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Research paper

Rhizosphere microbiome metagenomics of gray mangroves (*Avicennia marina*) in the Red Sea

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ABSTRACT

Mangroves are unique, and endangered, coastal ecosystems that play a vital role in the tropical and subtropical environments. A comprehensive description of the microbial communities in these ecosystems is currently lacking, and additional studies are required to have a complete understanding of the functioning and resilience of mangroves worldwide.

In this work, we carried out a metagenomic study by comparing the microbial community of mangrove sediment with the rhizosphere microbiome of *Avicennia marina*, in northern Red Sea mangroves, along the coast of Saudi Arabia. Our results revealed that rhizosphere samples presented similar profiles at the taxonomic and functional levels and differentiated from the microbiome of bulk soil controls. Overall, samples showed predominance by Proteobacteria, Bacteroidetes and Firmicutes, with high abundance of sulfate reducers and methanogens, although specific groups were selectively enriched in the rhizosphere. Functional analysis showed significant enrichment in 'metabolism of aromatic compounds', 'mobile genetic elements', 'potassium metabolism' and 'pathways that utilize osmolytes' in the rhizosphere microbiomes.

To our knowledge, this is the first metagenomic study on the microbiome of mangroves in the Red Sea, and the first application of unbiased 454-pyrosequencing to study the rhizosphere microbiome associated with *A. marina*. Our results provide the first insights into the range of functions and microbial diversity in the rhizosphere and soil sediments of gray mangrove (*A. marina*) in the Red Sea.

1. Introduction

Mangroves are unique inter-tidal ecosystems that cover nearly 60%–70% of the Earths' tropical and subtropical coasts (Andreote et al., 2012). These ecosystems are of ecological importance as they buffer seagrass beds and coral reefs against the impact of river-borne siltation, as well as protect coastal communities from sea-level rise, storm surges, and tsunamis (Basak et al., 2014; Dias et al., 2012; Dahdouh-Guebas et al., 2005; dos Santos et al., 2011; McLeod and Salm, 2006). Despite such widely recognized benefits, mangroves across the globe are seriously threatened, with reported losses exceeding 35% during the last quarter century (UNFAO, 2003) and being critically endangered or approaching extinction in 26 out of the total 120 countries with mangrove forests (Duke et al., 2007). As coastal ecosystems, they are particularly exposed to the convergence and accumulation of toxic compounds generated by anthropogenic activities (Basak et al., 2014; dos Santos et al., 2011). Current urban and industrial developments along the coast combined with the effects of climate change increase the threat of extinction of mangroves as well as the need to conserve, protect, and restore them (Basak et al., 2014; Dias et al., 2012; dos Santos et al., 2011; Duke et al., 2007, Parmesan and Yohe, 2003).

The Red Sea coast lies at the geographical limits of the mangrove growth, hosting the northernmost mangals in the Indo-Pacific (Fouda and Gerges, 1994). Studies on these mangroves remain relatively scarce. Nevertheless, it is well established that *Avicennia marina* is the predominant, and frequently the only, plant species present (Price et al., 1987). Surveys along the Red Sea coast report a northwards decrease in

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mangrove development with forests becoming patchier and plants displaying decreased height- signs of population subjected to higher stress such as lower temperatures and nutrient availability, as well as higher salinities and pollution levels (Khan et al., 2010; Kumar et al., 2010; Price et al., 1987).

A. marina, also known as gray mangrove, grows in *multiple* areas across the globe. It is known for its particularly pronounced tolerance to extreme and harsh environmental conditions, such as: salinity, aridity, water temperature, and frost frequency (Nguyen et al., 2015). Furthermore, its capacity to absorb and accumulate high quantities of heavy metals plays a vital role in cleansing the coastal environment and makes it a promising candidate for the purpose of phytostabilization of industrially polluted coastal shores (Almahasheer et al., 2013).

The sturdiness of mangrove plants, such as *A. marina*, has been related to their inherent physiological resistance and beneficial interactions with associated and specific microbial communities (Gomes et al., 2010). Nonetheless, little is known about the microbiology of mangroves. Most surveys have been based on culture-dependent techniques, fingerprinting methods, and use of clone libraries to analyze phylogenetic and functional genes (e.g. Basak et al., 2014; Dias et al., 2011, 2012; Ghizelini et al., 2012; Gomes et al., 2010; Liang et al., 2007). Yet, due to sampling and methodological limitations, results from such studies are known to be biased and not representative of the total microbial diversity (Basak et al., 2014). Andreote et al. (2012) made a substantial contribution in this field with the characterization of the total microbial diversity of a pristine mangrove ecosystem through metagenomic analysis. However, to date, a comprehensive description of the microbial life in the mangrove ecosystem is lacking and more studies are in need to have a complete understanding of the functioning and resilience of mangroves worldwide.

In this study we compare the microbiome associated with the rhizosphere of *A. marina* with control bulk soil collected from locations in the northern Red Sea. Our approach was based on metagenomic analysis, obtained by 454-pyrosequencing, which provide new insights into the functional and microbial diversity in mangroves and factors that might condition them. To our knowledge, this is the first metagenomic study on the microbiome of mangroves in the Red Sea, and the first application of unbiased 454-pyrosequencing to study the rhizosphere microbiome associated with *A. marina*.

2. Materials and methods

2.1. Site description and soil sample processing

Sediment samples were collected along a 978 m transect of mangrove shore, in Thuwal, Saudi Arabia, in December 2011. Sampling locations (Fig. 1) include four different *A. marina* rhizosphere samples (RSMgr01: 22°18′691″N, 39°5′444″E; RSMgr02: 22°18′722″N, 39°5′462″E; RSMgr03: 22°19′108″N, 39°5′450″E; RSMgr04: 22°19′133″N, 39°5′449″E), and two bulk soil control samples (CS01: 22°18′693″N, 39°5′401″E; CS02: 22°18′712″N, 39°5′401″E). At each site, samples were collected from a 10 cm depth.

After collection into sterile plastic bags, the samples were stored at 4 °C prior to processing within 12 h.

2.2. Chemical analysis

Environmental parameters were measured for samples RSMgr01, RSMgr02, CS01 and CS02. A 1:10 soil to water ratio was used to homogenize the sample, which was stirred for 10 min. Then, Temperature, pH and conductivity were measured using the 5 Star pH/ISE/ORP/DO Conductivity Portable Meter (Thermo Scientific, USA). Salinity was calculated according to the Standard Methods for the Examination of Water and Wastewater (APHA, 1998). Nitrate was measured by

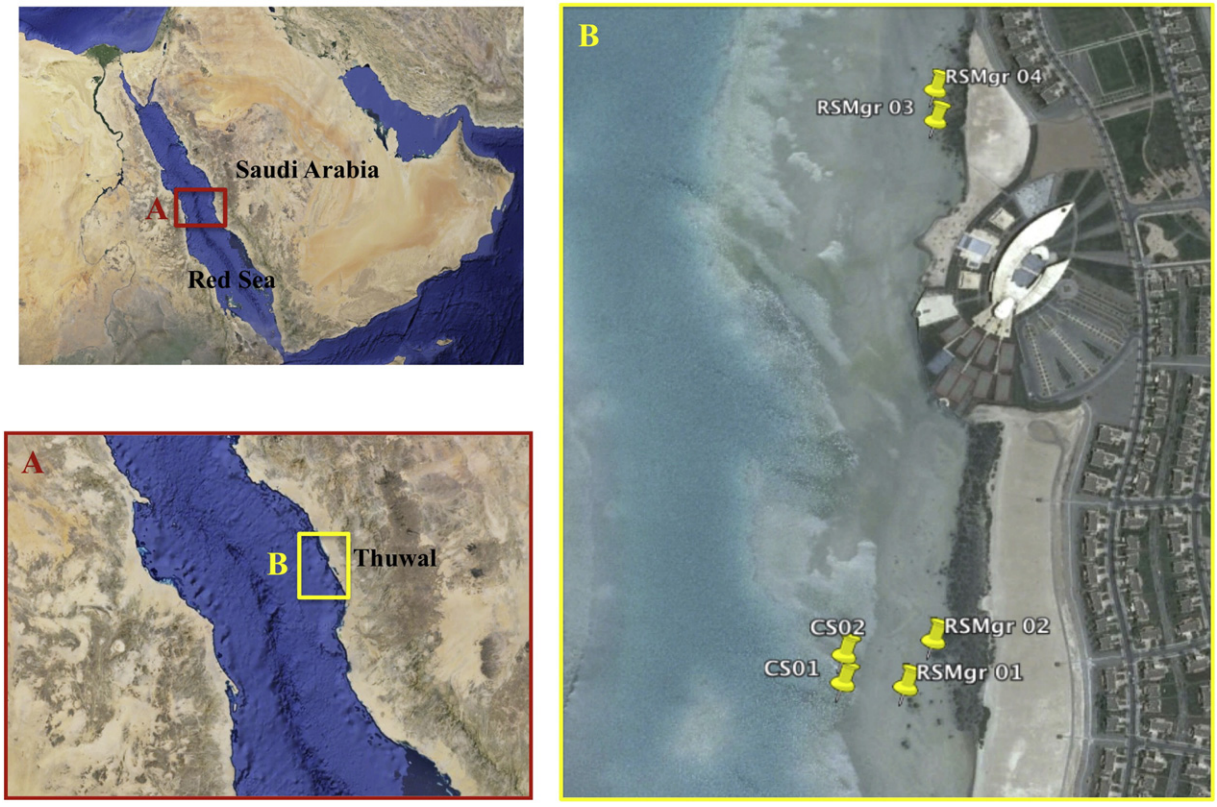

Fig. 1. Satellite map of the Red Sea and detail of: A) Saudi Arabia and B) Thuwal coast, the source of Mangrove samples used in this study. Pictures were generated on Google Earth, retrieved on January 26, 2015. Yellow marks represent the sampling sites of bulk soil sediment (CS) and rhizosphere soil sediment of *A. marina* (RSMgr) samples.

suspending 3 g of air dried soil in 30 mL of $CaSO_4$ (0.01 M) for 10 min, followed by filtration and measurement using the Autoanalyzer/Photometric Analyzer, Aquakem250 (Thermo Scientific, USA). Magnesium, Potassium, Sulfur and Phosphorous were measured by microwave-assisted digestion method, using freshly prepared aqua regia (1:3 volume mixture of 37% HCl (trace metal grade, Fisher Scientific) and 69% HNO_3 (trace metal grade, Fisher Scientific), respectively) that was added to the pre-weighed samples.

For digestion, 0.3 g of each sample were weighed into a Teflon digestion container; and, 1 mL of HNO_3 and 3 mL of HCl were added. After, the containers were closed and the samples were heated on microwave (Milestone–Ethos 1) (program: heating at 10 min to 175 °C and holding at 175 °C for 10 min; power: 1000 W for both steps). After digestions, samples were transferred to volumetric test tubes and diluted to a final volume of 50 mL with 18.2 MΩ·cm (milliQ element) water. The concentrations of trace metals were determined, using Inductively Coupled Plasma Optical Emission Spectrometer (Varian 720-ES ICP OES, Australia). The instrument conditions were: power (KW) 1.2, plasma flow (L/min) 1.65, auxiliary flow 1.5, nebulizer flow (L/min) 0.7, sample uptake delay (L/s) 70, pump rate (rpm) 15 and rinse time (s) 45. Organic matter was measured using the Loss of Ignition Method (Dean, 1974). A 30 g wet soil sample was weighed into a tared quartz crucible, dried for 1 h at 105 °C and weighed, then ashed at 550 °C for 1 h and reweighed. The resulting loss of weight was the estimate of the organic matter.

2.3. DNA extraction and sequencing

DNA was extracted from 5 g of soil using the ZR Soil Microbe DNA MidiPrep kit (Zymo Research Corporation, Irvine, CA, USA), and in accordance with the manufacturer's protocol. The extracted DNA was quantified using NanoDrop (Thermo Scientific, USA) spectrophotometer. The DNA samples were subjected to pyrosequencing using 454 GS FLX Titanium technology at the Bioscience Core Laboratory in King Abdullah University of Science and Technology (Thuwal, Saudi Arabia). Each sample was deeply sequenced to capture broad ranged taxonomic variation. Half of each 454 plates were allocated per sample.

2.4. Taxonomic assignment of metagenomic sequences

Unassembled raw pyrosequencing data was processed using the MG-RASTv3.3 pipeline (Meyer et al., 2008). Low quality reads were removed using Dynamic Trim at a Phred score of 15 (Cox et al., 2010), followed by dereplication using the algorithm described by Gomez-Alvarez et al. (2009). A table of the frequency of hits to each individual taxon (taxonomy) or subsystem (function) for each metagenome dataset was generated. Obtained data were normalized against the total number of reads in each dataset. Reads encoding SSU rDNA sequences were aligned against the SSU SILVA database (Pruesse et al., 2007) and ribosomal sequences retrieved were filtered with an e-value of 10^{-20}, 97% identity cutoff and 50 bases minimum alignment length. Similarly, for taxonomic assignment of the complete metagenome datasets, reads were aligned against the M5nr database (Wilke et al., 2012). Sequences retrieved were filtered with e-value of 10^{-10}, 60% identity cutoff and 50 bases minimum alignment length. Reads were classified using the Representative Hit algorithm (Meyer et al., 2008).

Results were further analyzed using the Statistical Analyses of Metagenomic Profiles (STAMP) (Parks and Beiko, 2010) tool to identify biologically relevant differences between the microbial populations of the studied samples. As recommended by the STAMP developer, statistical assessment of the data was performed, using Two-sided Fisher's Exact Test, and Storey's FDR method was used to correct for multiple tests. Taxa with q-value = <0.05 were deemed as significant at genus and species levels. The Data is available on the MG-RAST server with the following accession numbers: 4523017.3-4523020.3, for RSMgr01-RSMgr04 and 4506447.3-4506448.3 for CS01 and CS02 respectively.

In addition to MG-RAST's functional annotation, we also used our own KAUST Automatic Annotation of Microbial Genomes (AAMG) pipeline (Alam et al., 2013), optimized for metagenomic data (MetaAAMG). This pipeline filtered the input metagenomic data using prinseq (Schmieder and Edwards, 2011) for low quality sequence reads and shorter read length. Prokaryotic RNA is then predicted using metaRNA (Huang et al., 2009) and tRNAscan-SE (Schattner et al., 2005) and Infernel (Nawrocki and Eddy, 2013). Open Reading Frames (ORFs) were predicted using metagenomic mode of prodigal (Hyatt et al., 2012) and MetaGeneAnnotator (Noguchi et al., 2008). RNA predictions were compared with latest public RNA databases such as SILVA (Pruesse et al., 2007), GreenGenes (DeSantis et al., 2006), RDP (Maidak et al., 1996) and Rfam (Burge et al., 2013) using Nucleotide BLAST. ORFs were compared to latest version of microbial UniProt/Trembl (The UniProt, 2014), KEGG (Kanehisa et al., 2014) and Conserved Domain Database (CDD) (Marchler-Bauer et al., 2011). Furthermore, Domains and Gene Ontology assignment was performed using high throughput Interproscan (Apweiler et al., 2000; Hunter et al., 2009) setup. The computing of intensive BLAST jobs was accelerated by parallel processing of metagenomic reads by first making sequence subsets (e.g. 10,000 sets), each compared to public databases using a cycle of multi-core jobs (job array) with a large number of these job arrays being run all at once.

2.5. Functional analysis of metagenomic sequences

The complete set of metagenome reads was functionally annotated using the SEED Subsystems database (Overbeek et al., 2005). Identification of enriched functions was performed using the STAMP tool as stated above.

Osmolytes and their associated pathways were identified using an in-house developed tool: Dragon Explorer of Osmoprotectant associated Pathways (DEOP) (http://www.cbrc.kaust.edu.sa/deop/).

3. Results and discussion

3.1. Environmental parameters at Red Sea mangrove study sites

Chemical composition and environmental analysis were determined for two *A. marina* rhizosphere samples (RSMgr01 and RSMgr02), and control samples (CS01 and CS02) (Table 1).

For all sites, the measured temperature was constant at 21.2 °C. The pH of the rhizosphere samples was 8.14 ± 0.6, conductivity ranged from 27.86 to 34.20 (mS/cm) and salinity ranged from 18.65 to 23.38 psu. These parameters for the control samples presented lower values. The pH of the control samples was 9.39 ± 0.4, conductivity ranged from 13.40 to 21.76 (mS/cm) and salinity ranged from 8.40 to 14.23 psu.

Values for organic matter content and for Nitrate concentration were more than three times higher in the rhizosphere than in the control samples. In general, all other measured chemical components (Magnesium, Potassium, Sulfur and Phosphorous) were slightly increased for the rhizosphere samples (Table 1).

3.2. Total community structure and diversity

A total of 5,804,251 raw pyrosequencing reads was obtained from the six environmental samples (four RSMgr and two CS). After quality trimming, a total of 4,945,550 sequences was obtained, including: 1,089,202 reads for RSMgr01 (average read length of 566 bp), 1,211,004 reads for RSMgr02 (average read length of 563 bp), 762,883 reads for RSMgr03 (average read length of 558 bp), 894,444 reads for RSMgr04 (average read length of 542 bp), 570,547 reads for CS01 (average read length of 256 bp) and 417,470 reads for CS02 (average read length of 255 bp) (Table 2).

Principle Component Analysis (PCA) was used to reduce the number of variables of the data and maintain as much variance as possible.

Table 1
Environmental parameters of the sampling locations in this study.

Site	Sal (psu)	pH	Cond (mS/cm)	Mg (ppm)	K (ppm)	S (ppm)	P (ppm)	NO_3^- (mg/L)	OM (%)
CS01	14.23	9.34	21.76	7457 ± 0.431	1278 ± 0.133	1991 ± 0.203	168 ± 0.020	1.253	3.19
CS02	8.40	9.43	13.40	7252 ± 1.018	1389 ± 0.137	1796 ± 0.180	186 ± 0.031	1.412	2.53
RSMgr01	23.38	8.20	34.20	8215 ± 0.218	1770 ± 0.155	2126 ± 0.155	220 ± 0.044	5.542	9.21
RSMgr02	18.65	8.08	27.86	10,934.6 ± 0.537	2216 ± 0.113	1833 ± 0.360	215 ± 0.030	3.598	10.12

Abbreviations: Cond, conductivity; Sal, salinity; psu, practical salinity unit; NO3-, nitrate; ppm, part permillion; Mg, magnesium; K, potassium; S, sulfur; P, phosphorous; OM, organic matter.

For this analysis, eukaryotic reads were excluded and prokaryotic communities were analyzed separately at both the phylum and genus level (Fig. 2). At the phylum and genus level, prokaryotic communities of RSMgr and CS were distinct from each other, but displayed lower variability within sample groups.

The distinction between the prokaryotic communities of different samples was further augmented at the genus level as can be seen with the rarefaction curves (Fig. 3). These also highlighted the greater species richness of the rhizosphere soil sediment of *A. marina* (RSMgr) samples over bulk soil sediment (CS) samples, while pointing to some underestimation of total diversity. Such an increased microbial diversity would be consistent with the rhizosphere effect. Briefly, root exudates condition their associated microbial community by selecting specific groups at the taxonomic level but most importantly, at the functional level. Exudation provides a valuable source of Carbon, while microorganisms that colonize the rhizosphere help plants to acquire Phosphorus and Potassium, and some enhance Nitrogen uptake, or even help the plants to cope with infection, toxic compounds, and other sources of stress (Kristensen et al., 2008; Singh et al., 2004).

Phylogenetic analysis of the metagenomic libraries revealed a preponderance of prokaryote taxa ~82% of the soil community in the CS and 75.9%–84% of the RSMgr community. The proportion of partial SSU rDNA gene sequences derived from Archaea decreased in the bulk soil samples while the Eukaryotic fraction increased (Fig. 4A). The Archaeal dominant phyla determined from taxonomic affiliations based on SSU rDNA gene sequences showed the enrichment of *Methanosarcinales*, *Thermococcales*, *Methanococcales* and *Methanobacteriales* in CS, and *Halobacteriales* and *Nitrosopumilales* (Thaumarchaeota) in RSMgr (Fig. 5A). For bacteria, the taxonomic affiliations were determined based on SSU rDNA gene sequences and the complete datasets (TACD) using sequences from M5nr. Bacterial phyla across all samples were dominated by Proteobacteria, Bacteroidetes and Firmicutes, while 35% of the sequences were unclassified (Figs. 4A and 5B).

Metagenomic data from mangroves are very scarce, not allowing for comparative studies, but our results are in general agreement with previous studies that use different isolation-based methodologies, particularly regarding dominant microbial groups. Comparison of our results with the pioneer metagenomic study of Brazilian mangrove sediment microbiomes by Andreote et al. (2012) show that, although prokaryotes were still dominant in our samples, bacteria were detected at slightly lower numbers, while archaea were at a comparable level. We also recorded a relative decrease in archaea present in the rhizosphere samples when compared to the bulk soil. While the factors involved in regulating the diversity and abundance of archaea in soils are still poorly understood, such variation can arise from differences in the samples. The rhizosphere likely provides a richer environment when compared to bulk soil, which is less subjected to chronic energetic stress conditions that are usually associated with archaeal dominance over bacteria (Valentine, 2007). Furthermore, another study revealed that the abundance and diversity of archaea in soils were conditioned by C/N ratios and specifically inhibited by high levels of Nitrogen availability (Bates et al., 2011), seemingly agreeing with our chemical data (Table 1).

Further comparison with Andreote et al. (2012), revealed noteworthy discrepancies particularly in their reported high abundance of Acidobacteria and absence of Thaumarchaeota (*Nitrosopumilales*). Such differences can be a reflection of the different geographical location, plant species, and/or physico-chemical characteristics of the samples, which would lead to different community compositions. Additional divergence could also be attributed to differences in sample collection and processing (e.g. sampling depth, DNA extraction methodology). However, it should be noted that some recent reports pointed to erroneous detection and frequent overestimation of groups such as Acidobacteria in metagenomic studies due to contamination with DNA extraction kit reagents (Salter et al., 2014), which might explain such a sharp difference. Likewise, the presence of Thaumarchaeota in mangroves and the importance of this group of archaeal ammonia oxidizers have been reported in several other studies (e.g. Dias et al., 2011; Otero et al., 2014).

The microbial taxa detected in our samples (Figs. 4A, B, 5A, B) point to a predominance of sulfate reducers (most importantly Deltaproteobacteria), together with other groups linked with the biogeochemical Sulfur cycle, as well as several methanogens (Methanobacteria, Methanococci, Methanomicrobia, and Methanopyri). The typical conditions in mangroves with anoxic conditions and very low redox potential provide an ideal setting that selects for sulfate reducers and methanogens (Dar et al., 2008; Taketani et al., 2010b). Although traditionally sulfate reducers outcompete methanogens and inhibit their numbers, the co-occurrence of both groups in mangroves can been linked to the use of different, non-competitive substrates (Lyimo et al., 2002; Muyzer and Stams, 2008; Oremland et al., 1982).

Sulfate reduction in mangroves is fueled by the high concentrations of sulfate in the seawater that regularly floods them and this metabolic process is generally perceived as one of the most important in these ecosystems (Sherman et al., 1998). Sulfate reducers play a vital role in the oxidation of organic matter, degradation of toxic compounds such as long-chain and aromatic compounds (Muyzer and Stams, 2008), and the resulting production of H_2S reacts with insoluble iron phosphates (Alongi et al., 1992), releasing phosphate and other ions which are essential for plant nutrition (Rodríguez and Fraga, 1999).

Table 2
Statistics of sequenced samples using Pyrosequencing 454 technology.

Samples	No. of raw reads	Failed QC (%)	Average length	Average GC (%)
CS01	705,326	19.10	256	50
CS02	514,784	18.90	255	50
RSMgr01	1,267,409	14.10	566	50
RSMgr02	1,416,928	14.50	563	51
RSMgr03	854,451	10.70	558	51
RSMgr04	1,045,353	14.40	542	51

3.3. Microbial abundance in mangrove rhizospheres

A method based on the assignment of taxonomic affiliation for complete data sets, TACD, was used to measure the microbes that are specifically enriched in the rhizophere, by comparing the obtained sequences with sequences from the M5nr database via the MG-RAST pipeline (Meyer et al., 2008) (Fig. 5B). Furthermore, the MetaAAMG pipeline (Alam et al., 2013) that has been modified for metagenomic data and uses the latest versions of microbial UniProt/Trembl (The

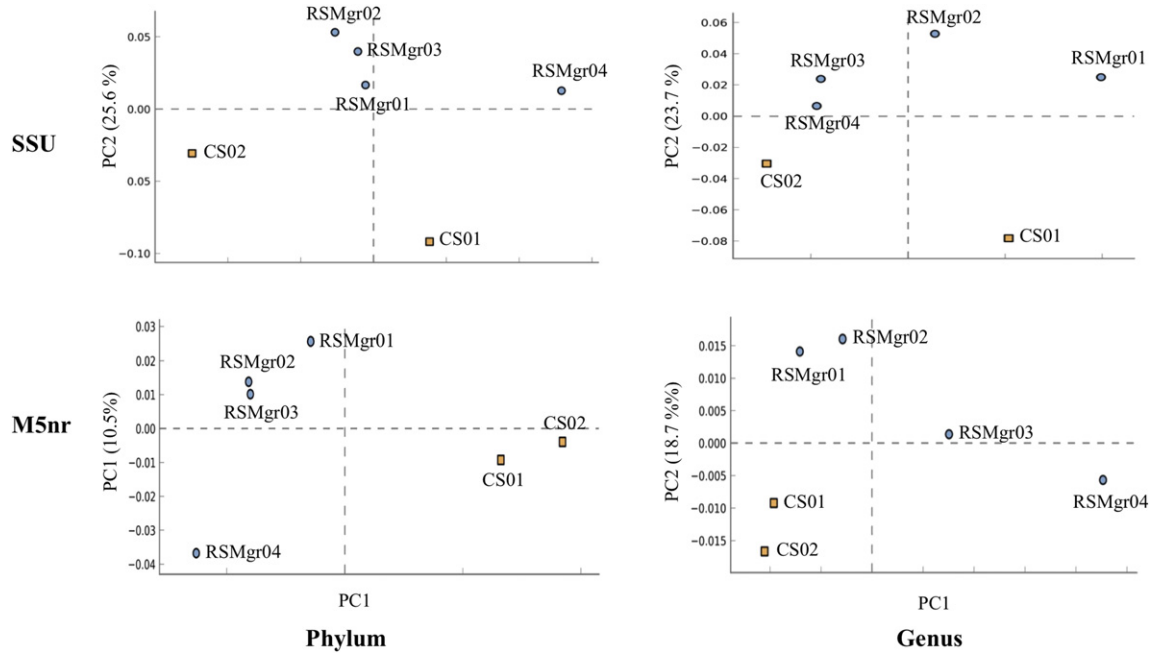

Fig. 2. Principle Component Analysis (PCA) of prokaryotic communities of the samples, calculated at the Phylum and Genus taxonomic levels, for reads against the SSU and M5nr databases. (PC = Principal Component).

UniProt, 2014), KEGG (Kanehisa et al., 2014) and CDD (Marchler-Bauer et al., 2011), was also used to confirm the taxonomic affiliations based on TACD. Results demonstrated that taxonomic distribution of phyla across RSMgr and CS has profiles that are similar to the results obtained from MG-RAST.

Of the 47% of sequences that matched these databases, 92.1%, 3.5% and 2.89% were affiliated with Bacteria, Archaea and Eukaryota, respectively, and the dominant phyla were similar to taxonomic affiliation determined by SSU rDNA gene sequences. Furthermore, a comparison of SSU data and TACD showed an significant enrichment of Bacteroidetes, and a moderate one for Lentisphaerae and Verrucomicrobia in the RSMgr, contrasting with enrichment of Euryarchaeota, Chlorobi and Actinobacteria in the CS (Fig. 6).

The encountered differences are likely caused by negative, and positive selection effects for these microbial groups in the rhizosphere microbiomes, particularly associated with the presence of Oxygen and growth substrates. The leaky roots and pneumatophores of *A. marina* would allow for some oxygen to seep into the rhizosphere, making proximal areas one of the few oxic ones in the mangroves. The presence of oxygen would have an inhibitory effect on anaerobes while allowing the proliferation of aerobic groups. This would support the observed enrichment patterns for the strictly anaerobic Chlorobi, and methanogenic Euryarchaeota — whose metabolism is hampered by the presence of Oxygen; the aerobic archaeal ammonia oxidizer of the *Nitrosopumilales* (Thaumarchaeota); and the Halobacteria, which usually prefer aerobic conditions.

Bacteroidetes are very frequent in tidal mudflats or near-shore sediments, as well as hydrocarbon-contaminated environments, although only a few were confirmed as hydrocarbon degraders (Kim and Kwon, 2010). Their hypothesized role as initiators of the mineralization of high-molecular-weight organic matter (Kirchman, 2002) would fit well with their preferential placement in the proximity of *A. marina*, and enrichment in the rhizosphere. The increased abundance of Bacteroidetes in the rhizospheres of mangroves has been previously

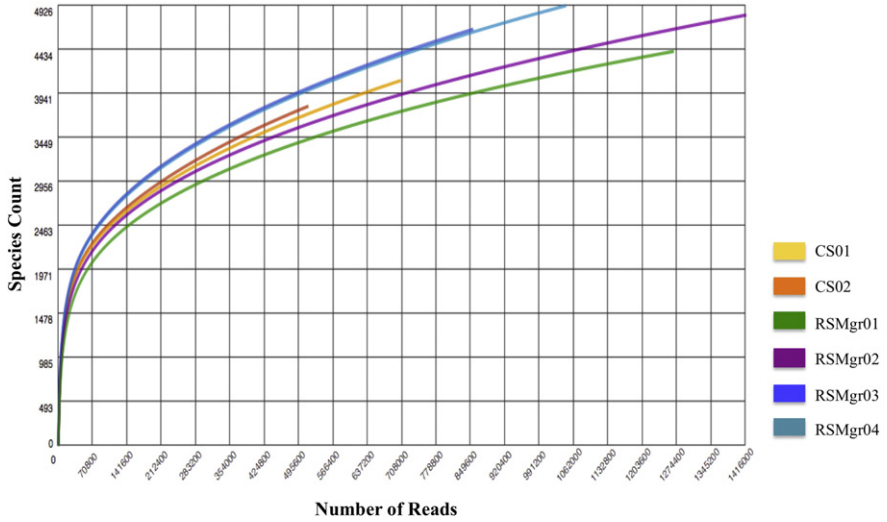

Fig. 3. Rarefaction analysis of the collected samples. Each curve compares the number of sequence reads with the number of detected species.

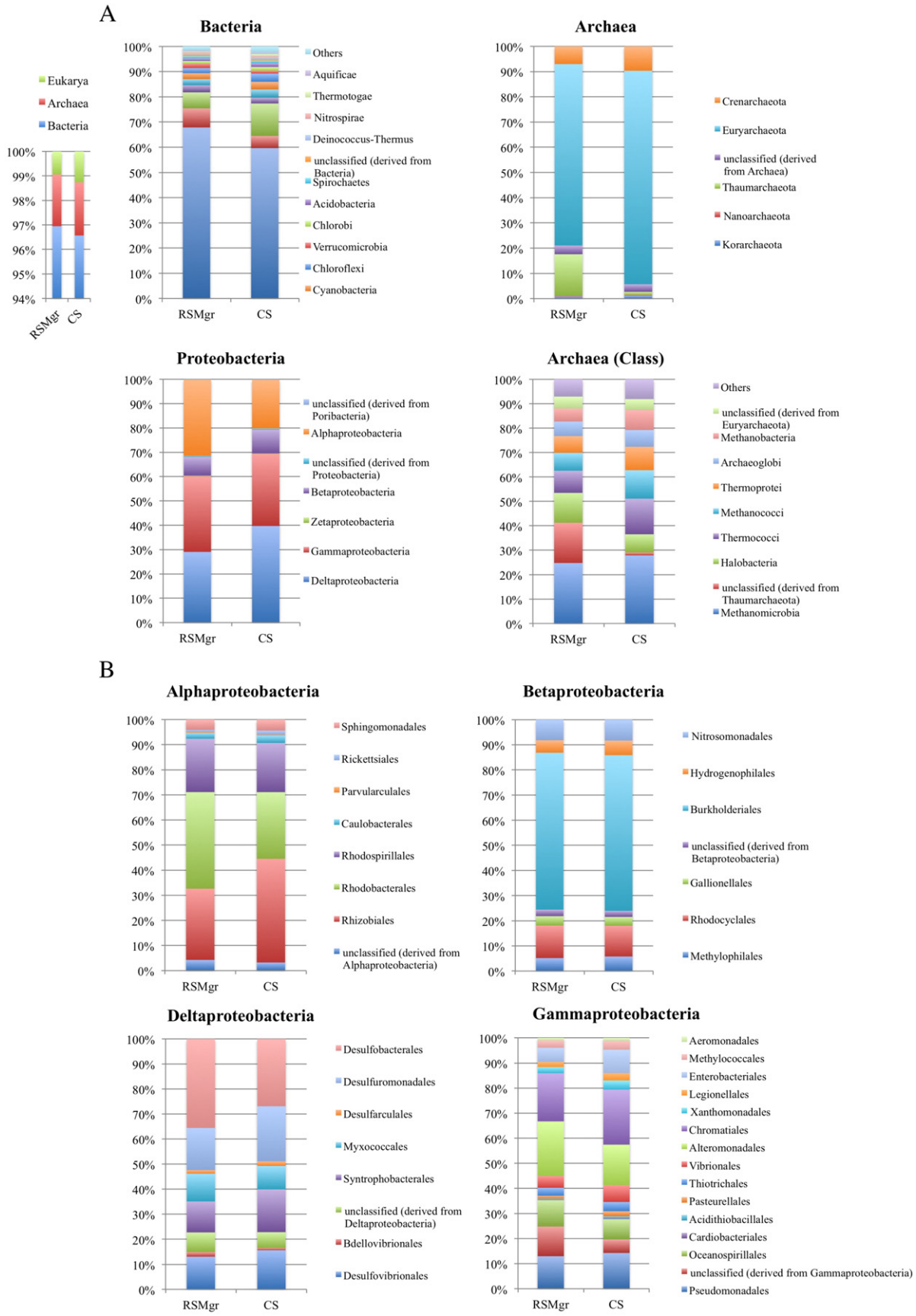

Fig. 4. Relative abundance of the most dominant taxa. Each panel plots the relative abundance of different taxa inhabiting rhizosphere sediment samples (RSMgr) and bulk soil samples (CS), based on M5nr database. A. Total counts, Bacteria, Archaea, Proteobacteria and Archaea (Class). B. Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria and Gammaproteobacteria.

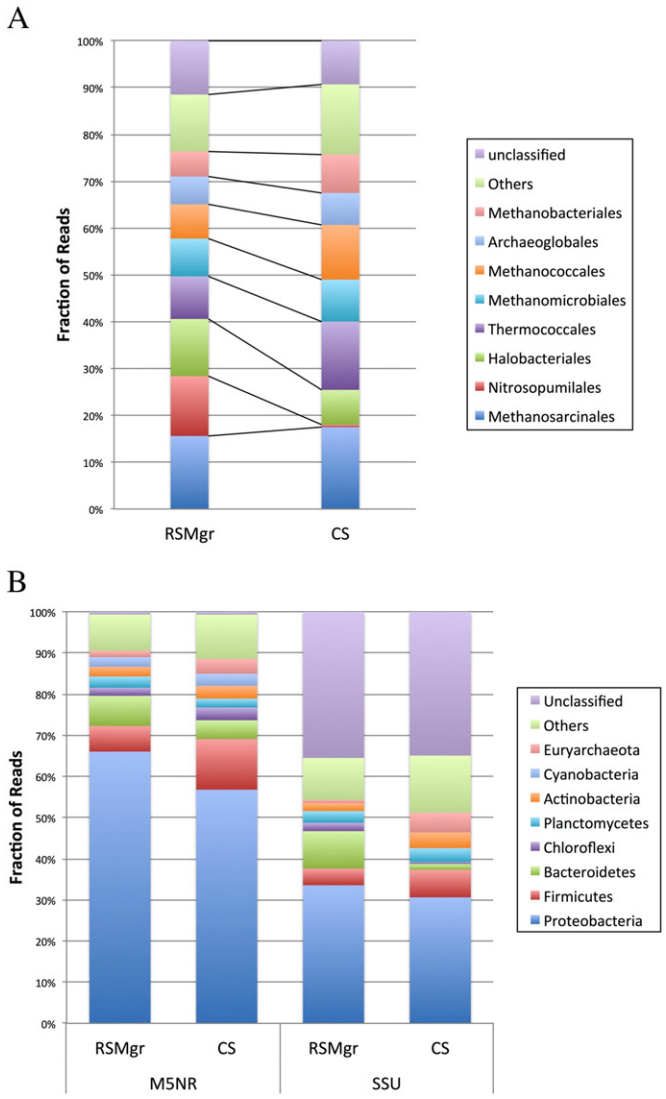

Fig. 5. Taxonomic distribution of phyla across the 6 samples, averaged into two groups: rhizosphere sediment samples (RSMgr) and bulk soil samples (CS). A. 16S rDNA reads of Archaea shown at the Order level. B. Total prokaryotic reads mapped against two different databases: M5nr for total DNA and SSU for 16S rDNA.

noted in other plant species (Gomes et al., 2010). The Actinobacteria are known to include mostly soil-borne microbes, which might explain their enrichment in the sediments. Despite their frequent detection in soils, little is known about the possible role of Lentisphaerae and Verrucomicrobia.

A more detailed analysis of data based on M5nr results on the genus level revealed that the top 30 genera (8.40% of to total genus) were affiliated with 38.57% of bacterial sequences. Moreover, these results demonstrated concurrence with findings based on SSU rDNA gene sequences, as all of the sequences commonly found in at least three of the four RSMgr samples but not found in the CS were identified in the top 30 genera except Marinoscillum (35th genus), Chloroherpeton (38th genus) and Legionella (70th genus). However, bacteria present in the RSMgr samples on the species level revealed that the top 30 species (5.2% of to total species) were affiliations with 34.10% of bacterial sequences. These species include *Victivallis vadensis* (Lentisphaerae), *Pseudomonas filiscindens* (Proteobacteria), *Salinibacter ruber* (Bacteroidetes), *Planctomyces maris* (Planctomycetes), *Ktedonobacter racemifer* (Chloroflexi), *Prolixibacter bellariivorans* (Bacteroidetes), *uncultured Desulfobacteraceae bacterium* (Proteobacteria), *Francisella noatunensis* (Proteobacteria), *Robiginitalea biformata* (Bacteroidetes), *Pseudomonas aeruginosa* (Proteobacteria), *Pelagibaca bermudensis* (Proteobacteria), *Dokdonia donghaensis* (Bacteroidetes), *Flexithrix dorotheae* (Bacteroidetes), *Erythrobacter litoralis* (Proteobacteria) *and Marinoscillum furvescens* (Bacteroidetes), all of which belong to the genera exclusively sequences from the rhizosphere microbiome (Fig. 7). The identification of these 15 species provides a good starting point for further research on their associated function, and thus increase our understanding of the interplay of the enriched species of this rhizosphere microbiome.

3.4. Functional analysis of the A. marina rhizosphere microbiome

Functional analysis of the complete set of metagenomic reads was assessed using the Subsystems and DEOP databases. In order to derive a population scale model of the metabolic potential of the mangrove rhizosphere and bulk soil microbial populations, the metagenomic data was interrogated for matches to the Subsystems (Overbeek et al., 2005) and DEOP databases (http://www.cbrc.kaust.edu.sa/deop/) in search of rhizosphere-specific enrichments.

Results for the Subsystems database revealed that RSMgr sequences were enriched in pathways related to 'metabolism of aromatic compounds', 'genetic mobile elements', 'potassium metabolism' (Fig. 8A), three essential functional cores that likely shape the *A. marina* rhizosphere-specific microbiome.

Subgroups of metabolism of aromatic compounds enriched in the RSMgr include pathways associated with the catabolism of aromatic amino acids or other aromatic compounds. According to the M5nr data analysis, they include: Gentisate degradation, Phenylacetyl-CoA catabolic pathway, Homogentisate pathway of aromatic compound degradation, N-heterocyclic aromatic compound degradation, Protocatechuate branch of beta-ketoadipate pathway, Salicylate and gentisate catabolism, Chloroaromatic degradation pathway, Phenylpropanoid compound degradation and Quinate degradation.

When analyzing data through DEOP, results revealed that RSMgr sequences were enriched with enzymes from pathways that utilize osmolytes as compared to CS (Fig. 8B). Pathways that utilize osmolytes considered as more relevant, for having a higher percentage of completion, were the ones that were found in at least three of the four RSMgr samples but not in the CS, being a total of 11.

3.4.1. Metabolism of aromatic compounds

Anthropogenic activities have caused an accumulation of several toxic compounds in the environment, with elevated concentrations commonly found in marine and coastal regions near urban and industrial areas (Santos et al., 2011). Common pollutants in the Red Sea include oil spills from ships that use the Suez Canal, and from local oilfields (Dicks, 1986). As mangroves are coastal ecosystems, they are particularly exposed to the convergence and accumulation of toxic compounds, as confirmed by studies pointing to contamination with oil-derived compounds (e.g. Dicks, 1986).

Aromatic hydrocarbons of low molecular weights have a particularly high damaging potential in mangroves as they are phytotoxic and affect all stages of plant growth (Ghizelini et al., 2012). The role of microbes in degrading toxic hydrocarbons next to plant roots in mangroves is seen as essential for their survival (Ghizelini et al., 2012).

In line with our findings (Fig. 8A), Toyama et al. (2006) demonstrated accelerated aromatic compound degradation in aquatic environments due to interaction between *Spirodela polyrrhiza* and its rhizosphere microbiome. They demonstrated that the mechanisms for accelerated removal of aromatic compounds were quite different depending on the substrates. *S. polyrrhiza* showed selective accumulation of phenol-degrading bacteria in its rhizosphere fraction, implying that aniline- and 2,4-DCP-degrading bacteria were not much accumulated. However, *S. polyrrhiza* secreted peroxidase and laccase to attract the desired phenol-degrading bacteria to it rhizosphere microbiome. Thus, the composition of plant exudate released by *A. marina* is likely to attract bacteria with complete pathways for catabolism of aromatic amino acids or other

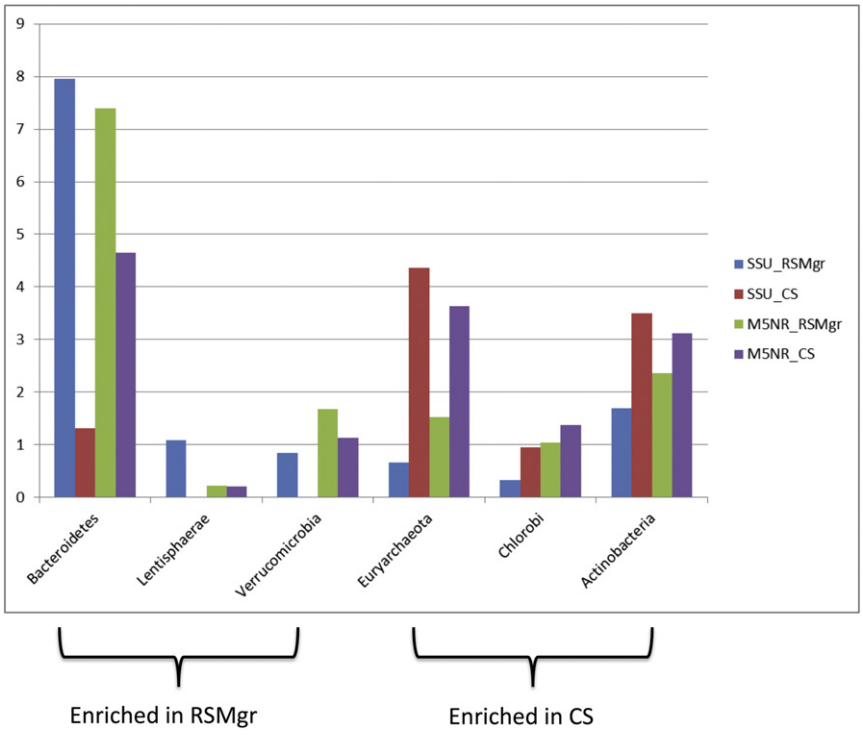

Fig. 6. Comparison of sequences obtained from the M5nr database via the MG-RAST pipeline, with identification of enriched phyla based on 16S rDNA, with adj.Pvalue of 0.05.

aromatic compounds found to be enriched in the rhizosphere, as aforementioned in the Subsystems database results.

3.4.2. Mobile genetic elements

Mobile genetic elements (phages, prophages, transposable elements, and plasmids) are very often carriers of hydrocarbon degradation genes, and the spread of these elements has been postulated as an important factor in rapid adaptation to pollution (Taketani et al., 2010a; Marri et al., 2007). Increased values in the rhizosphere (Fig. 8A) are likely beneficial for the plants and are in accordance with the results mentioned in the previous section.

3.4.3. Potassium metabolism

Since plants require potassium for numerous physiological processes such as growth and development, protein synthesis, phloem solute transport, maintenance ion balance in the cytosol and vacuole, attention is drawn to the importance of bacteria, such as *Pseudomonas*, which are capable of mobilizing potassium in accessible form in the soil. Interestingly, the RSMgr showed increased concentrations of potassium and enrichment of potassium metabolism compared to CS (Fig. 8A). Thus, a key function for a subset of *A. marina* rhizosphere-specific microbial communities should be potassium cycling. Interestingly, in cases of potassium deficiency, plants exude molecules that signal low potassium levels such as phytohormones, auxin, ethylene and jasmonic acid, as

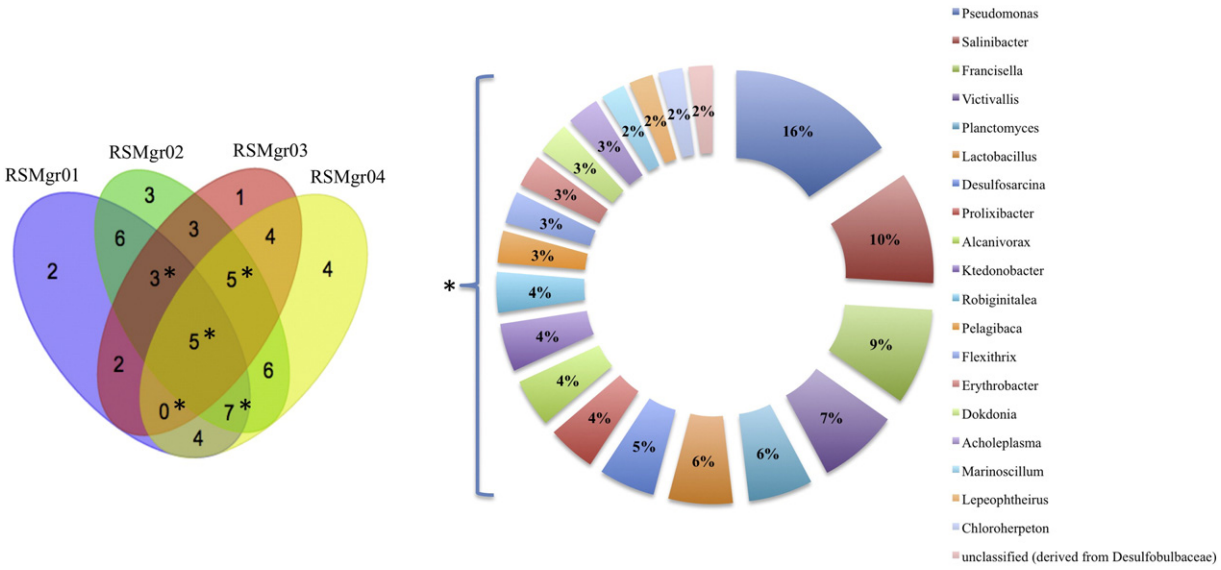

Fig. 7. Venn diagram depicting genera exclusively identified in at least three of four rhizosphere sediment samples (RSMgr), but in none of the bulk soil samples (CS) samples.

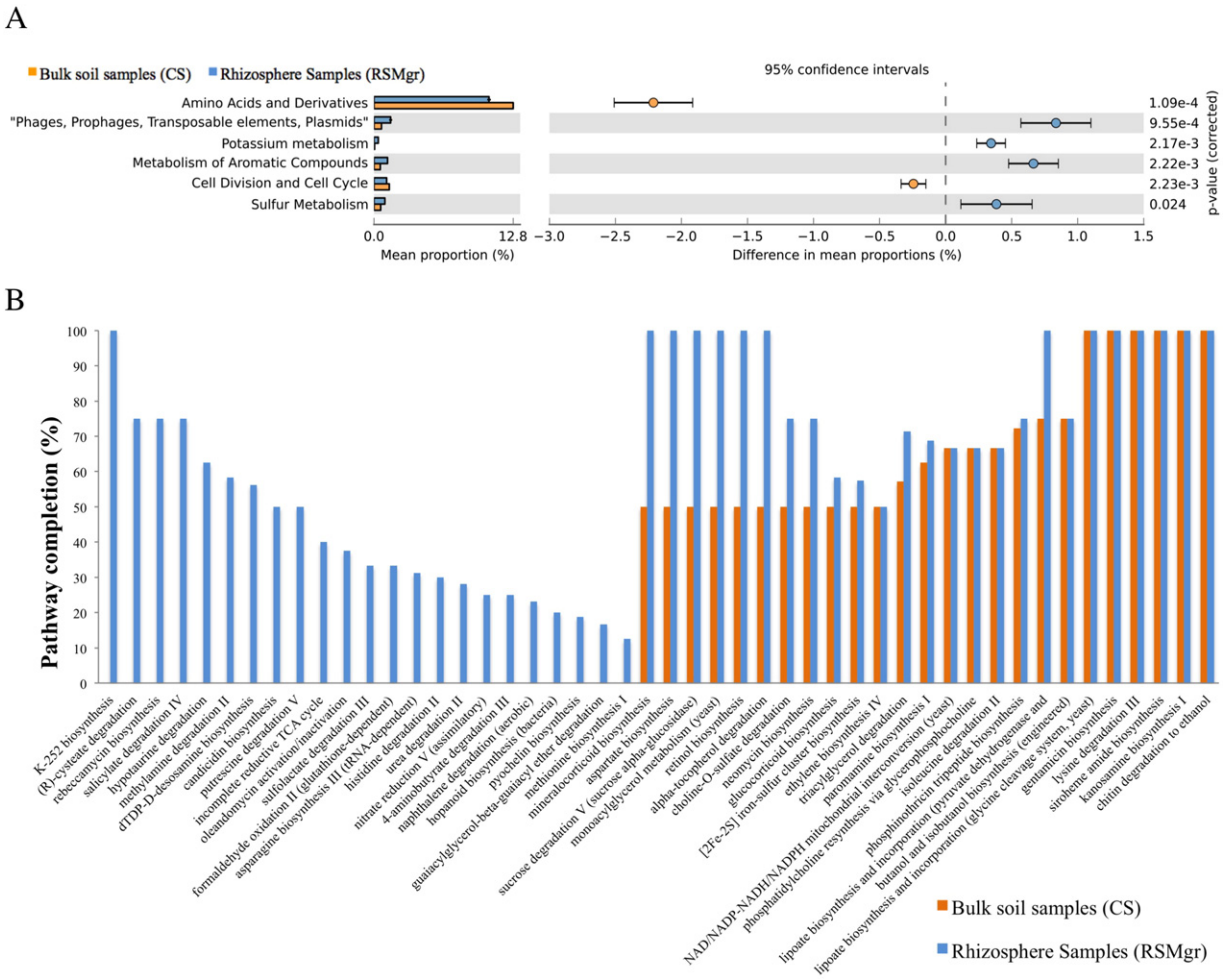

Fig. 8. A. Enriched pathways as per the Subsystems and DEOP databases on the bulk soil samples (CS) and the rhizosphere sediment samples (RSMgr). Pathways with adj.Pvalue < 0.05. B. Osmolyte utilizing pathways found in rhizosphere sediment samples (RSMgr) and bulk soil samples (CS). For each pathway a percentage score reflecting its level of completion in a sample was computed. A pathway was given the value 100, if all of its gene-members were found in that sample. And, 0 was given when none of the genes involved in the specific pathway were found. Percentage scores were averaged across RSMgr and across CS.

well as trigger developmental responses in roots (Ashley et al., 2006). These strategies allow plants to survive, compete and shape the rhizophere microbiome as well as demonstrate the importance of potassium cycling.

Furthermore, potassium could play an important role as it has been reported that it can alleviate iron toxicity to a certain degree (Wang et al., 2013). Despite being an essential trace element in plants, excessive iron, which is particularly pronounced in tropical and subtropical regions, is a major abiotic stress factor that inhibits growth (e.g. Benckiser et al., 1984; Gao et al., 2014).

3.4.4. Osmoprotection

A. marina is known to absorb salt that is later deposited on the leaves to form a layer over the stomata, thereby preventing dehydration (Rippey, 2004). This fact supports our environmental parameter findings (Table 1), which revealed an increase in salt concentration in RSMgr when compared to CS. Salt is drawn towards the roots leading to an increase in the osmolytes available for utilization at the RSMgr, implying that, bacteria at the rhizosphere required the accumulation of osmolytes to alleviate the salt stress. Taken together, these remarks clarify the identification of 11 relevant osmolyte utilizing pathways that are specifically enriched in the rhizophere microbiome. These include: K-252 biosynthesis (osmolytes used in this pathway include dimethylglycine and glycine betaine), (R)-cysteate degradation (L-glutamate), rebeccamycin biosynthesis (β-D-glucose), salicylate degradation IV, hypotaurine degradation (hypotaurine and L-alanine), methylamine degradation II (L-glutamate and N-methyl-L-glutamate), dTDP-D-desosamine biosynthesis (L-glutamate), candicidin biosynthesis (L-glutamate, L-glutamine), putrescine degradation V (4-aminobutanoate and L-alanine), incomplete reductive TCA cycle ((S)-malate) and oleandomycin activation/inactivation (β-D-glucose). From these, K-252 biosynthesis, rebeccamycin biosynthesis, candicidin biosynthesis and oleandomycin activation/inactivation are all antibiotic production pathways. Also, dTDP-D-desosamine is an essential component of macrolide antibiotics revealing the availability of the osmolytes used in these pathways to provide osmoprotection. And, the secondary use of these osmolytes is to produce antibiotics that provide an additional advantage of eliminating competitors that do not have resistance to them.

Putrescine is an exudate that has also been reported to impact the rhizophere microbiome as increased uptake of putrescine causes decreased growth rate and consequently decreased ability for competitive colonization (Kuiper et al., 2001). We found abundant unclassified species (derived from Gammaproteobacteria) and also found increased 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase in the RSMgr metagenome dataset. Curiously, it has been demonstrated that a Gammaproteobacterium (*Pseudoalteromonas maricaloris*) isolated from mangrove *A. marina* rhizosphere along the Abu Dhabi coastline, which produced relatively high levels of ACC deaminase in vitro, reduced endogenous levels of ACC in the roots and shoots, and significantly increased the levels of plant growth regulators including

indole-3-acetic acid, indole-3-pyruvic acid, putrescine, spermidine and spermine in roots and shoots compared with control *A. marina* (El-Tarabily and Youssef, 2011).

Furthermore, the genome of members of our identified *A. marina* rhizosphere-specific community, such as *F. noatunensis* displays the complete module for putrescine transport system. Also, the genomes of *S. ruber* and *R. biformata* have a complete and incomplete module, respectively, for GABA biosynthesis in which putrescine is used to produce GABA. The *P. aeruginosa* genome has complete modules for both the putrescine transport system and GABA biosynthesis. These suggest that putrescine may be one of the *A. marina* rhizosphere exudates that influenced its rhizosphere microbiome.

Hypotaurine and cysteate is likely being utilized as nitrogen, carbon and energy source as has been demonstrated by *Paracoccus denitrificans* PD1222 (Bruggemann et al., 2004, Felux et al., 2013) and *Paracoccus pantotrophus* NKNCYSA (Mikosch et al., 1999), respectively. While, *Streptomyces* sp. WA46 has been shown to degrade salicylate via the salicylate degradation IV pathway in which salicylate is converted to gentisate via salicyloyl-CoA and gentisyl-CoA (Ishiyama et al., 2004). Functional analysis using Subsystems also showed enrichment of salicylate and gentisate catabolism. On the other hand, methylamine is a substrate of methanogenesis and Archaea are the only microbes that have been identified as being capable of producing methane.

3.5. Conclusions

Our results provided the first insights into the range of functions and microbial diversity in the rhizosphere and soil sediments of the northernmost mangals of the Indo-Pacific:

Overall, Proteobacteria, Bacteroidetes, and Firmicutes were dominant, with high abundance of sulfate reducers and methanogens.

Specific microbial taxa underwent possible positive and negative selection in the rhizosphere and soil, likely influenced by the presence of oxygen and growth substrates provided by the roots;

The identification of specifically enriched taxa, particularly at the species level, provides a good starting point for further research on their associated functions, and increases our understanding of the interplay of the enriched species of rhizosphere microbiome.

Functional analysis showed significant enrichment in the rhizosphere microbiomes for: 1\ metabolism of aromatic compounds, 2\ mobile genetic elements, 3\ potassium metabolism and 4\ pathways that utilize osmolytes. These functions help the plants in their nutrient uptake and coping with infection, toxic compounds, and other sources of stress.

Several factors shape rhizosphere microbiome and, based on our findings, we suggested that primary mechanisms include: 1\ plant exudates, that attract the required microbiome; and, 2\ production and accumulation of specific osmolytes to alleviate salt stress. Secondary mechanisms include: antibiotic resistance, metabolism of aromatic hydrocarbons, potassium cycling, as well as putrescine, hypotaurine, cysteate and methylamine utilization, all of which are mechanisms that require the accumulated osmolytes.

Conflict of interests

The authors declare no conflict of interest.

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