Metagenomics as a preliminary screen for antimicrobial bioprospecting

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1 Introduction

Mangroves and coastal microbial mat ecosystems are recognized as high biodiversity hotspots (Kennedy et al., 2008; Bolhuis et al., 2014). 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 (Alongi, 1988; Canfield and Des Marais, 1993; Varin et al., 2010; Gallo et al., 2013). In the same context, the structure of sediment microbial communities is strongly influenced by the availability of nutrients, anthropogenic and ecological properties (Cordova-Kreylos et al., 2006; Ghosh et al., 2010). The microbial inhabitants of both mangrove mud (MN) and microbial mat (MM) ecosystems have been assessed through several cultivation approaches. However, metagenomics combined with phylogenetic studies reveals that < 1% of bacterial diversity has been successfully cultured to date (Amano et al., 1995). Metagenomic approaches have facilitated a more comprehensive and culture-independent description of the microbial populations present in mangroves (Andreote et al., 2012; Xu et al., 2014; Basak et al., 2015; Simoes et al., 2015; Alzubaidy et al., 2016) and coastal MM ecosystems (Bauersachs et al., 2011; Bolhuis and Stal, 2011; Armitage et al., 2015; Simoes et al., 2015; Alzubaidy et al., 2016) and coastal MM ecosystems (Bauersachs et al., 2011; Bolhuis and Stal, 2011; Armitage et al., 2015; Simoes et al., 2015; Alzubaidy et al., 2016). Heterologous cloning and expression of metagenomic samples have been used for functional-based or sequence-based screening (Couto et al., 2010; Ye et al., 2010; Li et al., 2012; Mai et al., 2014; Goncalves et al., 2015). 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. (2011) used mangrove metagenomics to identify bacterial genera that could be possible signals for the biomonitoring of oil pollution of mangroves. Abed et al. (2011) 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 (Hemala et
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 (Fig. 3). For each location, samples were collected from two mangrove- and microbial mat-associated sediment samples and 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 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 Rabigh harbor lagoon (RHL). The second site, Al Kharrar lagoon (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. 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, MKN, MMR, and MMK.

2.3. Data trimming and quality control

Pre-assembly quality control (QC) was performed on the Illumina HiSeq raw reads using the Trimmomatic ver. 0.33 (Bolger et al., 2014). 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 (Andrews, 2010) was also used to visualize the data quality and assurance, pre and post trimming.

2.4. Metagenome assembly and post assembly quality control

Metagenome paired end read data was assembled using a MegaHit de-novo assembler (Li et al., 2015). In total, 12 metagenome samples were assembled. We opted to assemble each of the replicates separately, because by pooling the duplicates together we would lose all information on variability and hence will be of little use for statistical purposes (Thomas et al., 2012). The assemblies were performed using the metasensitive option - k-mers of 21, 41, 61, 81 and 99. In the post assembly stage, we removed all contigs with lengths of ≤1000 bp in order to remove fragmented sequences which can introduce false positives during downstream analyses. Additionally, we used the UCHIME module in MOTHUR (Schloss et al., 2009) 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 (MSNR) (Wilk et al., 2012) using the Burrows-Wheeler Alignment tool (BWA) (Li and Durbin, 2009) in METAGENAssist (Amdt et al., 2012).

2.5. 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 (Alam et al., 2013). The AAMG includes annotation from various databases such as InterProScan (Quevillon et al., 2005), Gene Ontology (The Gene Ontology, 2000), KEGG (Mao et al., 2005), MetaCyc (Krieger et al., 2004) and Pfam (Bateman et al., 2004). 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 (Storey, 2002). 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 polyketide synthase (PKS), non-ribosomal peptide synthetase (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, MNR, MNN and MNRK 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 (Zimin et al., 2008; Yao et al., 2012). 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 (Treangen et al., 2011). 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 (Rho et al., 2010) and Meta_RNA (Huang et al., 2009) 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 (Alam et al., 2013). We opted for this method because of its 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 (Huson et al., 2007; Fancello et al., 2012). 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 Supplementary 5.

2.6. Diversity calculation

To compare the diversity between the AKL and RHL environments, we used the Whittaker concept of diversity (Whittaker, 1972). 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 (Whittaker, 1972). 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 (Whittaker, 1972). 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 (Shannon, 2001). 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.

2.7. BTEX analysis

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, 2 g from each sediment sample were added to NaCl/water liquid. The total run time to analyze the samples via GC was 22 min, with a split ratio of 20:1 using the J&W 29505-USL8-JL60-SUPERLCO column (250 °C: 30 m × 250 μm × 0.2 μm). For acquisition and SIM parameters, refer to Supplementary 4.

3. Results and discussion

3.1. 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 101 bp. After trimming, a total of 419.33 million sequences were obtained: 67.22 million reads for MNK, 59.85 million reads for MMR and 58.20 million reads for MNR (see Table 1). 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, MMK and MMR 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 MNK, MMK, MMR and MMR were distinct from each other, with replicates displaying lower variability at the genus level (Fig. 1a). The distinction between microbial communities of the four samples is further augmented at the species level as depicted by the rarefaction curves (Fig. 1b), and sediment specific and location specific alpha, beta and gamma diversity calculations (Table 2).

Our results reveal that MNK has a significantly higher species diversity compared to MMR (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.

Our observation supports a previous study (Saintilan, 2009), 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 (King et al., 1983). Additionally, the low marsh tends to be dominated and influenced by autotrophs that limit nutrient resources available to non-autotrophic species (Zedler et al., 2008).

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) to 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 Halobacteria (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 Methanobacteria (Euryarchaeota) and Thermococcales (Euryarchaeota) were only enriched in the AKL samples. Moreover, Crenarchaeales (Thaumarchaeota), Nitrosopumilales (Thaumarchaeota) and Thermoproteales (Crenarchaeota) were specifically enriched in MNK. For bacteria, the phyla dominant in RHL and AKL

Table 1
The assembly statistics.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pre-trimming #Reads</th>
<th>Post-trimming - #Reads</th>
<th>% #Reads dropped</th>
<th>N50</th>
<th>Total size (Mb)</th>
<th>Min length (bp)</th>
<th>Max length (bp)</th>
<th>Average length (bp)</th>
<th>#contigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNK1</td>
<td>19,964,711</td>
<td>19,882,619</td>
<td>0.41</td>
<td>1901</td>
<td>16,785,778</td>
<td>1000</td>
<td>43,022</td>
<td>1802</td>
<td>8874</td>
</tr>
<tr>
<td>MNK2</td>
<td>25,051,699</td>
<td>24,925,293</td>
<td>0.50</td>
<td>2448</td>
<td>13,844,845</td>
<td>1000</td>
<td>63,525</td>
<td>2340</td>
<td>5916</td>
</tr>
<tr>
<td>MNK3</td>
<td>190,682,699</td>
<td>189,259,143</td>
<td>0.75</td>
<td>2410</td>
<td>502,912,101</td>
<td>1000</td>
<td>107,678</td>
<td>2236</td>
<td>224,901</td>
</tr>
<tr>
<td>MMK1</td>
<td>22,887,831</td>
<td>21,802,193</td>
<td>1.29</td>
<td>3592</td>
<td>34,092,493</td>
<td>1000</td>
<td>50,463</td>
<td>2815</td>
<td>12,110</td>
</tr>
<tr>
<td>MMK2</td>
<td>14,861,974</td>
<td>14,771,142</td>
<td>0.61</td>
<td>3504</td>
<td>33,811,219</td>
<td>1000</td>
<td>173,647</td>
<td>2774</td>
<td>12,195</td>
</tr>
<tr>
<td>MMK3</td>
<td>21,734,064</td>
<td>21,629,985</td>
<td>0.48</td>
<td>2421</td>
<td>62,646,981</td>
<td>1000</td>
<td>60,925</td>
<td>2225</td>
<td>28,159</td>
</tr>
<tr>
<td>MNR1</td>
<td>22,163,310</td>
<td>21,993,504</td>
<td>0.77</td>
<td>2406</td>
<td>49,170,138</td>
<td>1000</td>
<td>79,639</td>
<td>2257</td>
<td>21,784</td>
</tr>
<tr>
<td>MNR2</td>
<td>24,396,180</td>
<td>24,101,284</td>
<td>1.21</td>
<td>2591</td>
<td>84,012,947</td>
<td>1000</td>
<td>127,238</td>
<td>2389</td>
<td>35,160</td>
</tr>
<tr>
<td>MNR3</td>
<td>21,300,172</td>
<td>21,123,037</td>
<td>0.88</td>
<td>1544</td>
<td>16,962,471</td>
<td>1000</td>
<td>44,153</td>
<td>1587</td>
<td>10,690</td>
</tr>
<tr>
<td>MMR1</td>
<td>22,292,713</td>
<td>22,068,422</td>
<td>1.01</td>
<td>3080</td>
<td>60,115,822</td>
<td>1000</td>
<td>162,858</td>
<td>2551</td>
<td>23,863</td>
</tr>
<tr>
<td>MMR2</td>
<td>17,468,092</td>
<td>17,128,022</td>
<td>1.95</td>
<td>2387</td>
<td>32,813,842</td>
<td>1000</td>
<td>81,568</td>
<td>2264</td>
<td>14,492</td>
</tr>
<tr>
<td>MMR3</td>
<td>20,793,784</td>
<td>20,650,379</td>
<td>0.69</td>
<td>2786</td>
<td>72,564,776</td>
<td>1000</td>
<td>104,985</td>
<td>2443</td>
<td>29,706</td>
</tr>
</tbody>
</table>
samples differed (Fig. 2). 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) (Fig. 2). The complete list of taxonomy for each of the four samples is given in Supplementary 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 (Alzubaidy et al., 2016). This mangrove located in Thuwal represents a pristine mangrove. Our results are not in general agreement with this study regarding 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

Table 2

<table>
<thead>
<tr>
<th>Sediment specific diversity</th>
<th>Location specific diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>β</td>
</tr>
<tr>
<td>MNR</td>
<td>319.47</td>
</tr>
<tr>
<td>MNK</td>
<td>620.78</td>
</tr>
<tr>
<td>MMR</td>
<td>217.82</td>
</tr>
</tbody>
</table>
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 (Bolhuis and Stal, 2011; Babauta et al., 2014; Bolhuis et al., 2014). 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 (Abed et al., 2002; Grotzschel et al., 2002). Also, Acosta-Gonzalez et al. (2013) 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 MM 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.

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 3). Also, when comparing MMR and MMK, 19 genera were enriched in one of these samples compared to the other (Table 4).

For the MNR sample we found an enrichment of the Cyanobacterial class Oscillatorioğphyceae (genera Dactylococcopsis and Halothece), while for the MMK sample we found only an enrichment of the Cyanobacterial class Gloeobacteria (genus unclassified) (Table 3). MMK samples showed the same enrichment as MNK, while MM samples also showed enrichment of Cyanobacteria genera from class Oscillatorioğphyceae (genera Microcoleus, Coleofasciculus and Lyngbya) when compared to MMK samples. Studies have reported increased cyanobacterial counts and dramatic shifts in the composition of the cyanobacterial community, induced by exposure to hydrocarbon contamination (Abed et al., 2002; Grotzschel et al., 2002). Moreover, studies have reported the degradation of aromatic compounds by cyanobacteria, such as Anabaena cylindrica, Phormidium faveolarum and Oscillatoria sp. strain JCM (Cerniglia et al., 1980; Radwan and Al-Hasan, 2002). Cyanobacteria Microcoleus chthonoplastes and Phormidium corium, isolated from oil-contaminated sediments, were also shown to degrade n-alkanes (Al-Hasan et al., 1998), as well as Aphanathece halophytica, Dactylococcopsis salina, Halothecce strain EPUS, Oscillatoria strain OSC, and Synechocystis strain UNIGA (Abed and Köster, 2005). Cyanobacteria 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 nitrogen 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 (OREN, 2006; Garby et al., 2013).

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,*

![Fig. 3. Maritime map showing location of two sample sites depicted by the red flags.](image)

Table 3  
Log Odds Ratio for mangrove (MNK versus MNR) enriches microbial classes and genera.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Genus</th>
<th>Log Odds Ratio</th>
<th>p-Value</th>
<th>Q-value (FDR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroidetes</td>
<td>Flavobacteria</td>
<td><em>Flavobacterium</em></td>
<td>−1.11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Sphingobacteria</td>
<td><em>Rhodothermus</em></td>
<td>−3.074</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Sphingobacteria</td>
<td><em>Salinibacter</em></td>
<td>−4.531</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Cytophagia</td>
<td><em>Pontibacter</em></td>
<td>−1.004</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Cytophagia</td>
<td><em>Fulvivirga</em></td>
<td>0.129</td>
<td>1.00E-04</td>
<td>1.00E-04</td>
</tr>
<tr>
<td>Chloroflexi</td>
<td>Caldilineae</td>
<td><em>Caldilinea</em></td>
<td>1.767</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>Oscillatorophyceae</td>
<td><em>Dactylococcopsis</em></td>
<td>−4.652</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>Oscillatorophyceae</td>
<td><em>Halothecae</em></td>
<td>−5.386</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gemmatimonadetes</td>
<td>Gemmatimonadales</td>
<td><em>Gemmatimonas</em></td>
<td>0.654</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Delta-proteobacteria</td>
<td><em>Desulfovococcus</em></td>
<td>3.493</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>Delta-proteobacteria</td>
<td><em>Desulfoboacterium</em></td>
<td>3.607</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Spirochaetes</td>
<td>Spirochaetales</td>
<td><em>Spirochaeta</em></td>
<td>2.941</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 4
Log Odds Ratio for microbial mat (MMK versus MMR) enriches microbial classes and genera.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Genus</th>
<th>Log Odds Ratio</th>
<th>p-Value</th>
<th>Q-value [FDR]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroidetes</td>
<td>Sphingobacteria</td>
<td>Rhodothermus</td>
<td>−0.832</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Sphingobacteria</td>
<td>Salinibacter</td>
<td>−0.446</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>Cytophagia</td>
<td>Cyclobacterium</td>
<td>−2.019</td>
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<td>0</td>
</tr>
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<td>Cologfuscicula</td>
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<tr>
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<td>Planctomycetca</td>
<td>Planctomyces</td>
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<td>Alpha-proteobacteria</td>
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</tr>
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<td>Delta-proteobacteria</td>
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<td>Spirohaetales</td>
<td>Spirohaeta</td>
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</table>

Cesiribacter and Cyclobacterium, Flavobacteria (Nonlabens), Sphingobacteria (genera Salinibacter and Rhodothermus) and Bacteroidia (genus unclassified) compared to the MMK sample (see Table 4). 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) (Quero et al., 2015). There, results revealed that the core microbiome included Clostridia, Cytophaga, Flavobacteria, Archaea (within the classes Methanobacteria and Methanomicrobia) and several classes within the phylum Proteobacteria. Abed et al. (2006) reported a similar microbiome (Beta-, Gamma- and Delta-proteobacteria, Cytophaga-Flavobacteriia-Bacteroidiia group and Spirochaetes) for a cyanobacterial mat, which degraded petroleum compounds at elevated salinity and temperature, sampled from the Saudi Arabian coastline. Abed et al. (2014) also reported the significant Sphingobacteria counts in oil-contaminated cyanobacterial mat in a constructed wetland. Hemalatha and VeeraManikandan (2011) 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 Cytophaga, Flavobacteria and Sphingobacteria, are key contributors in the hydrocarbon-degrading microbiome.

The MNK showed an enrichment of Proteobacteria genera when compared to the MNR sample, specifically from the class Delta-proteobacteria (Desulfococcus, Desulfobacterium), followed by a genus from the class Zeta-proteobacteria, Beta-proteobacteria, Alpha-proteobacteria and Gamma-proteobacteria (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 Alpha-proteobacteria (genera Skermanella and Azospirillum). Likewise, MMK showed enrichment of Proteobacteria genera when compared to the MMR sample, specifically from the class Delta-proteobacteria (Desulfococcus) and followed by a genus from the class Zeta-proteobacteria, Beta-proteobacteria and Gamma-proteobacteria (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 Delta-proteobacteria dominated the deep-sea plume (Hazan et al., 2010; Baelum et al., 2012; Mason et al., 2012; Redmond and Valentine, 2012; Gutierrez et al., 2013).

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 (Plotnikova et al., 2001; Sass et al., 2008). 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 (Kumar et al., 2007). In the same report, no growth was observed when culturing was done on toluene, phenol, 2-hydroxyquinoline and carbazole.

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 (Li et al., 2015). 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) (see Supplementary 4 for results), 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.

4. Metabolism of aromatic compounds

The commonly reported pollutants in the Red Sea (Dicks, 1986) 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 hints 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 Supplementary 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 arctica)” and Benzoate degradation (see Supplementary 2). Also, “Styrene degradation” is significantly enriched in the MMK sample compared to MMR. Other aromatic compound degradations were also observed such as Toulene degradation, Ethylbenzene degradation and Xylene degradation. These findings make sense as anaerobic hydrocarbon-degrading Delta-proteobacteria were 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.

5. 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, cyto-toxins, toxins and pigments (Gallo et al., 2013; Manivasagan et al., 2014). We assessed the percentage of sequences that matched: 1/ PKS and NRPS enzymes, 2/ enzymes involved in antibiotic synthesis and antibiotic resistance pathways. Table 5 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 resistance 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 Supplementary 3.

The results described in Tables 5, 6 and 7 show high microbial species diversity. For example, in Table 6, we observed 243 sequences associated with antibiotic synthesis enzymes derived from 45 species. Similarly, in Table 5, we observed ~13% or 28 of MMR enzymes sequences for PKS being derived from 13 Cyanobacteria species (see Supplementary 6 and 7).

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 Tables 6–7). 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 ring in the phyla of interest compared to the odds of antibiotic biosynthesis activity occurring in Actinobacteria, represented as the odds ratio in Table 5. The odds ratio was determined for MNK, MNK, Thuwal – mangrove (MNT) (Alzubaidy et al., 2016), Lake Washington - Freshwater lake (LWF) (Wakeham, 1977) and Lake Tyrell - Hypersaline lake (LTHS) (Podell et al., 2014as).

We observe that locations associated with exposure to hydrocarbon contamination (MMK, 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 Actinobacteria. 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 9). Here it should be noted that Mason et al. (2014) 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. (2013) 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. (2013) 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.

### Table 5

<table>
<thead>
<tr>
<th>Phylum</th>
<th>MNK (n = 30)</th>
<th>MNR (n = 140)</th>
<th>MMR (n = 209)</th>
<th>MNT (n = 209)</th>
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<tbody>
<tr>
<td>% antibiotic synthesis</td>
<td>3.33%</td>
<td>5.00%</td>
<td>10.05%</td>
<td>9.05%</td>
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<tr>
<td>% antibiotic resistance</td>
<td>81.33%</td>
<td>70.71%</td>
<td>67.94%</td>
<td>64.57%</td>
</tr>
<tr>
<td>% PKS</td>
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<td>17.86%</td>
<td>13.40%</td>
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<tr>
<td>% NRPS</td>
<td>3.33%</td>
<td>7.86%</td>
<td>9.09%</td>
<td>9.05%</td>
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### Table 6

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<th>Phyla</th>
<th>Number of sequences associated with antibiotic synthesis enzymes</th>
<th>Number of sequences associated with antibiotic resistance</th>
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<td>MNK</td>
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### Table 7

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contamination levels are still high) and the dominant phyla in RHL
phyla in AKL being consistent with early hydrocarbon exposure (when
displayed more contamination than RHL. This may be due to dominant
phyla that should be targeted are increased at site exposed to hydrocar-
being consistent with late hydrocarbon exposure (when contamination
levels are lower as a result of an extended period of hydrocarbon degra-
dation). Additionally, RHL samples showed higher counts for PKS and
NRPS matches in comparison to AKL samples, even though differences
were minimal. However, both MNK 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 an-
timicrobial 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 phylum for antimicro-
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 hydrocar-
bon 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.

For future work, we will use traditional culturing methods to experi-
mentally screen 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.

6. 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 homogenous; each particular locality ex-
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Specifically, we show a shift in dominant phyla consistent with a his-
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synthesis enzymes.
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2016.09.021.

Availability of data

The assembled metagenomic contigs can be found in BioProject ID: PRJNA321092.

Conflict of interest

The author(s) declare that they have no competing interests.

Funding

This publication is based upon work supported by the King Abdullah University of Science and Technology (KAUST) Office of Sponsored Research (OSR) under Awards No URF/1/1976-02 and FCS/1/2448-01.

Acknowledgements

Not applicable.

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