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Host-association as major driver of microbiome structure and composition in Red Sea seagrass ecosystems

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Originality-Significance Statement

This work contributes new knowledge about the seagrass microbiome and is the result of a large and extensive sampling effort, including six seagrass species sampled in several stations along the Red Sea coast. We comprehensively characterize seagrass-associated bacterial communities including vegetated and bare sediments, and seagrass leaves, rhizomes, and roots. Moreover, we describe the bacterial community in the endophytic compartment separately from the total bacterial community associated to each tissue. We combine community network analyses and taxonomic analysis to characterize the structure and identify key members within the seagrass microbiome. One of the most significant outcomes of this study is the finding of highly similar OTUs in well-defined sub-clusters, which suggests the co-occurrence of highly connected key members within bacterial communities. We believe that this results provide a major advance towards the understanding of seagrass ecosystem functioning and building the seagrass holobiont concept as well as combining cutting edge analytical methods.

Summary

The role of the microbiome in sustaining seagrasses has recently been highlighted. However, our understanding of the seagrass microbiome lacks behind that of other organisms. Here, we analyze the endophytic and total bacterial communities of leaves, rhizomes, and roots of six Red Sea seagrass species and their sediments. The structure of seagrass bacterial communities revealed that the 1% most abundant OTUs accounted for 87.9 and 74.8 % of the total numbers of reads in sediment and plant tissue samples, respectively. We found taxonomically distinct bacterial communities in vegetated and bare sediments. Yet, our results suggest that lifestyle (i.e. free-living or host-association) is the main driver of bacterial community composition. Seagrass bacterial communities were tissue- and species-specific and

differed from those of surrounding sediments. We identified OTUs belonging to genera related to N and S cycles in roots, and members of Actinobacteria, Bacteroidetes, and Firmicutes phyla as particularly enriched in root endosphere. The finding of highly similar OTUs in well-defined sub-clusters by network analysis suggests the co-occurrence of highly connected key members within Red Sea seagrass bacterial communities. These results provide key information towards the understanding of the role of microorganisms in seagrass ecosystem functioning framed under the seagrass holobiont concept.

1. Introduction

Seagrasses comprise about 60 different marine angiosperm species distributed among four families with a polyphyletic origin (Les *et al.*, 1997) that evolved from freshwater plants and colonized the marine environment in at least three independent events (Olsen *et al.*, 2016).

Seagrasses are highly productive, supporting intense nutrient and carbon cycling and a large fraction of subsistence fisheries in many countries, especially in tropical regions (Unsworth and Cullen, 2010). In addition, seagrass ecosystems are key habitats for nitrogen loss via denitrification, removing excess nitrogen inputs in sub-tropical and temperate estuarine systems (Eyre *et al.*, 2011, 2016) and tropical and temperate coastal lagoons (Reynolds *et al.*, 2016; Garcias-Bonet *et al.*, 2018). Due to their intense and effective carbon sink capacity (Fourqurean *et al.*, 2012), the conservation and restoration of seagrass ecosystems have been put forward as a promising strategy for climate change mitigation (Nellemann *et al.*, 2009; Duarte *et al.*, 2013; Serrano *et al.*, 2019).

Biogeochemical cycling in seagrass ecosystems is supported by a rich and diverse microbiome, with enriched bacterial abundance and activity in seagrass relative to adjacent bare sediments (Hemminga and Duarte, 2000). The associated microbial communities can also play a key role in allowing seagrass to cope with stress, such as elevated sulfide (Kusel *et al.*, 2006). Therefore, microbial communities play a role in the resilience of seagrass ecosystems (Trevathan-Tackett *et al.*, 2019), similar to recent findings on corals (Ziegler *et al.*, 2018). Indeed, the importance of investigating the seagrass holobiont has been highlighted as a fundamental research program to better understand seagrass dynamics and resilience (Tarquinio *et al.*, 2019; Trevathan-Tackett *et al.*, 2019).

The importance of the microbiome in shaping the fitness of the host organism has been shown for many organisms, spanning from animals (McFall-Ngai *et al.*, 2013) to plants (Vandenkoornhuyse *et al.*, 2015). In terrestrial plants, there is ample evidence of the importance of the microbiome in promoting plant growth and plant resistance to biotic and abiotic factors (Vandenkoornhuyse *et al.*, 2015). However, our understanding of the seagrass microbiome lacks behind that of other organisms (Trevathan-Tackett *et al.*, 2019). Despite the scarcity of data for seagrasses compared to terrestrial plants, there is evidence of higher bacterial abundances in sediments colonized by seagrasses compared to bare sediments (Hemminga and Duarte, 2000; Duarte *et al.*, 2005). The patchy nature of seagrass sediments supports a diverse and complex microbial community (Herbert, 1999; Devereux, 2005), fueled by root organic exudates (Donnelly and Herbert, 1999; Devereux, 2005). Indeed, Martin *et al.* (2018) demonstrated that changes in root organic exudates, due to a reduction in light availability, modified the composition of the root microbiome of three seagrass species in a mesocosm experiment. Recent studies, using high-throughput sequencing techniques, demonstrate that the structure and taxonomic composition of the microbial communities associated to seagrasses differ from that in the surrounding sediments (Cúcio *et al.*, 2016; Ettinger *et al.*, 2017; Fahimipour *et al.*, 2017; Martin *et al.*, 2018). Similarly, the microbiome differs among seagrass tissues (Garcias-Bonet *et al.*, 2012; Mejia *et al.*, 2016; Ettinger *et al.*, 2017; Fahimipour *et al.*, 2017; Rotini *et al.*, 2017, 2020), pointing out the active selection of specific bacterial groups from the surrounding environment. Moreover, microbial communities in seagrass tissues show daily variations, with shifts in dominant groups, especially in leaves (Rotini *et al.*, 2020). As in terrestrial plants, the seagrass rhizosphere microbiome shapes the plant fitness by increasing nutrient availability (i.e. new supply of nitrogen by diazotrophs, Donnelly and Herbert, 1999; Welsh, 2000; Devereux,

2005; Sun *et al.*, 2015; Garcias-Bonet *et al.*, 2016), detoxifying sediments (i.e. removal of toxic sulfides by sulfur-oxidizing bacteria, Kusel *et al.*, 2006; Cúcio *et al.*, 2016; Fahimipour *et al.*, 2017) and providing resistance against invasive macroalgae colonization (Gribben *et al.*, 2017).

In a recent review on seagrass-associated bacteria, Tarquino *et al.* (2019) highlighted the need to advance towards the understanding of seagrass ecosystem functioning from a holobiont perspective and proposed the inoculation of specific bacteria to enhance seagrass resilience to environmental stressors and successfully restore seagrass habitats as promising future strategies. However, information regarding seagrass microbiomes is still lacking across regions and species, as most studies on seagrass have focused on few seagrass species, mostly from temperate areas, and on specific habitat compartments. Therefore, there is a need to further increase our knowledge on seagrass-associated bacterial communities, specifically increasing the number of seagrass species studied and combining the description of the microbiome in tissues and surrounding habitats. The Red Sea holds a high seagrass species diversity (Short *et al.*, 2007; Qurban *et al.*, 2019), with up to 12 species described (El Shaffai, 2011), and, therefore, it offers a unique opportunity to perform a comparative study of the microbiome of the seagrass species adapted to its extreme environmental conditions, including very high water temperature and salinity (Surface T_{max} up to 33 °C (Chaidez *et al.*, 2017)) and salinity (from 37 ‰ in the South to 41 ‰ in the North (Rasul and Stewart, 2019)).

Here, we test the main hypothesis that i) bacterial communities in Red Sea seagrass sediments differ from those of adjacent bare sediments; and the subsidiary hypotheses that ii) Red Sea seagrass bacterial communities are tissue-specific and differ from those of the surrounding sediments; and iii) Red Sea seagrass endophytic bacterial communities differ from those on seagrass tissue surfaces. We do so by analyzing the microbiome of six seagrass species

Halophila stipulacea, *H. decipiens*, *Enhalus acoroides*, and *Thalassia hemprichii* belonging to the family *Hydrocharitaceae*; and *Thalassodendron ciliatum* and *Halodule uninervis* belonging to the family *Cymodoceaceae*) found along the East coast of the Red Sea (Supplementary Fig. S1, Table S1) based on next-generation 16S rRNA gene sequencing using the Illumina MiSeq platform. Specifically, we analyze: i) bacterial communities in seagrass vegetated and bare sediments; ii) the endophytic bacterial community, i.e. endosphere microbiome; and iii) the total bacterial community, i.e. both endosphere and ectosphere microbiome, associated with seagrass leaves, rhizomes, and roots. We apply a tissue surface-sterilization protocol to describe endophytic bacterial community, i.e. bacteria that reside inside the plant tissues mainly in intercellular spaces, but also in intracellular spaces and interior of vascular tissues. We combine community network analysis and taxonomic analysis to characterize the structure and identify key members within the seagrass microbiome and we discuss the potential functional role of prevalent taxa components of the seagrass microbiome.

2. Results

We identified a total of 27,861 different OTUs (after removing singletons). The structure of bacterial communities in sediment and plant tissue samples followed a Pareto-like distribution, with few abundant OTUs and mostly rare OTUs (Supplementary Fig. S2). This implies that the 1% most abundant OTUs accounted for 87.9 % of the total numbers of reads in sediment samples (84.2 % and 89.5 % in vegetated and bare sediments, respectively), and 74.8 % of the total numbers of reads in plant tissues (74.8 % and 60.9 % in non-sterilized and surface-sterilized plant tissues, respectively). The fitted heavy-tailed power-law distributions revealed higher evenness in plant-associated bacterial communities than in sediment samples, with mean α ($\pm$ SD) = 1.60 ± 0.01 and 1.77 ± 0.05 , respectively (Fig. 1A). While vegetated and bare

sediment samples showed similar evenness (α ($\pm$ SD) = 1.77 ± 0.04 and 1.78 ± 0.03 , respectively; Fig. 1B), within plant-associated bacterial communities, those in surface-sterilized plant tissues were characterized by the highest evenness (1.45 ± 0.01) followed by non-sterilized plant tissue samples (1.68 ± 0.01) (Fig. 1C).

We identified 17,609 OTUs in sediment samples and 12,082 OTUs in plant tissue samples, with only 8.8 % of total OTUs being shared between sediment samples and seagrass tissues (Supplementary Fig. S3A), indicating a clearly distinct bacterial microbiome between plants and the surrounding sediment. About half of the total number of OTUs identified in sediment samples were unique of vegetated sediments, 32.5 % was shared between vegetated and bare sediment, and 18.1 % was only found in bare sediment (Fig. S3B), delineating the influence of seagrasses on the sediment bacterial community composition. The percentage of unique OTUs was higher in non-sterilized samples compared to unique OTUs in surface-sterilized tissues (Fig. S3C). The percentage of OTUs shared between the non-sterilized and surface-sterilized tissues was higher for the most abundant OTUs (Fig. S3D, E) than in the less abundant OTU fraction (Fig. S3F).

The bacterial community from vegetated sediment samples exhibited the highest observed diversity (Supplementary Fig. S4A); while the observed diversity in surface-sterilized plant samples was lower than in non-sterilized plants, vegetated sediment, and bare sediment samples (non-parametric Kruskal-Wallis test, $p < 0.001$, $p < 0.0001$, and $p < 0.0001$, respectively). The non-sterilized plant samples exhibited the highest Shannon's and Simpson's indices (Fig. S4B, C). Shannon's and Simpson's indices in non-sterilized plant samples were higher than in vegetated (non-parametric Kruskal-Wallis test, $p < 0.01$, and $p < 0.01$, respectively) and bare (non-parametric Kruskal-Wallis test, $p < 0.0001$, and $p < 0.001$, respectively) sediments.

Similarly, Shannon's and Simpson's indices in surface-sterilized plant samples were higher than in bare sediments (non-parametric Kruskal-Wallis test, $p < 0.05$, and $p < 0.05$, respectively). Surface-sterilized plant samples showed the highest evenness, which was significantly higher than in vegetated and bare sediments (non-parametric Kruskal-Wallis test, $p < 0.0001$, and $p < 0.0001$, respectively); followed by non-sterilized plant samples, which also showed higher evenness than vegetated and bare sediments (non-parametric Kruskal-Wallis test, $p < 0.001$, and $p < 0.001$, respectively) (Fig. S4D). Alpha diversity estimates did not differ among leaf, rhizome, and root samples (Fig. S5).

Canonical Analysis of Principal Coordinates revealed a clear distinction between plant tissues and sediment bacterial communities (Supplementary Fig. S6A; PerMANOVA test, $F_{1,97} = 14.2$, $p < 0.001$), in agreement with their clearly different pattern in the 3,000 most abundant OTUs (Supplementary Fig. S8). Differences in *in situ* environmental conditions (i.e. depth, temperature, and salinity) across stations (Supplementary Table S1), were not associated with changes in bacterial community composition between plant tissues and sediment samples (Supplementary Fig. S7A, ANOVA test, $F_{3,94} = 1.275$, $p = 0.067$). Vegetated and bare sediments held similar bacterial communities (Fig. S6B; PerMANOVA test, $F_{1,36} = 1.02$, $p > 0.05$). Considering only the subset of plant samples, bacterial communities differed based on the type of tissue, with leaf samples clustering together and separately from the rhizome and root samples (Fig. S6C; PerMANOVA test, $F_{2,60} = 1.99$, $p < 0.001$); and treatment, with non-sterilized plant samples clustering together and separately from surface-sterilized plant samples (Fig. S6D; PerMANOVA test, $F_{1,60} = 2.9$, $p < 0.001$). Bacterial communities of plant tissue samples differed based on *in situ* environmental conditions (Supplementary Fig. S7C, ANOVA test, $F_{3,57} = 1.7$, $p < 0.001$); and among seagrass species and stations (PerMANOVA tests; seagrass

species, $F_{5,60} = 1.7$, $p < 0.001$; stations, $F_{10,60} = 1.4$, $p < 0.001$) in contrast with sediment samples (Supplementary Fig. S7B; ANOVA test, *in situ* environmental conditions, $F_{3,33} = 0.9$, $p = 0.536$; PerMANOVA tests; seagrass species, $F_{5,36} = 1.2$, $p = 0.232$; stations, $F_{10,36} = 1.2$, $p = 0.226$). Bray Curtis distance among samples increased linearly with increasing spatial distance between locations in surface-sterilized rhizomes and roots (linear regression; $p < 0.001$ and $p < 0.0001$, respectively, Supplementary Fig. S9). However, this was not the case for the rest of plant tissues and sediment samples.

OTUs clustered based on the type of sample by network analysis (Fig. 2), showing the four largest components, in number of OTUs, as cluster I, II, III, and IV and the largest component dominated by surface-sterilized plant tissue samples (cluster XIII). The first cluster contained OTUs belonging to vegetated and bare sediment samples, while the third cluster mainly contained OTUs belonging to vegetated sediment samples. The second and fourth clusters exclusively contained OTUs belonging to non-sterilized plant tissue samples. Finally, the OTUs belonging to surface-sterilized plant tissue samples appeared in the 13th cluster, the first cluster dominated by surface-sterilized plant tissue samples. The clustering pattern indicated a clear separation of main OTUs based on sample type. In all five clusters, we identified sub-clusters of highly similar OTUs, likely indicating co-occurrence. The structural analysis of sub-clusters revealed OTUs acting as cluster hubs, i.e. OTUs with the highest number of connections (Fig. 3). The number of cluster hubs in each sub-cluster ranged from 1 to 8, and were mainly OTUs belonging to the phylum Proteobacteria (full taxonomic information of cluster hubs is provided in Supplementary Table S2).

Bacterial communities in plant tissue and vegetated sediment samples were dominated by Proteobacteria phylum in all stations (Fig. 4A). In bare sediments, Proteobacteria phylum

relative abundance was higher than 95% in all stations, except in stations S1 (*H. stipulacea*) and S6 (*T. hemprichii*), where it was 31 and 43%, respectively. In fact, in bare sediment samples from station S1 and S6 we detected other phyla at high relative abundances, such as Firmicutes (37 and 42%, respectively) and Chloroflexi (10 and 11%, respectively). In vegetated sediments, Proteobacteria phylum relative abundance was higher than 95% in stations S1, S2, S3, S6, S8, S11, and S12. In vegetated sediments from stations S4, S7A, S7B, and S10, Proteobacteria phylum relative abundance ranged from 66% in S7B (*H. uninervis*) to 88% in S4 (*H. uninervis*), with relevant relative abundances of Bacteroidetes, Firmicutes, and Chloroflexi phyla. In non-sterilized tissues, Proteobacteria phylum was the most abundant, ranging from 74% in S3 (*H. decipiens*) to 98% in S10 (*H. stipulacea*). Other phyla with relevant relative abundances in non-sterilized tissues were Cyanobacteria (8% in S11, *H. stipulacea*), Actinobacteria (7% in S2, *T. ciliatum*), Chloroflexi (6% in S7B, *H. uninervis*) and Firmicutes (5% in S6, *T. hemprichii*). In surface-sterilized tissues, Proteobacteria phylum relative abundance ranged from 59% in S4 (*H. uninervis*) to 99% in S7A (*T. hemprichii*), with relevant abundances of other phyla such as Actinobacteria, Cyanobacteria, Firmicutes, and Bacteroidetes. The relative abundance of Actinobacteria phylum accounted for up to 16 % in S6 (*T. hemprichii*), 13% in S4 (*H. uninervis*), and from 7 % to 10% in S10, S11, S8, and S2. Cyanobacteria phylum accounted for up to 13% in S4 (*H. uninervis*), Firmicutes accounted for up to 10% in S11 (*H. stipulacea*), and Bacteroidetes accounted for up to 9% in S2 (*T. ciliatum*) and 8% in S4 (*H. uninervis*). Same seagrass species sampled at different stations showed different relative abundances at the phylum level both in non-sterilized and surface-sterilized tissues (i.e. *H. stipulacea*, *H. uninervis*, and *T. hemprichii*, Fig. 4A). Similarly, sediments colonized by the same seagrass species but different locations held different bacterial communities at the phylum level. Moreover, we identified the OTUs

responsible for differences in bacterial communities between plant tissues and vegetated sediments (Supplementary Fig. S10). Rhizobiales, *Rhodobacteraceae*, and Clostridiales were particularly enriched in plant tissue samples (information at the genus level is given in Fig. S10). Indeed, *Rhodobacteraceae* was the most frequently identified family within the cluster hubs from non-sterilized tissue samples (Fig. 3, Table S2). Contrary, cluster hubs from vegetated sediment sub-clusters were OTUs mainly related to S cycle, such as *Desulfobulbus* and *Desulfovibrio* genera (cluster I.a and III.a, respectively; Table S2), *Chromatiaceae* family and Desulfuromonadales order (cluster I.a; Table S2). The taxonomic analysis of sub-clusters revealed higher diversity at the phylum level in those sub-clusters belonging to vegetated sediment samples compared to those found in plant tissues (Fig.3). Proteobacteria was the most abundant phylum in sub-clusters from plant tissue samples, together with Bacteroidetes (cluster II.a, II.b, III.c) and Actinobacteria (cluster IV.b).

Bacterial communities in non-sterilized and surface-sterilized leaf, rhizome and root samples were dominated by Proteobacteria, with other phyla such as Actinobacteria, and Bacteroidetes especially relevant in surface-sterilized roots; and Actinobacteria, Armatimonadetes, and Chloroflexi especially relevant in non-sterilized rhizomes and roots (Fig. 4B). In non-sterilized root samples, Proteobacteria phylum ranged from 68% in *T. ciliatum* to 100% in *H. stipulacea*. Actually, in non-sterilized roots of *T. ciliatum*, Actinobacteria phylum showed a relative abundance of 22%. In surface-sterilized root samples, the average relative abundance of Proteobacteria phylum ranged from 29% in *H. uninervis* to 82% in *E. acoroides*. Actinobacteria phylum was the second most abundant phylum in surface-sterilized roots samples: 29% in *H. uninervis*, 25% in *T. hemprichii*, 14% in *H. decipiens*, 13% in *T. ciliatum*, 9% in *H. stipulacea*, and 4% in *E. acoroides*. Relevant relative abundances of Bacteroidetes

(22%) and Firmicutes (17%) phyla were found in *H. uninervis* and *H. decipiens*, respectively (Fig. 5A). Overall, surface-sterilized root samples showed higher taxonomic diversity at the phylum level than non-sterilized root samples. We identified the OTUs responsible for differences in bacterial communities between non-sterilized and surface-sterilized root samples (Fig. 5B). OTUs belonging to Actinobacteria, Bacteroidetes, and Firmicutes phyla were particularly enriched in surface-sterilized root samples. Within the Proteobacteria phylum, we identified genera related to the S cycle in both non-sterilized and surface-sterilized root samples, such as *Arcobacter*, *Desulfobulbus*, *Desulfomonile*, and *Desulfovibrio* (Fig. 5B).

3. Discussion

We found distinct bacterial communities in sediment and seagrass-associated samples, regarding both structure and taxonomy. Sediment bacterial communities strongly differed from seagrass-associated bacterial communities, as hypothesized, pointing out that the environment type (i.e. free-living or host-associated) is a stronger driver of community structure than geographic distances with the associated broad environmental conditions. This is consistent with results from the earth microbiome project meta-analysis (Thompson *et al.*, 2017) and previous work on seagrasses (Cúcio *et al.*, 2016; Ettinger *et al.*, 2017; Fahimipour *et al.*, 2017; Martin *et al.*, 2018). In addition, host (i.e. seagrass species), tissue type (i.e. leaf, rhizome, and root), location, and environmental conditions (i.e. depth, temperature, and salinity) also contributed to shape bacterial communities in seagrass tissues.

A recent study confirmed that plant-driven changes in the rhizosphere due to radial oxygen loss are responsible for changes in surrounding microbial community composition (Brodersen *et al.*, 2018). In this regard, we found that bacterial communities from sediment surrounding seagrass differed from that in the adjacent bare sediment in all stations. Moreover, the same

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seagrass species held different bacterial communities at different locations, in agreement with Cúcio *et al.* (2016), reporting that the rhizosphere microbiome of three European seagrass species differed from bare sediment and between France and Portugal but it was not species specific. Similarly, the two seagrass species (*T. hemprichii* and *H. uninervis*) sampled in the same location (S7A and S7B), showed different bacterial community composition in their tissues and in the vegetated sediments, in agreement with (Martin *et al.*, 2018) who also found different bacterial communities in roots among three different seagrass species growing in a mixed meadow. These findings suggest that there is a differential selection based on host species, and that this selection depends on the environment as well. Changes in environmental variables, such as temperature and light availability, have been related to changes in the microbiome of seagrass leaves (Rotini *et al.*, 2020). As expected, seagrass-associated bacterial communities also differed among leaf, rhizome, and root tissues, in agreement with previous seagrass studies (Garcias-Bonet *et al.*, 2012; Mejia *et al.*, 2016; Ettinger *et al.*, 2017; Fahimipour *et al.*, 2017; Rotini *et al.*, 2017, 2020; Crump *et al.*, 2018). Moreover, the bacterial community in the endophytic compartment differed from the total tissue-associated community, pointing out at the endosphere as a unique environment.

The decrease in diversity and increase in evenness from sediment to tissue-associated bacterial communities indicates that the rare fraction of bacterial OTUs is bigger in sediment than in tissues. Similarly, Fahimipour *et al.* (2017) found that the microbial diversity, expressed as the Shannon Index, was higher in seagrass sediments than in root surface of *Zostera marina*; and Ettinger *et al.* (2017) found that sediment bacterial communities showed higher diversity compared to *Z. marina* roots and leaves. Altogether, these findings corroborate the idea of an

active selection of specific bacterial groups from the surrounding environment and tissue surfaces.

The endophytic bacterial community in seagrass roots was mostly enriched in genera belonging to the phylum Actinobacteria. This is in agreement with relatively higher abundances of Actinobacteria in the endosphere of *Arabidopsis* than in the rhizosphere (Vandenkoornhuyse *et al.*, 2015). OTUs belonging to Actinobacteria were also identified as part of the core microbiome in three European seagrass species (Cúcio *et al.*, 2016). The phylum Actinobacteria is related to an endophytic lifestyle, with many representatives being endophytes of terrestrial plants, and known to provide biotic resistance in terrestrial plants through antimicrobial compounds (Govindasamy *et al.*, 2014; Suman *et al.*, 2016). Specifically, the genera *Rhodococcus* and *Blastococcus*, found here to be the most and the second most enriched Actinobacteria in surface-sterilized root tissues, have been previously described as endophytes in terrestrial plants. The genus *Rhodococcus* has been previously found in roots of herbaceous, medicinal, and rice plants (Govindasamy *et al.*, 2014). Similarly, the genus *Blastococcus* has been previously found in roots of woody plants (Qin *et al.*, 2009). Therefore, here we identify Actinobacteria as characteristic members of the seagrass root endosphere, for which we suggest a potential beneficial role based on the analogy from evidence of their role in terrestrial plants. Despite the sterilization of plant tissue surfaces has been extensively applied to describe endophytes in terrestrial plants (e.g. Bulgari *et al.*, 2009; Qin *et al.*, 2009; Govindasamy *et al.*, 2014; Reinhold-Hurek *et al.*, 2015); our results on endophytic bacterial communities need to be taken with caution, as some bacteria firmly attached to surfaces could remain after the sterilization protocol, and be included as endophytes in our analysis.

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Rhizobiales, *Rhodobacteraceae* (Proteobacteria phylum), and Clostridiales (Firmicutes phylum) were particularly enriched in plant tissue samples compared to sediment samples. Although our taxonomic identification did not reach the genus level in these groups, Rhizobiales include members with N fixing capabilities and Clostridiales include members involved in S cycling. Similarly, *Rhodobacteraceae*, found here to be a key structural member of seagrass tissues based on our network analysis, is a highly diverse family with key players in biogeochemical cycles (Simon *et al.*, 2017). The importance of N and S cycling in seagrass ecology is largely known and previous seagrass microbiome studies have already reported these potential functions. The core rhizobiome of *Z. marina*, *Z. noltii* and *C. nodosa* was composed mainly of Proteobacteria related to S cycle (Cúcio *et al.*, 2016). In a global study on *Z. marina*, a large fraction of OTUs enriched in roots compared to sediments, were predicted to be sulfur-oxidizing bacterial taxa (Fahimipour *et al.*, 2017). Similarly, in a local study on *Z. marina*, the possible function of the microbiome was related to N and S cycles (Ettinger *et al.*, 2017). Using metatranscriptomics, Crump *et al.* (2018) identified the expression of genes involved in S oxidation, S reduction, N fixation and denitrification in *Z. marina* and *Z. japonica* roots, suggesting potential mutualisms. In fact, we identified the S-oxidizing *Arcobacter* genus to be specifically enriched in surface-sterilized roots samples, in agreement with Crump *et al.* (2018) that pointed *Arcobacter* as one of the root indicator taxa in *Zostera*.

The description of the Red Sea seagrass microbiome provided here identifies Red Sea seagrass as holding a rich and diverse microbiome, highly structured across sites, species and tissues. This structure provides a richness of specialized environments that supports a high alpha diversity of prokaryotes. Indeed, the OTUs found here in association with Red Sea seagrass, 27,861 OTUs, were ten times larger than the number of prokaryote OTUs identified in 94

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samples collected within the photic layer from south to north of the Red Sea (2,708 OTUs; Pearman *et al.*, 2016) and compares, in number of prokaryote OTUs to those found in scleractinian corals in a recent review (up to 10,000 OTUs; Blackall *et al.*, 2015), thereby identifying seagrass as a major reservoir for microbial diversity in the Red Sea. However, comparisons among studies with different methodological approaches needs to be done with caution, as diversity in terms of number of OTUs, is methodological and sequencing depth dependent. This large diversity is rooted in the heavy-tailed power-law distribution of prokaryotes (Eguíluz *et al.*, 2019). Moreover, the finding of highly similar OTUs in well-defined sub-clusters based on our network analysis, suggests the co-occurrence of highly connected key members within the bacterial community of the Red Sea seagrass microbiome. These results provides key information towards the understanding of the role of microorganisms in seagrass ecosystem functioning framed under the seagrass holobiont concept. Although we cannot ascertain functionality of plant enriched OTUs found here, we highlight the need to elucidate the role of prokaryotes in seagrasses by metagenomic and metatranscriptomic sequencing and targeted experimental manipulations in order to advance our understanding of the functional role of the seagrass microbiome.

4. Experimental procedures

4.1. Study site and sample collection

This study was conducted along the East coast of the Red Sea in February 2016 onboard the R/V Thuwal. We sampled 6 different seagrass species (*Halophila stipulacea*, *H. decipiens*, *Thalassodendron ciliatum*, *Halodule uninervis*, *Enhalus acoroides*, and *Thalassia hemprichii*) distributed across 10 stations along a latitudinal gradient. Specifically, we sampled *Halophila stipulacea* and *Thalassia hemprichii* in three stations; *Halodule uninervis* in two stations; and *H.*

decipiens, *Thalassodendron ciliatum*, and *Enhalus acoroides* in one station (Supplementary Fig. S1, Supplementary Table S1). At each station we collected 3 types of samples by SCUBA diving: i) seagrass shoots containing leaves, rhizomes and roots, ii) vegetated sediment and iii) adjacent bare sediment. The seagrass shoots were carefully collected by hand in order to avoid damaging the roots, placed in Ziploc bags and transported immediately onboard to be processed. The vegetated and bare sediments were sampled by collecting triplicate cylindrical cores (I.D. = 4.5 cm, height = 40 cm) containing at least 10 cm of undisturbed sediment.

Once onboard, the samples were processed immediately after each sampling. Seagrass shoots were separated in leaves, rhizomes, and roots using sterile scissors and forceps. The sediment particles not firmly attached to the roots and rhizomes were carefully removed. Then, the seagrass tissues were divided into two parts and subjected to two different protocols. First, one half of the seagrass tissues was surface-sterilized in order to describe the seagrass endosphere microbiome. The surface-sterilization was performed following the protocol described by Coombs and Franco (2003) and adapted by Garcias-Bonet *et al.* (2012). Briefly, seagrass tissues were immersed in ethanol (99% for 1 min), then in NaOCl (3.125% for 6 min), then in ethanol (99% for 30 s) and finally rinsed with autoclaved seawater. Second, the other half of seagrass tissues were only rinsed with autoclaved seawater in order to describe the seagrass microbiome both in the endosphere and the ectosphere (i.e. tissue surface). The seagrass tissues were preserved in RNAlater at 4°C until further processing. The sediment samples were carefully extruded from the cylindrical cores and 5 ml sediment samples were collected from the 10 cm first sediment layer for each core. The sediment samples were preserved in LifeGuard Soil Preservation Solution (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) at 4°C until DNA extraction.

4.2. Bacterial Enrichment protocol from seagrass tissues samples

For the seagrass tissue samples, we performed a bacterial enrichment protocol previously described for terrestrial plants by Ikeda *et al.* (2009), based on differential centrifugation, in order to avoid seagrass chloroplast amplification and due to the low biomass and uneven distribution of bacteria within plant tissues. Briefly, 30 g fresh weight of seagrass tissues were homogenized in 200 ml of bacterial cell extraction (BCE) buffer (50 mM Tris-HCl, 1% Triton X-100, 2 mM β -mercaptoethanol) using a blender for 1 min at highest speed. We repeated the blending for three times, keeping the homogenate on ice for 1 minute between blending periods to avoid overheating. Then the homogenate was filtrated through a sterilized Miracloth filter (MilliporeSigma, Massachusetts, USA) using a vacuum filtration system. The filtrated homogenate was then centrifuged at $500 \times g$ for 5 min at 10°C (Beckman Coulter Avanti J-26XP, California, USA). The supernatant was centrifuged at $5,500 \times g$ for 20 min at 10°C and the resulting pellet was resuspended in 50 ml of BCE buffer using a vortex stirrer, filtrated through a sterilized Kimwipe (Kimtech Science) and centrifuged at $15,000 \times g$ for 10 min at 10°C . The pellet was resuspended in 6 ml of Tris-HCl (50 mM, pH 7.5). The suspension was overlaid on Nycodenz® solution (8 g of Nycodenz® in 10 ml of Tris HCl 50 mM pH 7.5). Specifically, 3 ml of the suspension was carefully overlaid on 2 ml of Nycodenz® solution avoiding mixing of the two resulting phases and centrifuged at $10,000 \times g$ for 40 min at 10°C (Thermo Scientific™ Sorvall WX Ultra Series on AH-650 Swinging Bucket Rotor). The band at interface between the Nycodenz® phase and the upper phase was collected, mixed with an equal volume of sterilized water, and centrifuged at $10,000 \times g$ for 1 min. The pellet was frozen at -80°C until further DNA extraction.

4.3. DNA extraction and 16S rRNA gene library preparation

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The DNA extraction from the bacterial pellet obtained from seagrass tissue samples was performed using NucleoSpin TriPrep Kit (Macherey–Nagel, Dürer, Germany), while the DNA extraction from sediment samples was performed using RNeasy PowerSoil DNA Elution Kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). Despite the use of two DNA extraction kits may introduce some bias, we chose these DNA extraction kits based on the nature of our samples to maximize DNA extraction yield and purification. Since sediment samples contained high organic matter content, including humic acids, which inhibit downstream PCRs, we used the commercial soil DNA extraction kit (RNeasy PowerSoil DNA Elution Kit; Mo Bio Laboratories, Inc., Carlsbad, CA, USA) for sediment samples. In contrast, DNA extraction in plant samples was performed directly on pellets containing bacterial cells after the bacterial enrichment protocol, using a more suitable DNA extraction kit. The V3 and V4 regions of 16S rRNA gene were amplified using the primers designed by Klindworth *et al.* (2013), containing Illumina adapter overhang nucleotide sequences. The sequence of the primer 341F is 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and the sequence of the primer 785R is 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'. We run two duplicate PCR reactions per sample (final volume 25 µl) using Qiagen multiplex PCR master mix (QIAGEN, Valencia, CA), 2.5 µl DNA and a final primer concentration of 0.3 µM. The following PCR conditions were used: initial denaturation at 95 °C for 15 min, followed by 30 cycles consisting of denaturation (95 °C for 40 s), annealing (55 °C for 30 s) and extension (72 °C for 30 s) and a final extension step at 72 °C for 5 min. Duplicate PCR products were pooled and visualized by gel electrophoresis (1.5 % agarose). The 16S rRNA gene library was prepared according to the Illumina 16S metagenomic sequencing library preparation guide.

Briefly, the PCR products were cleaned by AMPure XP magnetic bead based purification (Beckman Coulter, Brea, CA, USA) and MiSeq indexing adaptors were added via PCR. Indexed PCR amplicons were cleaned by AMPure XP magnetic bead based purification, quantified using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and pooled in equimolarity. The pool was quantified using KAPA SYBR FAST Universal qPCR kit with Illumina Primer Premix (Kapa Biosystems Ltd., London, UK) and the pooled amplicon size was assessed on Bioanalyzer (Agilent Technologies, Santa Clara, USA). Six pM of the pool was sequenced on one lane on the Illumina MiSeq platform with 25% PhiX control at KAUST Bioscience core laboratory. The libraries were sequenced using 2 x 300bp overlapping paired-end reads using MiSeq Reagent Kit v3 (Illumina, Inc.).

4.4. Sequence analysis

MiSeq reads were quality checked using FASTQC (Andrews, 2015). Adapters were removed and low-quality reads were quality-trimmed using Trimmomatic 0.36 (Bolger *et al.*, 2014) using default paired-end read settings and reads below length 36 were ignored. Reads were error corrected using SPAdes V3.5 (Bankevich *et al.*, 2012) in --only-error-correction mode. Error-corrected paired end reads were merged using PEAR V0.9.11 (Zhang *et al.*, 2013), with minimum overlap of 100 bps and a minimum length of 390. Chimeric sequences were removed using VSEARCH 2.3.4 (Rognes *et al.*, 2016). OTU picking was done using MOTHUR V1.39.5 (Schloss *et al.*, 2009) using SILVA v128 (Quast *et al.*, 2012) as a reference database. OTUs were clustered at 97% sequence identity. Whereas recent studies propose a change from traditional OTU definition based on the use of fixed dissimilarity thresholds to amplicon sequence variants (ASV, Callahan *et al.*, 2017) all papers on seagrass microbiomes published to-date are based on

OTUs. Hence, we chose to use traditional OTU methods to allow comparability between our results and those for reported seagrass microbiomes elsewhere.

All sequenced reads were deposited in the NCBI database under the Bioproject accession number PRJNA574590.

4.5. Bacterial community structure

The resulting OTU table was divided by sample type (vegetated sediment, bare sediment, non-sterilized leaves, non-sterilized rhizomes, non-sterilized roots, surface-sterilized leaves, surface-sterilized rhizomes, and surface-sterilized roots). We analyzed the abundance distribution of bacterial OTUs in sediment and seagrass tissue samples by performing rank-abundance plots. We fitted heavy-tailed discrete power-law distributions for each group of samples by maximum likelihood estimation using the *powerLaw* package v0.70.6 (Gillespie, 2014) in R. A power-law distribution of abundances follows the shape of $P(A) \sim A^{-\alpha}$ in the range of $A \in [X_{min}, \infty)$. We estimated X_{min} and α parameters, and the statistical significance of the fitted distribution for each group of samples by bootstrapping with 2500 simulations for each group. Shared and unique OTUs among sample types were calculated using Venn diagrams computed with the on-line resource <http://bioinformatics.psb.ugent.be>.

4.6. Alpha and beta diversity of seagrass bacterial community

We calculated Observed, Shannon's, Simpson's, and inverse Simpson's diversity indices for each sample using the *estimate_richness* function from *phyloseq* package (McMurdie and Holmes, 2013) in R software 3.4.1 (RCoreTeam, 2017) together with Prism 6 (GraphPad) software. Moreover, we calculated Simpson's evenness dividing the inverse Simpson's index by the observed number of OTUs for each sample (Morris *et al.*, 2014). Differences in alpha diversity estimates (i.e. Observed, Shannon's, Simpson's, and Simpson's evenness indices)

among sample types were tested by the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test. We used the Bray-Curtis distance among samples to perform Canonical Analysis of Principal Coordinates. We tested the significance of the resulting ordination by permutational MANOVA (PerMANOVA) tests (*adonis* function from *vegan* package in R) with 999 permutations and the null hypothesis that our groups have the same centroid. We tested the influence of environmental variables (Depth, Seawater salinity, and Seawater temperature) on seagrass bacterial community composition by performing a constrained ordination using Bray distances and combining the three environmental variables, followed by a permutational (999 permutations) ANOVA on the constrained axes used in the ordination. Canonical Analysis of Principal coordinates based on Bray-Curtis dissimilarity matrix was used to visualize the different abundance of the 3000 most abundant OTUs in seagrass tissue and sediment samples using the *plot_heatmap* function (Rajaram and Oono, 2010) in the *phyloseq* package. The enriched OTUs in each compartment were calculated and plotted using *edgeR* package (Robinson *et al.*, 2010).

4.7. Network analysis

We identified the main components of the network generated based on the pairwise similarity of OTU abundances per sample. To be specific, the abundance of OTU i in sample m is represented by A_{im} , and the total abundance of OTU i is given by $A_i = \sum_{m=1,S} A_{im}$, where S is the total number of samples. The vector $\vec{x}_i = (x_{i1}, x_{i1}, \dots, x_{iS})$, contains the relative abundances of OTU i in each sample $x_{im}=A_{im}/A_i$. Thus, the similarity between OTU i and j is calculated based on the Jensen-Shannon divergence: $w_{ij} = 1 - JSD(\vec{x}_i||\vec{x}_j)$, where JSD corresponds to the Jensen-Shannon divergence. This similarity is 1 when the abundance distribution is the same for the two OTUs. Precisely, the links with weight $w_{ij} = 1$ are not considered because they

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correspond to OTUs with prevalence in a single sample. For the remaining links we set a threshold of 0.8, that is, we consider only links with weights larger than this threshold. We identified the four largest components (i.e. clusters) consisting of OTUs found in vegetated and bare sediments, and non-sterilized tissues and the first component dominated by OTUs found in surface-sterilized tissues. For each main cluster, we identified the OTUs belonging to well-defined sub-clusters and we analyzed their structure and taxonomic composition. The network was obtained using a code written in Fortran 95, the community analysis was performed with *infomap* (available at www.mapequation.org/infomap) and the visualization with *Gephi* (available at <https://gephi.org>).

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Conflict of Interest Statement

The authors declare no conflict of interest.

References

- Andrews, S. (2015) Babraham bioinformatics-FastQC a quality control tool for high throughput sequence data. URL: <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/> (accessed 0612 2018).
- Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., et al. (2012) SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of computational biology* **19**: 455–477.
- Blackall, L.L., Wilson, B., and Oppen, M.J.H. van (2015) Coral—the world’s most diverse symbiotic ecosystem. *Molecular Ecology* **24**: 5330–5347.
- Bolger, A.M., Lohse, M., and Usadel, B. (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**: 2114–2120.
- Brodersen, K.E., Siboni, N., Nielsen, D.A., Pernice, M., Ralph, P.J., Seymour, J., and Kühl, M. (2018) Seagrass rhizosphere microenvironment alters plant-associated microbial community composition. *Environmental Microbiology*.
- Bulgari, D., Casati, P., Brusetti, L., Quaglino, F., Brasca, M., Daffonchio, D., and Bianco, P. (2009) Endophytic bacterial diversity in grapevine (*Vitis vinifera* L.) leaves described by 16S rRNA gene sequence analysis and length heterogeneity-PCR. *J Microbiol* **47**: 393–401.
- Callahan, B.J., McMurdie, P.J., and Holmes, S.P. (2017) Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *The ISME journal* **11**: 2639–2643.
- Chaidez, V., Dreano, D., Agusti, S., Duarte, C.M., and Hoteit, I. (2017) Decadal trends in Red Sea maximum surface temperature. *Scientific Reports* **7**: 8144.
- Coombs, J.T. and Franco, C.M.M. (2003) Isolation and identification of actinobacteria from surface-sterilized wheat roots. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* **69**: 5603–5608.
- Crump, B.C., Wojahn, J.M., Tomas, F., and Mueller, R.S. (2018) Metatranscriptomics and Amplicon Sequencing Reveal Mutualisms in Seagrass Microbiomes. *Frontiers in Microbiology* **9**: 388.
- Cúcio, C., Engelen, A.H., Costa, R., and Muyzer, G. (2016) Rhizosphere microbiomes of European seagrasses are selected by the plant, but are not species specific. *Frontiers in Microbiology* **7**..
- Devereux, R. (2005) Seagrass rhizosphere microbial communities. *Interactions between macro-and microorganisms in marine sediments* 199–216.
- Donnelly, A.P. and Herbert, R.A. (1999) Bacterial interactions in the rhizosphere of seagrass communities in shallow coastal lagoons. *Journal of Applied Microbiology* **85**: 151S–160S.
- Duarte, C.M., Holmer, M., and Marba, N. (2005) Plant–microbe interactions in seagrass meadows. *Interactions between macro-and microorganisms in marine sediments* 31–60.
- Duarte, C.M., Losada, I.J., Hendriks, I.E., Mazarrasa, I., and Marba, N. (2013) The role of coastal plant communities for climate change mitigation and adaptation. *Nature Clim Change* **3**: 961–968.
- Eguíluz, V.M., Salazar, G., Fernández-Gracia, J., Pearman, J.K., Gasol, J.M., Acinas, S.G., et al. (2019) Scaling of species distribution explains the vast potential marine prokaryote diversity. *Sci Rep* **9**: 1–8.

- Accepted Article
- El Shaffai, A. (2011) Field guide to seagrasses of the Red Sea, by: IUCN, Gland, Switzerland and Total Foundation, Courbevoie, France.
- Ettinger, C.L., Voerman, S.E., Lang, J.M., Stachowicz, J.J., and Eisen, J.A. (2017) Microbial communities in sediment from *Zostera marina* patches, but not the *Z. marina* leaf or root microbiomes, vary in relation to distance from patch edge. *PeerJ* **5**: e3246.
- Eyre, B.D., Ferguson, A.J.P., Webb, A., Maher, D., and Oakes, J.M. (2011) Denitrification, N-fixation and nitrogen and phosphorus fluxes in different benthic habitats and their contribution to the nitrogen and phosphorus budgets of a shallow oligotrophic subtropical coastal system (southern Moreton Bay, Australia). *Biogeochemistry* **102**: 111–133.
- Eyre, B.D., Maher, D.T., and Sanders, C. (2016) The contribution of denitrification and burial to the nitrogen budgets of three geomorphically distinct Australian estuaries: Importance of seagrass habitats. *Limnology and Oceanography*.
- Fahimipour, A.K., Kardish, M.R., Lang, J.M., Green, J.L., Eisen, J.A., and Stachowicz, J.J. (2017) Global-scale structure of the eelgrass microbiome. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* **83**: e03391-16.
- Fourqurean, J.W., Duarte, C.M., Kennedy, H., Marbà, N., Holmer, M., Mateo, M.A., et al. (2012) Seagrass ecosystems as a globally significant carbon stock. *Nature geoscience* **5**: 505–509.
- Garcias-Bonet, N., Arrieta, J.M., Duarte, C.M., and Marba, N. (2016) Nitrogen-fixing bacteria in Mediterranean seagrass (*Posidonia oceanica*) roots. *Aquatic Botany* **131**: 57–60.
- Garcias-Bonet, N., Arrieta, J.M., de Santana, C.N., Duarte, C.M., and Marba, N. (2012) Endophytic bacterial community of a Mediterranean marine angiosperm (*Posidonia oceanica*). *Frontiers in Microbiology* **3**.
- Garcias-Bonet, N., Fusi, M., Ali, M., Shaw, D.R., Saikaly, P.E., Daffonchio, D., and Duarte, C.M. (2018) High denitrification and anaerobic ammonium oxidation contributes to net nitrogen loss in a seagrass ecosystem in the central Red Sea. *Biogeosciences* **15**: 7333–7346.
- Gillespie, C.S. (2014) Fitting heavy tailed distributions: the powerLaw package. *arXiv preprint arXiv:14073492*.
- Govindasamy, V., Franco, C.M., and Gupta, V.V. (2014) Endophytic actinobacteria: diversity and ecology. In *Advances in endophytic research*. Springer, pp. 27–59.
- Gribben, P.E., Nielsen, S., Seymour, J.R., Bradley, D.J., West, M.N., and Thomas, T. (2017) Microbial communities in marine sediments modify success of an invasive macrophyte. *Scientific Reports* **7**: 9845.
- Hemminga, M.A. and Duarte, C.M. (2000) Seagrass ecology, Cambridge University Press.
- Herbert, R.A. (1999) Nitrogen cycling in coastal marine ecosystems. *Fems Microbiol Rev* **23**: 563–590.
- Ikeda, S., Kaneko, T., Okubo, T., Rallos, L.E., Eda, S., Mitsui, H., et al. (2009) Development of a Bacterial Cell Enrichment Method and its Application to the Community Analysis in Soybean Stems. *Microbial Ecology* **58**: 703–714.
- Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., and Glöckner, F.O. (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research* **41**: e1–e1.
- Kusel, K., Trinkwalter, T., Drake, H.L., and Devereux, R. (2006) Comparative evaluation of anaerobic bacterial communities associated with roots of submerged macrophytes

growing in marine or brackish water sediments. *Journal of Experimental Marine Biology and Ecology* **337**: 49–58.

- Les, D.H., Cleland, M.A., and Waycott, M. (1997) Phylogenetic studies in Alismatidae, II: evolution of marine angiosperms (seagrasses) and hydrophily. *Systematic Botany* 443–463.
- Martin, B.C., Gleeson, D., Statton, J., Siebers, A., Grierson, P., Ryan, M.H., and Kendrick, G.A. (2018) Low light availability alters root exudation and reduces putative beneficial microorganisms in seagrass roots. *Frontiers in Microbiology* **8**: 2667.
- McFall-Ngai, M., Hadfield, M.G., Bosch, T.C., Carey, H.V., Domazet-Lošo, T., Douglas, A.E., et al. (2013) Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences* **110**: 3229–3236.
- McMurdie, P.J. and Holmes, S. (2013) phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* **8**: e61217.
- Mejia, A.Y., Rotini, A., Lacasella, F., Bookman, R., Thaller, M.C., Shem-Tov, R., et al. (2016) Assessing the ecological status of seagrasses using morphology, biochemical descriptors and microbial community analyses. A study in *Halophila stipulacea* (Forsk.) Aschers meadows in the northern Red Sea. *Ecological Indicators* **60**: 1150–1163.
- Morris, E.K., Caruso, T., Buscot, F., Fischer, M., Hancock, C., Maier, T.S., et al. (2014) Choosing and using diversity indices: insights for ecological applications from the German Biodiversity Exploratories. *Ecol Evol* **4**: 3514–3524.
- Nellemann, C., Corcoran, E., Duarte, C.M., Valdés, L., De Young, C., Fonseca, L., and Grimsditch, G. (2009) Blue carbon: the role of healthy oceans in binding carbon: a rapid response assessment, UNEP/Earthprint.
- Olsen, J.L., Rouzé, P., Verhelst, B., Lin, Y.-C., Bayer, T., Collen, J., et al. (2016) The genome of the seagrass *Zostera marina* reveals angiosperm adaptation to the sea. *Nature* **530**: 331.
- Pearman, J.K., Kürten, S., Sarma, Y.V.B., Jones, B.H., and Carvalho, S. (2016) Biodiversity patterns of plankton assemblages at the extremes of the Red Sea. *FEMS Microbiol Ecol* **92**.
- Qin, S., Li, J., Chen, H.-H., Zhao, G.-Z., Zhu, W.-Y., Jiang, C.-L., et al. (2009) Isolation, diversity, and antimicrobial activity of rare actinobacteria from medicinal plants of tropical rain forests in Xishuangbanna, China. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* **75**: 6176–6186.
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2012) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research* **41**: D590–D596.
- Qurban, M.A.B., Karuppasamy, M., Krishnakumar, P.K., Garcias-Bonet, N., and Duarte, C.M. (2019) Seagrass Distribution, Composition and Abundance Along the Saudi Arabian Coast of Red Sea. In *Oceanographic and Biological Aspects of the Red Sea*. Springer Oceanography. Rasul, N.M.A. and Stewart, I.C.F. (eds). Cham: Springer International Publishing, pp. 367–385.
- Rajaram, S. and Oono, Y. (2010) NeatMap - non-clustering heat map alternatives in R. *BMC Bioinformatics* **11**: 45.
- Rasul, N.M.A. and Stewart, I.C.F. eds. (2019) *Oceanographic and Biological Aspects of the Red Sea*, Cham: Springer International Publishing.
- RCoreTeam (2017) R: A language and environment for statistical computing [Internet]. Vienna, Austria; 2014.

- Reinhold-Hurek, B., Bünger, W., Burbano, C.S., Sabale, M., and Hurek, T. (2015) Roots shaping their microbiome: global hotspots for microbial activity. *Annual review of phytopathology* **53**: 403–424.
- Reynolds, L.K., Waycott, M., McGlathery, K.J., and Orth, R.J. (2016) Ecosystem services returned through seagrass restoration. *Restoration Ecology* **24**: 583–588.
- Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**: 139–140.
- Rognes, T., Flouri, T., Nichols, B., Quince, C., and Mahé, F. (2016) VSEARCH: a versatile open source tool for metagenomics. *PeerJ* **4**: e2584.
- Rotini, A., Conte, C., Seveso, D., Montano, S., Galli, P., Vai, M., et al. (2020) Daily variation of the associated microbial community and the Hsp60 expression in the Maldivian seagrass *Thalassia hemprichii*. *Journal of Sea Research* **156**: 101835.
- Rotini, A., Mejia, A.Y., Costa, R., Migliore, L., and Winters, G. (2017) Ecophysiological plasticity and bacteriome shift in the seagrass *Halophila stipulacea* along a depth gradient in the Northern Red Sea. *Frontiers in Plant Science* **7**: 2015.
- Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., et al. (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* **75**: 7537–7541.
- Serrano, O., Lovelock, C.E., Atwood, T.B., Macreadie, P.I., Canto, R., Phinn, S., et al. (2019) Australian vegetated coastal ecosystems as global hotspots for climate change mitigation. *Nature communications* **10**: 1–10.
- Short, F., Carruthers, T., Dennison, W., and Waycott, M. (2007) Global seagrass distribution and diversity: A bioregional model. *Journal of Experimental Marine Biology and Ecology* **350**: 3–20.
- Simon, M., Scheuner, C., Meier-Kolthoff, J.P., Brinkhoff, T., Wagner-Döbler, I., Ulbrich, M., et al. (2017) Phylogenomics of Rhodobacteraceae reveals evolutionary adaptation to marine and non-marine habitats. *ISME J* **11**: 1483–1499.
- Suman, A., Yadav, A.N., and Verma, P. (2016) Endophytic microbes in crops: diversity and beneficial impact for sustainable agriculture. In *Microbial inoculants in sustainable agricultural productivity*. Springer, pp. 117–143.
- Sun, F., Zhang, X., Zhang, Q., Liu, F., Zhang, J., and Gong, J. (2015) Seagrass (*Zostera marina*) colonization promotes the accumulation of diazotrophic bacteria and alters the relative abundances of specific bacterial lineages involved in benthic carbon and sulfur cycling. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* **81**: 6901–6914.
- Tarquinio, F., Hyndes, G.A., Laverock, B., Koenders, A., and Sävström, C. (2019) The seagrass holobiont: understanding seagrass-bacteria interactions and their role in seagrass ecosystem functioning. *FEMS Microbiol Lett* **366**.
- Thompson, L.R., Sanders, J.G., McDonald, D., Amir, A., Ladau, J., Locey, K.J., et al. (2017) A communal catalogue reveals Earth's multiscale microbial diversity. *Nature* **551**.
- Trevathan-Tackett, S.M., Sherman, C.D.H., Huggett, M.J., Campbell, A.H., Laverock, B., Hurtado-McCormick, V., et al. (2019) A horizon scan of priorities for coastal marine microbiome research. *Nat Ecol Evol* 1–12.
- Unsworth, R.K.F. and Cullen, L.C. (2010) Recognising the necessity for Indo-Pacific seagrass conservation. *Conservation Letters* **3**: 63–73.

- Vandenkoornhuyse, P., Quaiser, A., Duhamel, M., Le Van, A., and Dufresne, A. (2015) The importance of the microbiome of the plant holobiont. *New Phytologist* **206**: 1196–1206.
- Welsh, D.T. (2000) Nitrogen fixation in seagrass meadows: Regulation, plant-bacteria interactions and significance to primary productivity. *Ecology Letters* **3**: 58–71.
- Zhang, J., Kobert, K., Flouri, T., and Stamatakis, A. (2013) PEAR: a fast and accurate Illumina Paired-End reAd mergeR. *Bioinformatics* **30**: 614–620.
- Ziegler, M., Eguíluz, V.M., Duarte, C.M., and Voolstra, C.R. (2018) Rare symbionts may contribute to the resilience of coral–algal assemblages. *ISME J* **12**: 161–172.

Figure headings

Fig. 1. Heavy-tailed power law distributions of seagrass-associated bacterial communities, showing the estimated scaling exponent (α) values in insets. (A) All samples together in black, sediment samples in orange and plant tissue samples in green. (B) All sediment samples together in black, vegetated sediment samples in brown and bare sediment samples in orange. (C) All plant samples together in black, non-sterilized plant samples in blue and surface-sterilized plant samples in green.

Fig. 2. OTU-based network analysis, showing the resulting five main clusters (I, II, III, IV, and XIII). Each node represents an OTU. Nodes are connected by lines when similarity > 0.8 between OTUs. Nodes are coded by color based on the percentage of reads in each type of samples: vegetated sediment (brown), bare sediment (orange), non-sterilized plant tissue (blue), and surface-sterilized plant tissue (green). Sub-clusters within each cluster are indicated as I.a, II.a, II.b, II.c, III.a, IV.a, and IV.b.

Fig. 3. Taxonomic composition at phylum level and structural schematic representation of main sub-clusters identified by network analysis as shown in Fig. 2. Cluster hubs are represented by thick line circles and color-coded based on phylum taxonomy. Cluster nodes are represented by thin line circles and colored in grey. Connections between cluster hubs are represented by thick lines and connections with nodes are represented by thin lines. Full taxonomic information of cluster hubs is provided in Supplementary Table S2.

Fig. 4. Stacked bar plot showing the bacterial community composition at the phylum level (A) in surface-sterilized tissue, non-sterilized tissue, vegetated sediment, and bare sediment samples for each sampled station. Seagrass sp. sampled on each station are indicated in parenthesis; and

(B) in surface-sterilized and non-sterilized leaf, rhizome, and root tissue samples. Only phyla with relative abundances higher than 2% are shown.

Fig. 5. Bacterial community in seagrass roots. A. Stacked bar plot showing the bacterial community composition at the phylum level in surface-sterilized and non-sterilized root samples for each seagrass species. Only phyla with relative abundances higher than 2% are shown. B. Differentially abundant OTUs between surface-sterilized and non-sterilized root tissue samples at the genus level, color-coded by phyla.

Bacterial community in Red Sea seagrass roots

Phylum Composition of root microbiome

Root compartment enriched genus