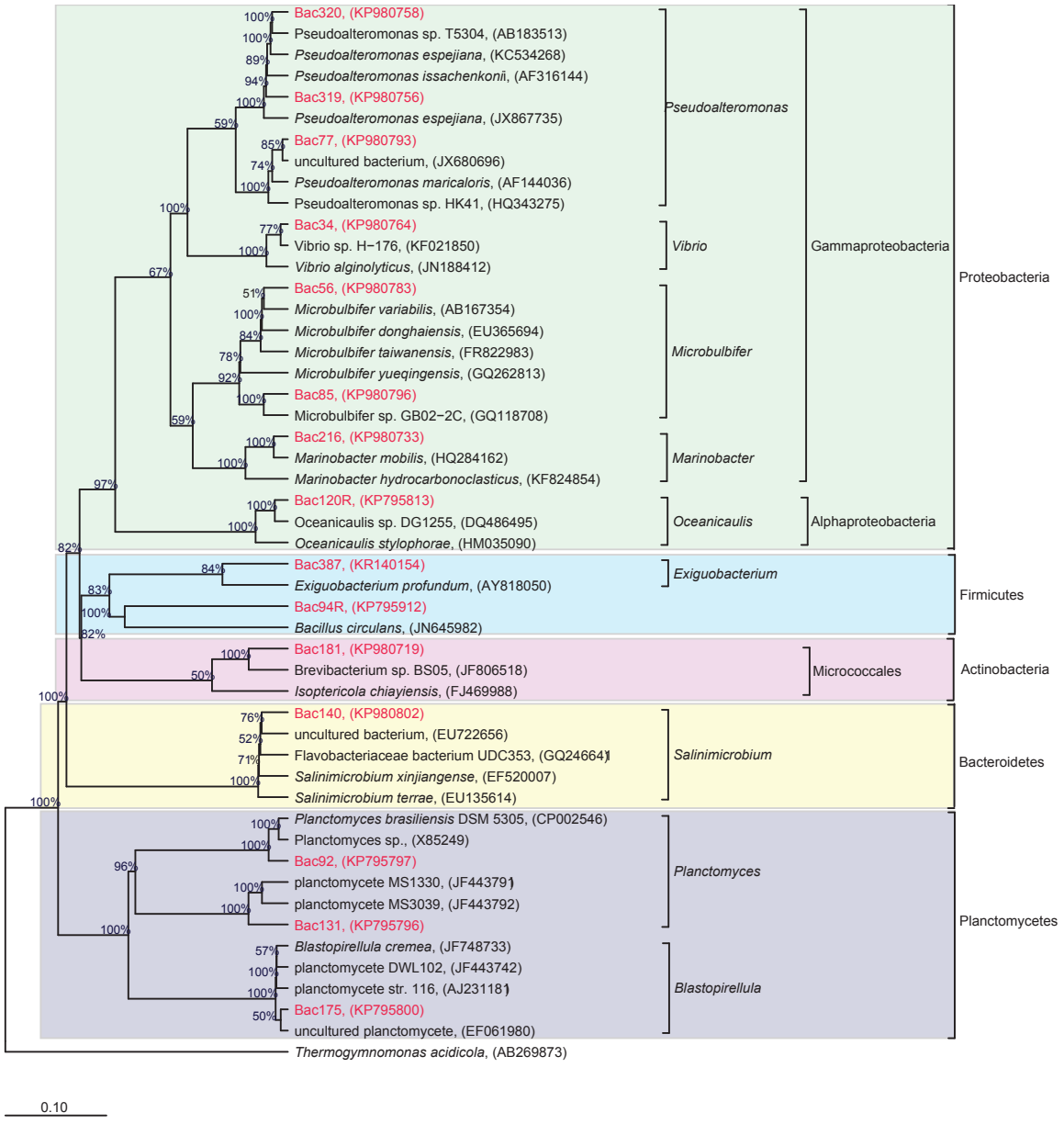

Figure 4.3 Phylogenetic tree showing the relationship of the 15 putative novel species. The sequence alignment was performed using the SINA online tool and trees were built in the software environment for sequence data called ARB, starting from the ribosomal RNA gene database called SILVA SSU dataset Ref NR 99 (version 119) using MrBayes with 100,000 Markov Chain Monte Carlo (mcmc) cycles.

4.4 Conclusion

Molecular characterization of the bacterial isolates indicates that our isolates (251 bacteria) fall under five different phyla such as Firmicutes, proteobacteria, actinobacteria, planctomycetes, and Bacteroidetes. The study has demonstrated that RHL has a diverse species of bacteria. In RHL, 79 isolates were obtained (27 isolates from MN, 22 isolates from MM, and 30 isolates from BS), while 52 isolates were obtained in AKL (19 isolates from MN, 15 isolates from MM, and 18 isolates from BS). Fifteen putative novel species were identified based on a 16S rRNA gene sequence similarity ≤98% to other strain sequences in the NCBI database.

Chapter 5

5 Screening the bacterial library for antimicrobial effects

5.1 Introduction

The imprudent use of antibiotics to treat infectious diseases, along with their widespread use in agriculture and food manufacturing industries, has caused an increase in pathogen resistance which now poses a global public health threat associated with an increase in mortality rates and health care costs [208]. Unfortunately, the successes of screening soil actinomycetes for diffusible broad-spectrum antibiotic agents in the 1940s–1960s has met with diminishing returns, as in several instances, active compounds identified from screening assays have led to previously described compounds. Since the 1960s, only two new classes of antibiotics, the cyclic lipopeptide daptomycin, identified by a classical screening approach [15] and linezolid (a completely synthetic oxazolidinone) [16], have been successfully introduced to the clinic [17]. Since type-I polyketide synthases (PKS-I) and NRPS have been found to support synthesis of secondary metabolites that act as antibiotics, immunosuppressants, toxins, siderophores, or antitumor agents, research is increasingly being focused on identifying microorganisms that harbor these multimodular enzymes [209] (see Table 5.1). The presence of these genes in the genome does not necessarily imply their expression or function, but does increase chances of identifying organisms capable of producing the above-mentioned bioactive compounds. Nonetheless, marine species that live in habitats

with stressful conditions such as low light, low or high temperature, high pressure and salinity, produce different arrays of bioactive secondary metabolites that help them to survive [210], provide defense against other organisms, and help in the competition for food. These species represent an insufficiently explored source of organisms with potential to produce compounds high with antimicrobial activity.

Several recent studies have screened for antimicrobial compounds produced by microorganisms isolated from natural environments such as in hydrothermal vents [18], sediments [19], plants [20], seawater [21], and eukaryotic marine organisms [22]. This broader screening approach has successfully identified promising new antibiotics [24-26]. Consequently, research that target sediments has sparked particular interest as soil compositions direct the diversity of the inhabiting microbial communities and their potential to produce antimicrobial compounds. Thus, microorganisms that inhabit unexplored unique soil compositions, such as in the RHL and AKL of the highly saline and hot Red Sea, represent untapped reservoirs of organisms that potentially produce novel bioactive compounds. Previously, studies related to the sediment configuration of these lagoons have been reported [57, 211-213]. Additionally, Red Sea mangrove-related studies [77, 78, 214, 215] have characterized biodiversity via metagenomics or bioactivity as induced by Red Sea derived bacteria [22, 216-218]. Nonetheless, both the lagoons are considered contaminated sites and have higher nutrient content and organic matter when compared to the open sea, making their environments more favorable for this type of research.

Thus far, a culture-dependent approach was applied to MN, MM, and BS samples collected from both RHL and AKL for bacterial isolation (Chapter 3) and identification

based 16S rRNA gene sequencing (Chapter 4). In this chapter, the 251 strains capacity to inhibit growth of four tested bacterial strains (*Escherichia coli* DH5α, *Staphylococcus aureus* ATCC 25923, *Pseudomonas syringae* pv. *tomato* dc3000, and *Salmonella typhimurium* dT2), as well as their genomic potential to produce secondary metabolites based on the identification of PKS and NRPS sequences are reported.

Table 5.1. NRPSs and PKSs compounds derived from microbiomes.

Compound	**Enzyme**	**Source**	**Activity**	**Reference**
Bacitracin	NRPS	*Bacillus* spp.	Antibacterial	[219]
Surfactin	NRPS	*Bacillus subtilis*	Antibacterial	[220]
Macrolactin	PKS	*Bacillus amyloliquefaciens*	Antibacterial	[221]
Mupirocin	PKS	*Pseudomonads fluorescens*	Antibacterial	[222]
Retimycin	NRPS	*Sallinispora arenicola*	Antitumor	[223]
Pederin	PKS	*Paederus fuscipes*	Antitumor	[224]
Salinosporamide K	NRPS	*Salinispora pacifica*	Antitumor	[225]
Salinilactam A	PKS	*Salinispora tropica*	Antitumor	[226]
Bryostatin	PKS	*Candidatus Endobugula sertula*	Antitumor	[227]

5.2 Materials and Methods

5.2.1 Media Preparation

5.2.1.1 LB Agar

For the media preparation, 40g of LB agar, Miller (Fisher Scientific, USA) was mixed with 500 µl of ddH_2O, then autoclaved on 121°C for 20min.

5.2.1.2 LB Broth with Antibiotic

Another preparation involved 40g of LB broth powder, mixed with 500 µl of ddH_2O then autoclaved at 121°C for 20min, the broth cooled to 50°C and 100ug of filtered Ampicillin was added.

5.2.1.3 S-Gal /LB Agar Blend

The medium was prepared using the β-galactosidase substrate S-Gal, IPTG and LB Agar (Sigma). The medium was prepared by suspending the contents of one packet in 500 ml of ddH_2O, autoclaved at 121°C for 20 min. Ampicillin (1000x stock) was added after the medium had cooled to approximately 50°C.

5.2.2 Screening for Antimicrobial Activity

Four tested bacterial strains (*Escherichia coli* DH5α*, Staphylococcus aureus, Pseudomonas syringae* DC3000, and *Salmonella typhimurium* DT2) were used to analyse their sensitivity towards the isolated stains. LB agar (Fisher BioReagents, Miller, USA) was prepared and autoclaved at 121°C for 20min. Stock cultures of the four target strains and all isolates were cultured individually in LB agar plates, then incubated overnight at 37°C. One of the grown colonies was collected and mixed individually with 1 mL of LB broth in a 1.5 µL tube and incubated at 37 °C, with shaking at 250 rpm overnight. A new LB agar plate was spread with a 100 µL aliquot of an individual pathogenic strain. Next, the 10 µL aliquot of isolate suspension (optical density 600 nm (OD_{600}) of 0.5) was

applied on the inoculated plate which was then incubated overnight aerobically at 37 °C. The inhibition zone of the annular radius and diameter was then been registered (Figure 5.1) after 24 h.

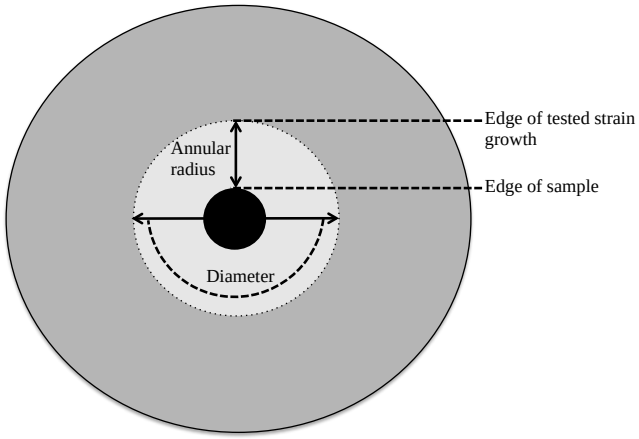

Figure 5.1. Sketch depicting the annular radius and diameter of the zone of inhibition.

5.2.3 Screening for PKS and NRPS Gene Fragments

Specific primers used to screen for domains associated with the PKS and NRPS genes, are listed in Table 5.2 [44, 46]. The PCR reaction was performed using the QIAGEN® Fast Cycling PCR Kit (Table 5.3). The PCR cycling conditions were as follows: 95°C for 15 min.; 94 °C for 30 sec.; 56°C (NRPS), 53°C (PKSI), 62.7°C (PKSII) for 90 sec.; 72°C for 90 sec.; repeated for 35 cycles; followed by 72°C for 10 min. The PCR products were electrophoresed on a 1% agarose gel, from which the bands of interest (NRPS: 700–800 bp; PKS-I: 1250–1400 bp; PKS-II: 800–900 bp) were cut out and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA). PCR products were cloned using pGEM®-T and pGEM®-T Easy vectors (Promega, Madison, Wisconsin, USA). The ligation reaction (Table 5.4) was briefly mixed and incubated at

RT for 1 hour. For the transformation process, 2µl of each ligation reaction was added to a sterile 1.5ml tube on ice. One Shot® Top 10 (Invitrogen) Competent cells were used, placed on ice 5 mins until just thawed. Cells were mixed by gently flicking the tube. Next, 25µl or 50µl was carefully transferred to the ligation reaction tube, the tubes were flicked gently, and incubated on ice for 20 min. Heat-shock was administered to the cell for 30 sec. in a water bath at 42°C without shaking, tubes were then returned to the ice immediately for 2 min. Of RT SOC medium, 250µl was added to the ligation reaction, samples were incubated for 1 hour at 37°C with shaking (~300 rpm). For each transformation culture, 100µl was plated onto LB/ampicillin/IPTG/X-Gal plates. Plates were incubated overnight at 37°C. Colonies were then checked on the next day, and only the white colonies were selected (Figure 5.2). One white colony was picked and was grown in 2ml of LB broth, with Ampicillin at 37°C overnight, and with shaking around 225rpm (3 -4 tubes were need for each sample). Plasmids that contain the PCR products were purified using the QIAprep® Spin Miniprep kit (Qiagen, Hilden, North Rhine-Westphalia, Germany). Digestions (Table 5.5) were applied to the miniprep samples to make sure that our plasmids contained the PCR products on the Gel. PCR products were then sequenced (using the T7 and SP6 primers) and compared with PKS and NRPS sequences in the NCBI database by using the Basic Local Alignment Search Tool X (BLASTX) [228].

Table 5.2. Primers targeting PKS and NRPS for this study.

Primer	Sequence	Target	Ref.
A7R	SASGTCVCCSGTSCGGTAS	NRPS	[46]
A3	GCST ACSYSA TST ACACSTCSGG		
K1	TSAAGTCSAACATCGGBCA	PKS I	[46]
M6R	CGCAGGTTSCSGT ACCAGT A		
KS**α**	TSGRCTACRTCAACGGSCACGG	PKS II	[45]
KS**β**	T ACSAGTCSWTCGCCTGGTTC		

Table 5.3. Experimental set up of the PCR reactions

Reagents	Amounts in 1X
Fast cycling PCR master mix 2X	10 µl
Forward primer (2µM)	2 µl
Reverse primer (2µM)	2 µl
dH2O	5 µl
DAN template (~ 30ng)	1 µl
Total reaction volume	20 µl

Table 5.4. Describes how the ligation reactions were set up.

Reagents	Standard reaction
2X Rapid ligation buffer	5µl
pGEM® -T Easy Vector (50ng/µl)	1µl
T4 DNA Ligase (3 units/µl)	1µl
PCR product	3µl

Table 5.5. Describes how the digestion reactions were set up.

Ragents	1X

10x fastDigest® Green Buffer (Fermentas)	1µl
EcoRI (20.000U/ml)	1µl
ddH_2O	5µl
Templets -Miniprep samples	3µl
Total volume	10µl

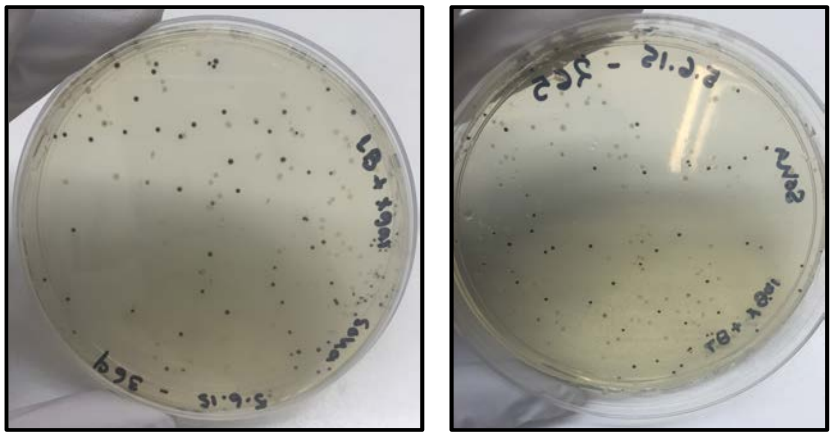

Figure 5.2. Show the white and blue colonies of the cloning.

5.3 Results and Discussion

5.3.1 Antimicrobial Screening

In order to defend themselves against other microorganisms, bacteria produce different compounds. Many of these metabolites are bioactive compounds and some have antimicrobial activity [229]. Within this context, we evaluated 251 isolates for their antimicrobial activity against four target bacteria: *Staphylococcus aureus* ATCC25923, *Pseudomonas syringae dc3000, Salmonella typhimurium* dT2 and Escherichia coli dh5α. The reason behind the selection of these bacteria was to cover different areas of antimicrobial use. *S. aureus* is the agent of several human infections such as skin and

wound infections, septicemia, endocarditis, food poisoning, toxic shock syndrome, meningitis, pneumonia and osteomyelitis [230]. Additionally, the strain *S. aureus* ATCC 25923 strain is commonly used as a control to test for antibiotic susceptibility, and as a quality control strain for commercial products [231]. *P. syringae* pv. *tomato* dc3000 is a phytopathogen used as a model to study plant-bacterial interactions [232]. *S. enterica serovar typhimurium* definitive type 2 (DT2) is host restricted to *Columba livia* (rock or feral pigeon). However, it is also closely related to *S. typhimurium* isolates that circulate in livestock and cause a zoonosis, that is, gastroenteritis in humans [233]. The strain of *E. Coli* that we used is not a pathogen, and was developed for laboratory cloning use [234]. Vanessa M D'Costa et al., 2007 shows that it is essential to include nonpathogenic microbes in antibiotic study, because it may be possible to find a way to predict resistance before it arises clinically and as well as improve diagnostic methods and therapeutic to counteract resistance before appearance in pathogens [235]. Of all isolates, 42 isolates inhibited the growth of *S. aureus* (30 of them belong to the phylum Firmicutes, 11 Proteobacteria, and one Bacteroidetes), 14 isolates inhibited the growth of *S. typhimurium (*12 of them belong to Firmicutes and 2 Protobacteria), 34 isolates inhibited the growth of *P. syringae (*29 of them belong to the phylum Firmicutes, and 5 Proteobacteria) and 23 isolates inhibited the growth against *E. coli* dh5α (19 of them belong to the phylum Firmicutes, 4 Proteobacteria). Only 10 isolates displayed zone inhibition against all of the three indicator laboratory pathogens (Table 5.6), of which nine isolates belong to the phylum Firmicutes, while one belongs to the phylum Proteobacteria (*Microbulbifer salipaludis* (Bac177)). Of these 10 isolates, only zone inhibition for *B. licheniformis* (Bac84) and *A. migulanus* (Bac271) showed an annular

radius of ≥3.0 mm (Diameter =10.8 mm) for all screenings, suggesting a higher activity against all four indicator laboratory isolates. However, the strain Bac177 of *M. salipaludis* displayed the most effective zone inhibition against *S. typhimurium*. Two strains, Bac57 and Bac376, belonging to the species *Bacillus amyloliquefaciens* and *Virgibacillus olivae,* respectively, displayed strain-specific activity as shown by an annular radius of ≥3.0mm (Diameter =10.8 mm) against *P. syringae.* Additionally, strains Bac389, Bac375 and Bac380 belonging to *Bacillus cereus*, *Pseudomonas fluorescens* and *Stenotrophomonas maltophilia* respectively, displayed strain-specific activity as shown by an annular radius of ≥3.0 mm (Diameter =10.8 mm) against *S. aureus.* Of the putative novel strains, only a strain (Bac319) of *Pseudoalteromonas espejiana* displayed zone inhibition as shown by an annular radius of ≥1.0 mm (Diameter = 6.7 mm) against *S. aureus*, and a strain (Bac320) of *Pseudoalteromonas atlantica* revealed zone inhibition as shown by an annular radius of ≥1.0 mm (Diameter = 6.7 mm) against *S. aureus* and *P. syringae.*

Some of the ten strains that inhibited the growth of the four tested strains are already known to be producers of bioactive compounds. For example, Gramicidin S, a cyclodecapeptide recently isolated from *A. migulanus* [236], was originally isolated from the Gram-positive *Bacillus brevis* and was shown to exhibit an antibiotic effect against some Gram-positive and Gram-negative bacteria, as well as some fungi [237]. In addition, *B. licheniformis* is used for several biotechnological applications for its capacity to produce degrading enzymes such as proteases, lipases, pectate lyases, and polysaccharides [238]. *B. licheniformis* has further been shown to exhibit potent

antimicrobial activity against indicator strains (*Lactococcus lactis*, *Lactobacillus bulgaricus* and *Listeria innocua*) and is clinically relevant (*Listeria monocytogenes*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Salmonella Typhimurium* and *Escherichia coli*) bacteria [239]. *B. subtilis* is a recognized producer of natural biocontrol agents, having high antifungal activity against *Alternaria solani*, *Botrytis cinerea*, *Monilia linhartiana* 869, *Phytophthora cryptogea* 759/1 and *Rhizoctonia* sp., and antibacterial activity against *Pseudomonas syringae* pv. tomato Ro and *Xanthomonas campestris* [240]. *B. sonorensis* has displayed antifungal activity against *Macrophomina phaseolina* [241] and has been shown to exhibit antibacterial activity against *Staphylococcus aureus* and *Listeria monocytogenes* as a potential food preservative [242]. *B. vallismortis* showed strong growth inhibition activity in vitro against the phytopathogens *Fusarium graminearum*, *Alternaria alternata*, *Rhizoctonia solani*, *Cryphonectria parasitica* and *Phytophthora capsici* [243]. So far, no specific antimicrobial activity has been detected for *B. borstelensis*, *P. dendritiformis* and *M. salipaludis*, which appears to be one of the novel findings of our study.

5.3.2 PCR-screening for PKS and NRPS Domains

The 251 isolated strains were screened for the detection of genes involved in the synthesis of bioactive compounds: PKS-I and PKS-II, and NRPS (Figure 5.3 and Table S5.1) [46]. Although these enzymes are known to support synthesis of bioactive secondary metabolites such as antibiotics [149, 244], their presence does not imply expression or functionality, but does increase the likelihood that the strain has the potential to produce antimicrobial compounds such as antibiotics, antitumor agents, or

immunosuppressants. For the 52 isolates that exhibited antimicrobial activity, at least one type of biosynthetic gene sequence was recovered from 25 strains. Of these 25 isolates, 56.0% were isolated from RHL, and 48.0% have a genetic affiliation to genus *Bacillus*.

Table 5.6. Isolates that displayed zone inhibition against all of the four indicator laboratory pathogens.

Strain ID	Closest phylogenetic relative by BLAST	Sequence similarity (%)	Zone of Inhibition (mm)							
			Staphylococcus aureus		*Salmonella typhimurium*		*Pseudomonas syringae*		*Escherichia coli*	
			Annular radius	Diameter	Annular radius	Diameter	Annular radius	Diameter	Annular radius	Diameter
Bac84	*Bacillus licheniformis*	99	4.5	14	3	11	3	11	3	10.5
Bac90	*Bacillus sonorensis*	99	3.8	12	2.8	10	3	11	3	10.7
Bac98	*Brevibacillus borstelensis*	99	5	15	0.5	6	4.3	13.5	4	12.5
Bac111	*Bacillus vallismortis*	99	2	9	4	13	0.5	6	5	15
Bac177	*Microbulbifer salipaludis*	99	3.3	11	6.5	18	1	7	3.8	12.1
Bac254	*Bacillus subtilis*	99	5	15	3	11	0.5	6	2.5	9.5
Bac270	*Aneurinibacillus migulanus*	99	4	13	2	9	3.3	11.5	3.5	11.7
Bac271	*Aneurinibacillus migulanus*	99	5.5	16	3	11	4.5	14	3.5	11.5
Bac363	*Paenibacillus dendritiformis*	99	0.5	6	3	11	1	7	5	14.5
Bac390	*Paenibacillus dendritiformis*	99	2	9	2	6	5	15	4	13

NRPS biosynthetic genes were identified in 40.8% of those selected strains, while PKS-I and PKS-II were identified in 28.5% and 20.4% of isolates respectively (Table S5.2). Six isolates were positive for all biosynthetic genes screened: *B. licheniformis* (Bac84), *B. vallismortis* (Bac111), *P. espejiana* (Bac319), *B. subtilis* (Bac254) and *P. dendritiformis*

(Bac363 and Bac390). For the isolates that displayed zone inhibition against all of the four indicator laboratory strains (Table 5.6), biosynthetic genes sequences were only found in five isolates: *B. licheniformis* (Bac84), *B. vallismortis* (Bac111), *B. subtilis* (Bac254), *P. dendritiformis* (Bac363 and Bac390). No biosynthetic gene sequences were recovered from the strains belonging to *B. borstelensis* (Bac98) *and M. salipaludis* (Bac177), bacteria for which antimicrobial activity has been reported in literature [245]. Additionally, from the 15 putative novel species (Table 4.4), biosynthetic gene sequences were recovered from nine strains belonging to the following species: *Microbulbifer gwangyangensis* (Bac85), Planctomyces brasiliensis (Bac 92), Bacillus simplex (Bac 94), *Blastopirellula cremea* (Bac175), *Brevibacterium avium* (Bac181), *Marinobacter xestospongiae* (Bac216), *P. atlantica* (Bac288), *P. espejiana* (Bac319) and *Exiguobacterium profundum* (Bac387).

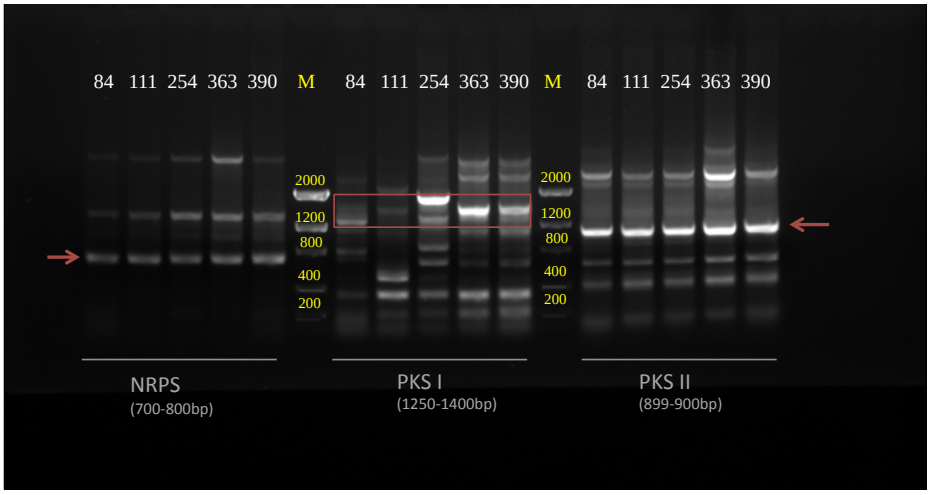

Figure 5.3. Show the PCR amplification of gene PKS I &II, NRPS.

Table 5.7. NRPS and PKS results of cultivated strains.

Isolated code	Closest Relative by BLAST X		
	NRPS	**PKS I**	**PKS II**
2	-	-	96% Spindolin [Vibrio parahaemolyticus] WP_029862754.1
4	99% transcriptional regulator [Bacillus licheniformis] WP_026580611.1	99% rhamnogalacturonyl hydrolase [Bacillus licheniformis] WP_003180899.1	-
13	47% hypothetical protein [Marinobacter manganoxydans] WP_008171913.1	98% Superfamily II DNA and RNA helicase [Vibrio alginolyticus] WP_005390478.1	-
26	98% succinate-semialdehyde dehydrogenase [Bacillus] WP_014416912.1	99% polyketide synthase [Bacillus] WP_029973687.1	-
71	95% non-ribosomal peptide synthase [Bacillus subtilis] KIN39535.1	92% 7-cyano-7-deazaguanine synthase [Bacillus sonorensis] WP_006638443.1	-
92	50% hypothetical protein [Blastopirellula marina] WP_040351748.1	86% hypothetical protein [Rubinisphaera brasiliensis] WP_013627469.1	96% hypothetical protein [Rubinisphaera brasiliensis] WP_013630520.1
123	83% non-ribosomal synthetase	98% ferrochelatase	-

	[Bacillus licheniformis] WP_025805844.1	[Bacillus sp. NSP9.1] WP_026586276.1	
164	98% non-ribosomal peptide synthetase FusAA [Paenibacillus polymyxa M1] WP_043921404.1	-	-
156	99% glucose starvation-inducible protein B [Bacillus] WP_023857883.1	-	-
174	98% adenylation domain of nonribosomal peptide synthetases [Pseudoalteromonas piscicida]WP_045965175.1	99% hypothetical protein [Pseudoalteromonas piscicida] WP_045965173.1	-
180	91% hypothetical protein [Vibrio alginolyticus]WP_047104361.1	98% deoxyguanosinetriphosphate triphosphohydrolase [Vibrio sp. 712i1] WP_017634779.1	100% hypothetical protein [Plasmodium chabaudi chabaudi] XP_731877.1
201	93% Six-hairpin glycosidase-like protein [Vibrio parahaemolyticus] WP_031817544.1	98% deoxyguanosinetriphosphate triphosphohydrolase [Vibrio alginolyticus] WP_047009478.1	-
202	-	98% deoxyguanosinetriphosphate triphosphohydrolase [Vibrio alginolyticus] WP_047009478.1	-

254	88% nonribosomal peptide synthase [Paenibacillus alvei] WP_021255906.1	97% trehalose permease IIC protein [Bacillus subtilis] WP_032722999.1	99% class A beta-lactamase [Bacillus cereus] WP_000027053.1
257	45% AMP-dependent synthetase [Smithella sp. F21] WP_037463319.1	98% deoxyguanosinetriphosphate triphosphohydrolase [Vibrio sp. 712i1] WP_017634779.1	-
258	-	96% deoxyguanosinetriphosphate triphosphohydrolase [Vibrio sp. 712i1] WP_017634779.1	-
265	59% nonribosomal peptide synthetase subunit [Paenibacillus alvei DSM 29] EJW17732.1	-	-
266	92% non- ribosomal peptidesynthetase [Bacillus licheniformis] KJH62268.1	93% Methyltransferase [Bacillus] WP_046130429.1	-
277	81% non-ribosomal peptide synthase [Bacillus licheniformis] AJO61325.1	94% trehalose permease IIC protein [Bacillus sp. NSP9.1] WP_026589597.1	-
282	94% nonribosomal peptide synthetase [Bacillus subtilis] WP_041352648.1	94% 7-cyano-7-deazaguanine synthase [Bacillus sonorensis]	-

		WP_006638443.1	
283	92% nonribosomal peptide synthetase [Bacillus subtilis]WP_041352648.1	94% 7-cyano-7-deazaguanine synthase [Bacillus sonorensis] WP_006638443.1	-
291	70 % nonribosomal peptide synthase [Burkholderia pseudomallei] WP_044368416.1	-	-
337	94% acetolactate synthase [Pseudoalteromonas sp. NW 4327] WP_024032406.1	-	-
369	65% nonribosomal peptide synthetase subunit [Paenibacillus alvei DSM 29] EJW20318.1	90% multidrug transporter AcrB [Pseudoalteromonas sp. NW 4327] WP_024034324.1	-
387	83% non-ribosomal peptide synthase [Bacillus licheniformis] AJO61326.1	53% hypothetical protein [Psychrobacter sp. JCM 18902] WP_045445323.1	-

5.4 Conclusion

Both Proteobacteria and Firmicutes were abundantly isolated from both the AKL and RHL sites. Approximately 40% of strains belonging to both the Firmicutes and Proteobacteria phylum showed potential to produce secondary metabolites via PKS and NRPS, while Firmicutes showed more potential to produce secondary metabolites with antimicrobial activity. Our study shows a rich biodiversity of culturable Firmicutes

(Table S5.3) from Red Sea ecosystems on the Saudi Arabian coastline. Of all isolated strains, we found that 52 exhibit potential antimicrobial activity. From these, 25 have at least one type of biosynthetic gene sequence, indicating that these isolates are a valuable resource for the potential discovery of bioactive effects. Moreover, 75% of putative novel species and 67% of strains that exhibited antimicrobial activity were isolated from RHL.

Chapter 6

6 Concluding Remarks

Since the composition of soil directs the diversity of the contained microbiome and its' potential to produce bioactive compounds, many studies has been focused on sediment types with unique features characteristic of extreme environments. However, not much is known about the potential of microbiomes that inhabit the highly saline and hot Red Sea lagoons. This case study (Chapter 2) explores MN and the MM of sediments collected from the RHL and AKL for antimicrobial bioprospecting. RHL appears the better location, and the best sediment type for this purpose is MN. On the other hand, AKL displayed increased anaerobic hydrocarbon degradation and an abundance of bacterial DNA associated with antibiotic resistance. Moreover, our findings show an identical shift in phyla associated with historic hydrocarbon contamination exposure reported in previous studies (that is, enrichment of Gamma- and Delta-proteobacteria), but we also report that bacterial DNA sequences associated with antibiotic synthesis enzymes are derived from Gamma-, Delta- and Alpha-proteobacteria. This suggests that selection pressure associated with hydrocarbon contamination tend to enrich the bacterial classes with DNA associated with antibiotic synthesis enzymes. Although Actinobacteria tends to be the common target for antimicrobial bioprospecting, our study suggest that Firmicutes (Bacilli and Clostridia) should be a target plylum for antimicrobial bioprospecting in all locations. While in hydrocarbon-contaminated sites, Bacteroidetes, Proteobacteria, Cyanobacteria and Firmicutes frequently appear to be better targets for antimicrobial bioprospecting than Actinobacteria.

To the best of our knowledge, this is the first metagenomic study that analyzed the microbiomes in Red Sea lagoons for antimicrobial bioprospecting.

Based on the above findings, laboratory experiments were designed to test the hypothesis that these ecosystems are suitable for bioprospecting antimicrobial agents from Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria. This was accomplished by, 1/ establishing a bacterial library cultured for sediment types such as MN, MM and BS from both RHL and AKL (Chapter 3), 2/ molecular characterization based on 16S rDNA gene sequences (Chapter 4) and 3/ screening for antimicrobial effect against known pathogens and for the presence of PKS and NRPS enzymes (Chapter 5).

In total a bacterial library of 251 isolates was established for which SPSS analysis was performed to show that the library of cultured strains clustered into five groups, indicating strain diversity. Further molecular characterization with respect to partial 16S rDNA gene sequences of all 251 bacterial isolates placed them in five different phyla including Firmicutes, Proteobacteria, Actinobacteria, Planctomycetes, and Bacteroidetes. For the two locations, RHL samples showed higher species diversity compared AKL samples based on the cultured bacterial library. Moreover, fifteen putative novel species were identified based on a 16S rRNA gene sequence similarity ≤98% to other strain sequences in the NCBI database. A detailed phylogentic tree was also constructed to establish their evolutionary status from the gene sequence data. All strains were then screened for antimicrobial effect and for PKS and NRPS enzymes.

Of all 251 isolated strains, we found that 52 strains exhibit potential antimicrobial activity against bacterial pathogens (*Staphylococcus aureus, Salmonella typhimurium, Escherichia coli* and *Pseudomonas syringae*). Additionally, 25 of these 52 strains have at least one type of biosynthetic gene sequence (PKS-I, PKS-II or NRPS), responsible for the synthesis of secondary metabolites, indicating that these isolates are a valuable resource for the potential discovery of bioactive effects.

Moreover, 10 of the isolates had a growth inhibition effect towards *Staphylococcus aureus*, *Salmonella typhimurium, Escherichia coli* and *Pseudomonas syringae*. We report the previously unknown antimicrobial activity of B. borstelensis, P. dendritiformis and M. salipaludis against all four-target bacteria. Moreover, 75% of putative novel species and 67% of strains that exhibited antimicrobial activity were isolated from RHL. This case study provides evidence that other phyla such as Firmicutes should also be targeted for their potential to produce antimicrobial compounds and that metagenomics analysis should be used as a preliminary screen to comprehensively identify target phyla for this type of bioprospecting instead of just following trend.

In this process we have also managed to short list 15 putative novel species that require further experimentation to verify species novelty such as whole genome sequencing. Also, 5 additional strains (Bac 84, 111, 254, 363 and 390) will be shortlisted for the bioassay-guided purification and identification of their antimicrobial compounds using High Performance Liquid Chromotography (HPLC), Nuclear Magnetic Resonance (NMR) and Mass Spectrometry**.**

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Supplementary Material

Text

Chapter 1 Supplementary

S1.1. Sampling Location

AKL and RHL are two lagoons found on the eastern coastal area of the Red Sea. There are a small number of studies relating to the sedimentological configuration of marine lagoon and/or Harbors [211, 246-248] including one recent study on bioactive compounds isolated from bacteria association with eukaryotes located in the Red Sea [216]. Research on marine sediment from lagoon environments offers an original research field, as their physical and chemical characteristics are markedly different from the rest of the Red Sea environment as will be discussed later.

The resulting document illustrates the research achieved so far on the culturability of microbes from three different sediments types: MN, MM, and BS two locations at AKL and RHL (Figure S1.1). To date there are no microbiological studies of such environments, and data on nutrient cycling and eutrophication levels are limited.

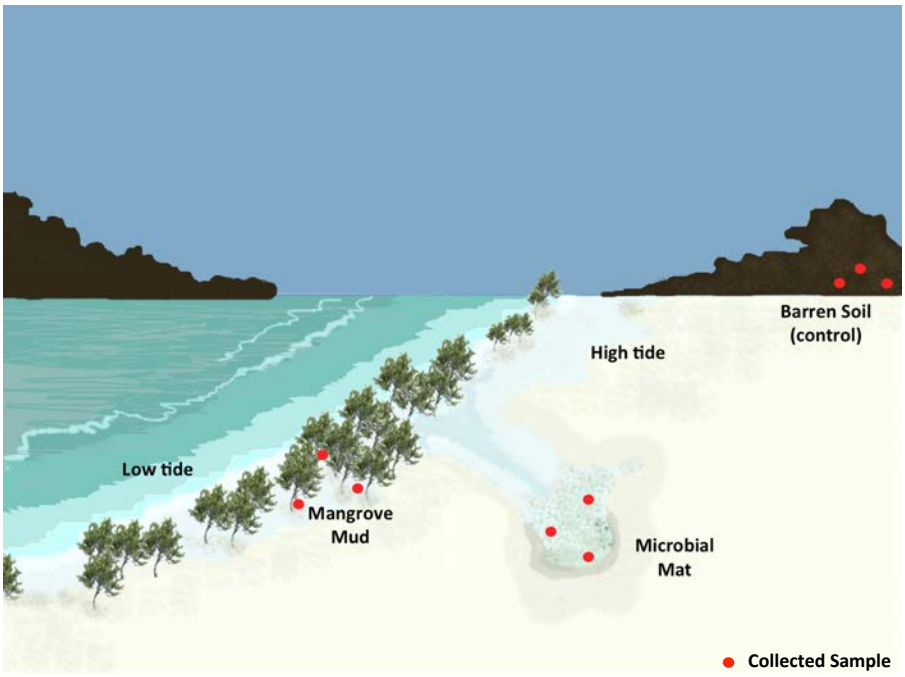


Figure S1.1. Lagoon schematic showing different component of lagoon system.

S1.2. Lagoon and Harbor environments

A lagoon is a slight body of water disconnected from a bigger body of water by reefs or barrier islands; it is a complex ecosystem with chemical, biological, and hydrological interactions. Different factors such as: salinity, tidal flow level, speed of wind, light concentration, and cargo of organic detritus, all act as constraints for the ecosystem's function. A change in any of these factors can have major results on the state of the lagoon ecosystem [249]. On the other hand, a harbor is an artificially constructed or natural part of a body of water that ships and boats can use to anchor to coastal features for protection from waves and wind [250]. A harbor is generally more affected by anthropogenic activity than lagoons because of sea vessel traffic. This harbor is 150 km north of Jeddah and 30 km north of King Abdullah Economic City, a large-scale development project aiming to be a commercial focus of the kingdom. This is a deep-

water port with an entry channel 28 m in depth, 1300 m in length, and 240 m in width. The weather is tropical with high humidity of up to 82 % in the summer, and with temperatures ranging is from 25 ° to 38 °C. The speed of the winds from North to Northwest is around 2.6- 12.9 m/s. The daily range of the tide cycle is 0.34 m and the temperature range of the seawater is from 22 ° to 29 ° C [251]. AKL lagoon and RHL harbor are two locations (Figure S1.2) that have had no microbiological studies. However, previous studies have focused on the chemical and textural structure of sediments in the lagoon [211, 246-248].

S1.3. Al-Kharrar Lagoon (AKL)

Al-Kharrar lagoon is connected to the Red Sea through a tight channel that extends for about 17 km in length and about 2-3 km in width over the northwestern side [246], with a lagoon depth ranging from 2-3 meters. High velocity tidal currents can enter AKL at speeds of up to 1 $m\text{-}s^{-1}$, but tidal range is low, only 20-30 cm at most [252], this tidal currents share in the growth and contribute to form microbial mats. The lagoon sediment structure is mainly white sandy mud in the northern part and muddy at the southern part of the lagoon [246].

S1.4. Rabigh Harbor Lagoon (RHL)

RHL is separated from AKL lagoon by almost 11 km of land and lies to its southeast. This Harbor is located at the northern perimeter of Petro Rabigh, the petrochemical and refining complex. The complex produces up to 400,000 barrels of crude oil per day [253], and contains millions of tons of ethane cracker, monoethylene glycol plant, linear low-

density polyethylene plant, polyethylene plant, and a polypropylene plant per year [254]. Whilst conducting this study, Petro Rabigh, had announced doubling their petrochemical complexes capacity for over the next three years [254]. This Harbor is located 150 km north of Jeddah and 30 km north of King Abdullah Economic City, a large-scale development project aiming to be a commercial focus of the kingdom of Saudi Arabia. This is a deep-water port with an entry channel 28m in depth, 1300m in length, and 240m in width. The weather in this region is tropical with high humidity of up to 82% in the summer, and the temperature ranges from 25°C to 38°C. The speed of the winds from north to northwest is around 2.6-12.9m/s. The daily range of the tide cycle is 0.34m and the temperature range of the seawater varies from 22°C to 29°C [251].

Figure S1.2. Al-Kharrar lagoon and Rabigh harbor location Map (a), satellite (b). Google maps.

S1.5. Sediments of Sample Collection:

S1.5.1. Mangrove Mud

Mangroves are coastal ecosystems; they grow at the edge between sea and land in

tropical and subtropical latitudes. They are woody plants with roots associated with microbes and fungi [255, 256]. Mangroves have adapted to conditions such as high salinity, high temperature, great tide variations, and anaerobic soils [256]. The microbial community of the mangrove muds represents a rich environment where the microbial community interacts with the mangrove roots. Bacterial groups found in association with the root system of mangroves include phosphate solubilizing bacteria, sulfate reducing bacteria, photosynthetic, and methanogenic bacteria. Mangroves are highly productive; it has been determined that 91% of the total microbial biomass is bacteria and fungi, while 7% is algae and 2% is protozoa [257]. So the question is: What are these microbes (Bacteria) doing? Studies have shown that the main roles of bacteria in mangroves are nutrient transformations and biogeochemical cycles [256], and they are responsible for carbon flux in this sediment [258, 259]. They are also the main players in the N cycle with transformation from fixation, nitrification, denitrification, and ammonification [260]. There is a gradient in the shift from aerobic to anaerobic conditions in the sediments of mangroves. It is aerobic on the sediment's surface and rapidly shifts to anaerobic at lower depths. This is a result of their organic matter. Organic matter degradation happens in the aerobic region by aerobic respiration, and the decomposition in the anaerobic layer happens by sulfate reduction [261, 262]. In view of the various bacterial taxonomic groups represented, and since they share parallel functional roles, the huge diversity observed in mangrove sediment environments shows that they can adapt to possible ecological impacts [263]. Each group of bacteria has its own important site location, and that depends on the changes of conditions such as PH, organic matter content, salinity, nutrient amount, and conductivity. The majority of the bacterial community in mangrove

sediments is made up of Alphaproteobacteria, Betaproteobacteria, Actinobacteria, Gammaproteobacteria, Bacteroidetes and Firmicutes [264]. Hiltner (1904) was the first to describe the rhizosphere as soil that surrounds plant roots. There is a wide diversity of bacteria in the rhizosphere such as *Phyllobacterium, Pseudomonas, Variovorax, Agrobacterium, Bacillus,* and *Azospirillum* [265]. Some studies show that bacteria and Actinomycetes diversity is higher in the rhizosphere than in the other soil outside this area [266].

S1.5.2. Microbial Mats in Salt Marshes

Salt marshes are one of the most productive systems inn the world and harbor diverse biological communities [75, 267]. The microbial communities found in this sediment decompose organic matter that come from plant residues and transform contaminants [268, 269]. They can also affect the availability of heavy metals [270], and execute other ecosystem services [271]. Microbial mats are found under the salt layer of the salt marshes**.** Microbial mats show large filamentous microorganisms, which form an entangled mass with sediment particles where they develop a sediment surface stabilizing both the sediment and themselves from eroding forces [272]. They develop in extreme conditions in their environments. Carbon autotrophy is the main mode of energy utilization in microbial mat systems, which means that microbial mats are developed by microbes that fix inorganic carbon. This can be done in two different ways, photosynthesis and chemosynthesis [272]. In Phototrophy light is the source of energy and microbes (usually cyanobacyteria) are the building blocks of microbial mats. In the case of chemosynthetic reaction, oxidation of a reduced compound (often sulfide or

methane) is usually the main energy source. This is usually the case in dark environments such as caves, deep oceans, cold seeps, and deep-sea hyperthermal vents where reduced compounds are available. Sometimes chemosynthetic bacteria (Chemoautotrophs) can also grow in illuminated environments, for example when a large amount of organic matter decomposes in seawater this creates high amounts of sulfide that is in direct contact with oxygen [272].

During the day, cyanobacteria oxygenic photosynthesis enhances the sediment with oxygen. At night, this oxygen is rapidly consumed and sulfide accumulates. This occurs as oxygen, light, and nitrate are absent while sulfate-reducing bacteria became active in producing sulfide, which is then oxidized during the day by accumulating oxygen. The succession of day and night and the subsequent activities of microbial mats result in the daily shifting of the sulfide and oxygen gradients. Also, there is a dramatic shift in a microbial mat's PH during the succession of day and night. In the day time, the fixation and the reduction of CO_2 causes PH to rise to a high level (>9). Conversely, at night the breakdown of organic matter causes the rise of CO_2 and reduces the PH level [272]. The organisms of mat-forming take place in these physicochemical gradients and form three micro-layers. First, the aerobic cyanobacteria forms a green layer on the top surface. Second, below the surface the anaerobic purple sulfur bacteria forms a pink layer. Often there is rusty layer that separates the top surface from the bottom; this layer is composed of iron that forms a barrier for sulfide and oxygen, which are toxic to the cyanobacteria and purple sulfur bacteria, respectively. When the sulfide diffuses to the top surface, it reacts with iron and oxidizes to sulfur, forming an insoluble iron sulfide and pyrite. In contrast, when oxygen diffuses downwards, it reacts with the iron and oxides it, forming

iron hydroxides. It has been assumed that sulfate-reducing bacteria are present in the anoxic layer of the microbial mat, i.e. that they are anaerobic microorganisms which can tolerate low levels of oxygen or may even be capable of respiring, but they are incapable of living by aerobic respiration [273, 274].

S1.5.3. Barren Soil

Barren soil is the soil that is filled with pore spaces and can mix with solids such as organic and mineral [275, 276] , water, and gases [277]. Soil is a complex system with the following special properties: First, Torsvik et al. (1996) found that the microbial community in soil is extremely diverse; this hypothesis was proven by finding 6000 different bacterial genomes per gram of soil. Second, soil is heterogeneous and the system is discontinuous, is usually a poor source of energy and nutrients, and the microbes live in separate microhabitats. The physical, chemical, and biological characteristics of these microhabitats are different in both space and time [278]. A third characteristic of soil as a microhabitat is the ability of the solid phase to absorb protein and nucleic acid as important biological molecules [279]. Finally, many reactions can be catalysed by the soils mineral components' surface, such as phenols oxidation, polyphenols with humic substances formation [280], polymerization, deamination, and ring cleavage [281]. Tiedje et al. (2001) suggested that with the spread of microbial species on the soil surface there is an absence of microbial community competition because several microbial species that live in soil are spatially separated for most of the time. They assumed that the interaction among microhabitats exists for a short time

directly after rain, which was confirmed by the fact that when soil was wet with water it resulted in a predominance of one or a few species [282].

Chapter 4 Supplementary

S4.1. Materials and Methods

S4.1.1. DNA Extraction Optimization

S4.1.1.1. Chemical lysis - SDS for 1hour

Chemical lysis is the process by which we gently lyse the microbial cell membranes without using freeze / thaw cycles and beads beating process.

1xTE buffer was prepared by adding 10ml of 0.1M Tris-HCL (pH 8.0) and 0.2ml of 0.5M EDTA (pH 8.0]) to 89.8ml ddH_2O

The main function of SDS is to dissolve hydrophobic material [283]. We took 1-3 loops of bacterial culture and added it to a 1.5ml tube, 500µl of TE Buffer was added to the bacterial culture, vortexed for 30sec., and then 25µl of lysozyme stock solution (20mg/ml) was added, which was then further mixed by overturning. It was incubated at 37°C for 30min with shaking. We then added 1% of SDS (25ul), placed it on spinning wheel at 4°C for 1h. After, 12.5µl proteinase-K stock solution 20mg/ml was added and then mixed thoroughly. We also Incubated at 65°C for 15min, briefly vortexed every 5min, then cooled to 37°C. Of the RNase stock solution (5µg/µl), 1µl was mixed thoroughly, and incubated at 37°C for 30min. Samples were placed on ice for 3-5 minutes before continuing with phenol extraction.

S4.1.1.2. Chemical lysis -SDS for overnight

The same as previous protocols (SDS for I hour), but the SDS was added on a spinning wheel at 4°C overnight [283].

S4.1.1.3. Centrifugation

We used centrifugation to separate bacteria from sediment particles before starting the process of the phenol extraction in order to get purer quality DNA for PCR amplification [284]. On the other hand, bacterial fractionation techniques may selectively remove cells that are easily dislodged, resulting in low efficiency of extraction, and bias in the composition of the purified DNA.

We added 1-3 loops of bacterial culture to 0.5 ml of TE buffer and vortexed for 30sec. The mixture was centrifuged at 1000g for 15min at 4°C. Then, we transferred the supernatant into a new tube, re-suspended the sediment with TE, vortexed for 30sec, and centrifuged again. We combined the supernatant from the two centrifugations and centrifuged them at 10,000g for 30sec at 4°C. The supernatant was later discarded. The Pellet was re-suspended by adding 0.5 ml of TE and vortexed for 30sec. The mixture was ready for direct process either by physical disruption (section S4.1.1.4.) or by physical and chemical disruption (section S4.1.1.5.). We then continued with the phenol extraction.

S4.1.1.4. Physical Disruption

Direct extraction helps to yield high amounts of nucleic acid more easily by directly lysing the bacteria that are in interaction with the sediment particles [283].

We added 0.5 ml of TE buffer to 1-3 loops of bacterial culture and vortexed well. Freeze and thaw steps were applied to our mixture, which caused the cell to swell and shrink, in the end breaking due to ice crystal formation during the freezing process. Several freeze-thaw cycles were needed to facilitate the cell membrane breakage, and this was done by adding the sample in -80°C for 10min, then in an 85°C water bath for 10min. Three freeze-thaw cycles were repeated. They were centrifuged for 5min at max speed, and the supernatant was transferred to a new tube for the phenol extraction process.

S4.1.1.5. Physical Disruption with Chemical

We followed the same steps as physical disruption (section S4.1.1.4.), but with added chemical process: 500µl of TE buffer and 25µl of lysozyme stock solution 20 mg/ml were added, and mixed by overturning. It was incubated at 37°C for 30min. After, 1% of the SDS was added and placed on spinning wheel at 4°C for 1h. We added 12.5µl of pro-K stock solution 20mg/ml and mixed thoroughly. It was incubated at 65-70°C for 15min, gently vortexed every 5min, then cooled to 37°C. Next, 1µl of RNase-A [5µg/µl] was added, mixed thoroughly, and incubated at 37°C for 30min. Samples were placed on ice for 3-5 minutes before continuing with phenol extraction [283].

S4.1.1.6. Beads

Beads are a harsher technique and is used to lyse membranes -more than chemical treatment alone- in order to improve the DNA of bacteria that is absorbed into sediment particles, especially from challenging soil types and gram-positive bacteria. One problem could be happened with using the beads, which is shearing the DNA into small fragments. This method started with 3 Sterilized beads using EtOH. The beads were placed in a 2ml screw cap tube once they were completely dry from the EtOH. Next, 0.5 ml of the Extraction buffer was added, with one sample tested alongside 1% CTAB, and one without 1% CTAB. We added 1-3 loops of bacterial culture with 0.5 ml TE buffer to the tube along with the beads. We used a tissue laser machine to shake the tube, using program 4, cycle 30, for 30sec and centrifuged at 13,000 rpm for 3min. The supernatant was transferred to a new 1.5 ml tube. A mixture of 25µl Lysozyme stock solution 20 mg/ml was added, mixed by overturning and incubated at 37°C for 30min. Finally, 1µl RNase- A [5µg/µl] was added, mix thoroughly, and incubated at 37°C for 30min, until ready to proceed with a phenol extraction.

S4.1.1.7. Hexadecyltrimethyl-ammonium bromide (CTAB)

CTAB is efficient in removing humic acid with no DNA loss [190]. The same steps as chemical treatment with SDS for 1h (section S4.1.1.1.) and SDS for overnight (section S4.1.1.2.), but with the addition of 1% CTAB (50µl) being added with the SDS stage Samples were placed on ice for 3-5min ready for phenol extraction.

S4.2. Results and Discussion

S4.2.1. DNA Extraction from Soils and Gel Analysis

To determine the optimal conditions for extracting high quality and quantity DNA, we first analyzed the ten different treatments used on cell lysis during the DNA extraction: chemical lysis (1 hour and overnight), centrifugation (with physical and with physical plus chemical treatment), physical disruption (freeze and thaw) or physical disruption with chemical lysis, Bead beatings, Bead beatings with CTAB, and the use of CTAB (one hour and overnight). DNA has been isolated from different sediment types such as, microbial mats, mangrove muds, and barren soil. By observing the absorbance of the 260 and 280 nm ($^{260}/_{280}$ ratio) of extracted DNA, we can get an idea about the purity of our DNA; it is accepted that a ratio of around 1.8nm ($^{260}/_{280}$ =1.8) is generally considered pure DNA. We found that the range of all $^{260}/_{280}$ ratios of our extracted DNA ranged between 0.22 and 1.6 nm, except for the ratio of the CTAB for overnight -based method which was between (1.87-1.96 nm), and is considered as 'pure' for DNA. By comparing the CTAB treatment to the other treatments, no difference in DNA yield was observed using the chemical, physical, Beads, and centrifugation treatments. On the other hand, with a CTAB treatment a high yield of DNA was observed with a high ratio of around 1.96, which represented the best-obtained result (Table S4.1) It is known that CTAB is important in removing humic acid [190, 285] and polysaccharides by employing high salt concentrations [286].

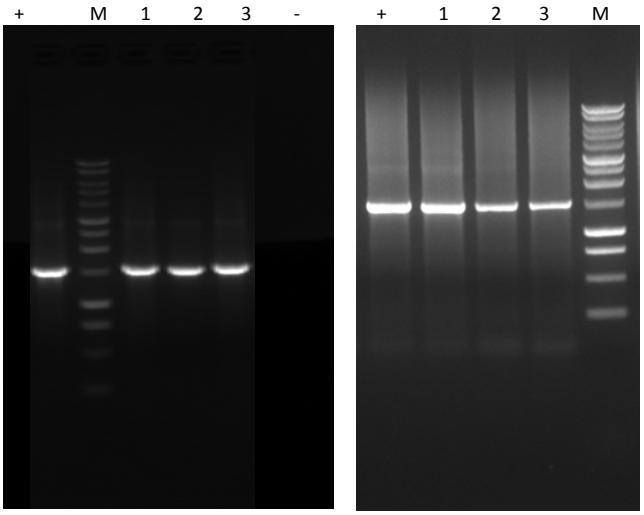
Table S4.1. Comparison of DNA yield from mangrove mud subjected to different treatment.

Treatment	**DNA yield (ng/µl)**	**A260/A280 ratio**
Chemical lysis 1h (C1)	29.3	1.12
C2	42.118	0.97

C3	30.334	1.02
Chemical lysis overnight (C1-ON)	17.629	1.15
C2-ON	14.688	1.31
C3-ON	15.95	1.08
Centrifugation with physical (Cent-P1)	13.072	0.99
Cent-p2	11.347	0.87
Cent-P3	14.147	0.87
Centrifugation with physical and chemical (Cent-PC1)	14.324	0.52
Cent-PC2	12.587	1.44
Cent-PC3	14.062	0.22
Physical (P1)	24.55	1.19
P2	12.875	0.74
P3	19.908	1.06
Physical and chemical (PC1)	19.322	1.43
PC2	15.688	1.59
PC3	10.61	1.36
CTAB for 1 h (CT1)	28.149	1.63
CT2	25.21	1.64
CT3	177.2	1.87
Beads with CTAB (B+CT1)	3.393	1.49
B+CT2	6.775	1.41
B+CT3	3.066	1.52
Beads only (B1)	4.771	0.8
B2	13.56	1.04
B3	6.846	0.82
CTAB overnight (CT1-ON)	23.02	1.96
CT2-ON	28.4	1.91
CT3-ON	48.36	1.87

S4.2.2. Universal amplification of bacterial 16s rRNA genes

Two universal primers were used to study bacteria in a diverse range of environments, such as 27f and 1492r, which allow amplification of the 16Sr RNA gene from the majority of known bacteria [287]. Figure S4.1 shows the PCR products for the ten DNA extraction treatments showing the best result with CTAB for overnight using the Qiagen® Fast Cycling PCR Kit. One band is observed as a result of the DNA amplification of the 16s rRNa genes (1465bp). Figure S4.1 represents an example of our PCR results showing products as a band size of ~1500 bp. Some of the samples were not successful in producing good amplifications with no band showing. PCR amplification of the 16S rRNA genes was successful, with DNA purified by PCR clean up using the NucleoSpin Extract II kit. This method produced DNA that was less pure. The $^{A260}/_{A280}$ ratio of DNA after the clean up was almost ≥ 1.8, indicating that the DNA was of good quality.



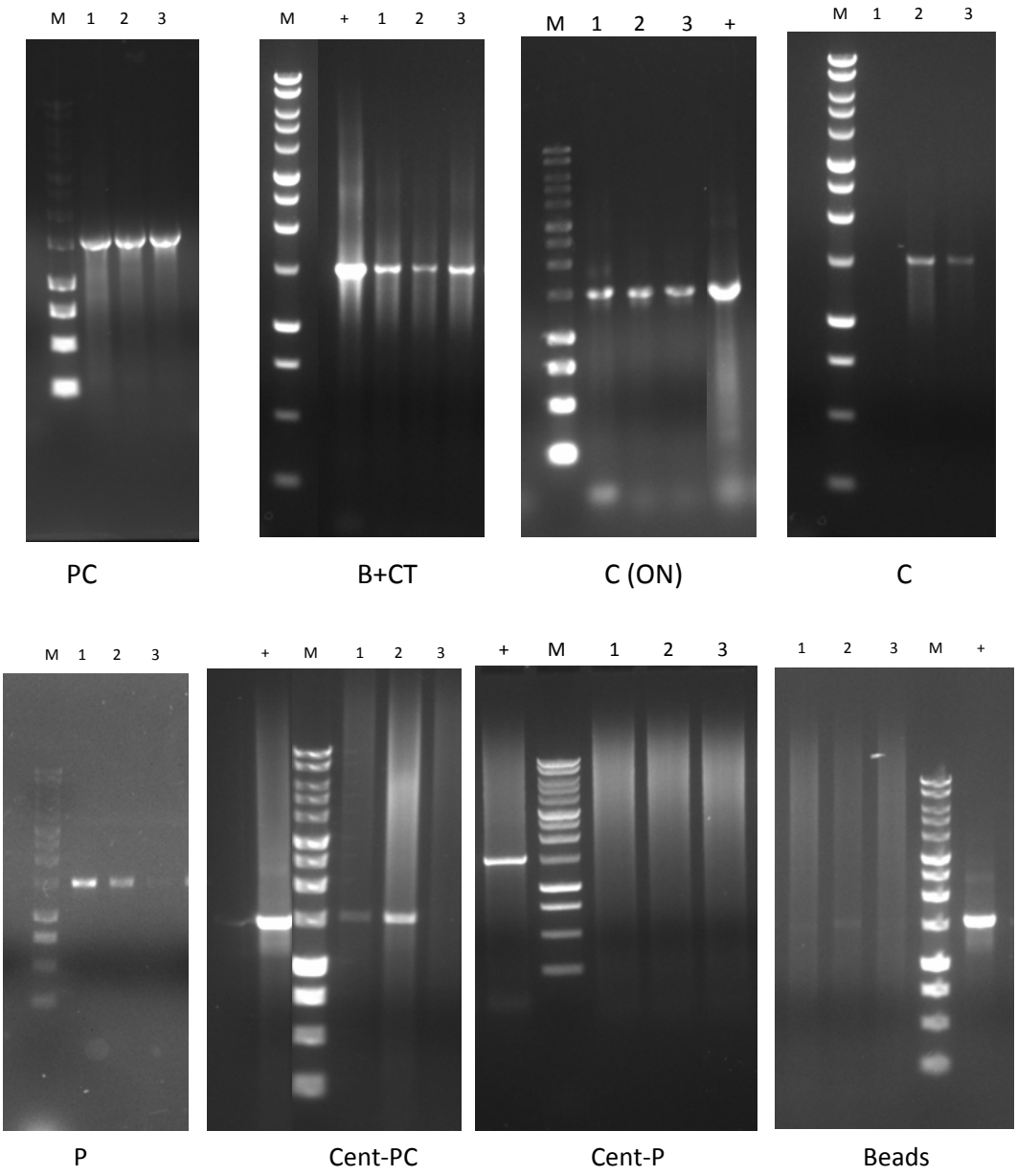


Figure S4.1. Agarose gel (1%) electrophoresis of 16S rRNA amplification products from bacteria found in Microbial Mat at Rhabigh Harbor from soil samples. positive control (E-coli). Bands mean genomic DNA was observed. No band mean PCR product was not observed.

Additional Files

This Supplementary contains the additional files for the Chapter 2, 3, 4 and 5

Supplementary Figures

Figure S2.1. Comparison between binning of predicted genes and 16s rRNA genes for taxonomic assignment.

Figure S4.2 Phylogenetic tree (Firmicutes phylum) shows the relationship among bacteria isolates The sequence alignment was performed using the SINA online tool and trees were built in the software environment for sequence data called ARB, starting from the ribosomal RNA gene database called SILVA SSU dataset Ref NR 99 (version 119) using MrBayes with 100,000 Markov Chain Monte Carlo (mcmc) cycles.

Figure S4.3 continued- Phylogenetic tree of Proteiobacteria phylum.

Figure S4.4 continued- Phylogenetic tree of Actenobacteria, Planctomycetes and Bacteroidetes phyla.

Supplementary Tables

Table S2.1. Taxonomy.

Table S2.2. Functions related to.

Table S2.3. Bioactive compounds.

Table S2.4. Metagenomic contigs associated with enzymes involved in antibiotic synthesis and resistance and the species from which they are derived**.**

Table S2.5. Metagenomic contigs associated with PKS and NRPS enzymes and the species from which they are derived.

Table S3.1. Morphological distinctiveness.

Table S5.1. Detailed information about all bacterial isolates.

Table S5.2. NRPS and PKS results of strains that exhibited antimicrobial activity.

Table S5.3. Genus abundance.