Vol. 87: 139–150, 2021
https://doi.org/10.3354/ame01976

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online October 7

The microbiome of the seagrass *Halophila ovalis*: community structuring from plant parts to regional scales

Bertrand Chengxiang Yan[1], Golam Rabbani[1], Nicole Li Ying Lee[2], Jillian Lean Sim Ooi[3], Jen Nie Lee[4], Danwei Huang[2,5,6], Benjamin J. Wainwright[1,*]

[1]Yale-NUS College, National University of Singapore, 16 College Avenue West, Singapore 138527, Singapore
[2]Department of Biological Sciences, National University of Singapore, 16 Science Drive 4, Singapore 117558, Singapore
[3]Department of Geography, Faculty of Arts and Social Sciences, Universiti Malaya, Kuala Lumpur, Malaysia
[4]Faculty of Science and Marine Environment, Universitiy Malaysia Terengganu, Kuala Nerus 21030, Malaysia
[5]Centre for Nature-based Climate Solutions, National University of Singapore, 16 Science Drive 4, Singapore 117558, Singapore
[6]Tropical Marine Science Institute, National University of Singapore, 18 Kent Ridge Road, Singapore 119227, Singapore

ABSTRACT: Seagrass meadows are critical marine ecosystems. They are significant carbon sinks and play numerous important roles in coastal areas. They help to prevent shoreline erosion and serve as nursery grounds for many marine species. Like their terrestrial counterparts, seagrasses form symbiotic relationships with diverse communities of bacteria that help to promote and maintain host fitness. In this study, we sampled the seagrass *Halophila ovalis* throughout Singapore and Peninsular Malaysia to characterise the associated bacterial communities and distributions in this acknowledged seagrass biodiversity hotspot. Three different parts of the seagrass (leaves, roots and rhizomes) were collected, and a sediment sample was collected in close proximity to each host. We used high-throughput 16S rRNA amplicon sequencing to examine the bacterial communities associated with each plant part and location. Our analyses indicated that bacterial assemblages associated with *H. ovalis* were distinct among locations, and different plant parts harboured divergent bacterial communities. We uncovered a significant distance–decay relationship, suggesting that dispersal limitations could explain the observed bacterial community structuring. We further identified bacterial indicator amplicon sequence variants (ASVs) that were associated with degraded or healthy seagrass meadows. The identification of indicator ASVs that are indicative of anthropogenically stressed seagrass, or a declining environment, could be used to implement proactive seagrass conservation and management schemes. This study addresses a current scientific gap within the characterisation of seagrass microbiomes, specifically of those from Southeast Asia, a region of acute seagrass losses, and provides a solid foundation for future seagrass research in the region.

KEY WORDS: Indicator species · Microbial ecology · Plant–microbe interactions · Seagrass microbiome · Southeast Asia · South China Sea · Strait of Malacca

1. INTRODUCTION

Seagrasses are marine angiosperms, and like their terrestrial counterparts, they form intricate relationships with diverse consortia of microorganisms that aid in plant growth, promote health and increase productivity (Tarquinio et al. 2019). These associations help make seagrass meadows some of the most productive ecosystems on the planet (Cullen-Unsworth & Unsworth 2013). Seagrass meadows are

*Corresponding author: ben.wainwright@yale-nus.edu.sg

significant carbon sinks, with some of the highest carbon stocks per unit area of all ecosystems, accounting for 10–18% of the oceans' annual carbon sequestration (Kennedy et al. 2010, Fourqurean et al. 2012, Schile et al. 2017). Seagrasses provide critical habitats for endangered species such as turtles and dugongs (Kendrick et al. 2012), act as nursery grounds for juvenile fish and invertebrates, help prevent coastal erosion, stabilise and prevent sediment resuspension, and reduce human exposure to waterborne pathogens (Harborne et al. 2006, Waycott et al. 2009, Kannan et al. 2010, Lamb et al. 2017).

Microorganisms account for an estimated 70% of all the biomass contained in marine ecosystems (Bar-On et al. 2018, Burgess & Gaines 2018), where they are responsible for about 98% of oceanic primary production and are involved in all of the biogeochemical cycling that takes place in marine habitats (Whitman et al. 1998, Sogin et al. 2006). Yet, 80% of these microorganisms remain unknown. In particular, seagrass meadows (especially those of Southeast Asia) are largely unexplored from a microbial perspective. These diverse coastal habitats present a remarkable opportunity to uncover many novel bacterial taxa and advance our understanding of microbial ecology (see Wainwright et al. 2018a, 2019a).

Work examining seagrass-associated bacteria and their distribution in Southeast Asia, the global epicentre of seagrass diversity (Short et al. 2007), remains in its infancy (Rabbani et al. 2021). Unfortunately, seagrasses in this biologically diverse region face some of the most challenging environmental and conservation issues, especially with regard to pollution, unsustainable aquaculture practices and rapid coastal urbanisation (Todd et al. 2010). Here, by characterising the microbial communities associated with different parts of the seagrass *Halophila ovalis* throughout the Malay Peninsula via 16S rRNA high-throughput sequencing, we provide a first glimpse into the bacterial communities associated with *H. ovalis* in the region and, by doing so, help fill a critical knowledge gap that exists in Southeast Asian seagrass research.

There is growing interest in the marine microbiome, but work in marine systems lags considerably in comparison to terrestrial environments, where microbiome manipulation is seen as an effective technique to mitigate and control disease outbreaks, improve drought resistance, promote growth and increase restoration success (Vandenkoornhuyse et al. 2015, O'Callaghan 2016, Zahn & Amend 2017, Jones et al. 2019, Egan et al. 2021). In marine systems the use of 'marine probiotics' has been proposed as a technique that can help decrease the incidence of coral bleaching and potentially increase coral health and resistance against stressors (Peixoto et al. 2017, 2019, Rosado et al. 2019). Like coral reefs, seagrass meadows are facing increasingly severe threats to their existence (Salinas et al. 2020). Climate change, sea level rise, poor land use practices, and the increasing urbanisation of coastal areas all have detrimental effects on seagrass cover. Globally, seagrass loss is estimated at 7% yr^{-1} (Waycott et al. 2009), with some areas experiencing losses of 45% (Yaakub et al. 2014, 2018). Research is now showing links between seagrass health and microbiome functioning, where microbe–plant mutualisms enhance growth and productivity through nitrogen fixation, mineralising of organic compounds, or the production of auxin to promote growth (Crump et al. 2018, Tarquinio et al. 2019, Martin et al. 2020).

Indicator species are species that can be used as ecological indicators of habitat conditions, environmental change and are frequently referred to as diagnostic species that can have applications in conservation and resource management (De Cáceres et al. 2010, 2012). The characterisation of microbial communities helps facilitate the identification of indicator species that can be representative of increasing anthropogenic stress, and once indicator species have been identified, it becomes possible to implement pro-active mitigation and management strategies (De Cáceres et al. 2010, 2012, Martin et al. 2020).

The present study tests the hypothesis that bacterial dispersal is limited between sample locations. Additionally, on account of the different roles they play, we hypothesise that seagrass leaves, roots and rhizomes will each contain different bacterial communities. As seagrass cover continues to decline in Southeast Asia and further afield, few, if any, seagrass conservation and restoration initiatives consider the contributions that microbes make for the maintenance of seagrass ecosystem health and functioning (Hurtado-McCormick et al. 2019, Tarquinio et al. 2019, Martin et al. 2020, Conte et al. 2021). It is becoming increasingly important that the roles played by microbes are acknowledged and included in conservation and restoration programmes. Failure to do so could mean that transplants are maladapted to their new environments, thus reducing the chance of restoration success. Work such as this is a vital first step in understanding the seagrass microbiome and, more generally, will advance our knowledge on the complex links that drive and structure the marine microbiome as a whole (Trevathan-Tackett et al. 2019, Archer et al. 2020).

2. MATERIALS AND METHODS

2.1. Sample collection, DNA extraction, PCR amplification, and sequencing

Ten individual *Halophila ovalis* plants free of any visible epiphytes were collected at low tide from each of 7 locations (Fig. 1). Each plant was briefly agitated in nearby seawater to remove any loosely attached sediment. To minimise the possible collection of clones, each plant was sampled from a location at least 10 m apart from the closest adjacent sample. Collected plants were separated into leaves, roots and rhizomes with a sterile razor blade. Additionally, a sediment sample (taken from approximately 4 cm below the surface) was collected in close proximity (<1 m) to each host plant. All samples were immediately immersed in a salt-saturated dimethyl sulfoxide (DMSO)-EDTA solution (Hernandez-Agreda et al. 2018) and stored at −20°C before they were transported to the laboratory on ice. Upon arrival they were placed at −80°C until sample processing occurred.

All samples were disrupted in an Omni Bead Ruptor 24 (Omni International) at 8 m s^{-1} for 2 min, and DNA was extracted with a Qiagen DNeasy PowerSoil Kit (Qiagen) following the manufacturer's protocol. PCR amplification of the V4 region of the 16S small subunit ribosomal RNA (SSU rRNA) was performed with the bacterial and archaeal primers 515F and 806R (515F-GTG CCA GCM GCC GCG GTA A; 806R-GGA CTA CHV GGG TWT CTA AT) (Caporaso et al. 2011) using a SC300, SuperCycler (Kyratec). Primers were modified to include Illumina adaptors, a unique barcode and a linker (Caporaso et al. 2011). Each reaction was performed in a total volume of 25 µl, containing 0.1 µl of KAPA 3G Enzyme (Kapa Biosystems), 12.5 µl KAPA PCR Buffer, 0.75 µl of each primer at 10 µM, 1 µl of undiluted template and water to 25 µl. PCR cycling was 94°C for 180 s, followed by 35 cycles of 94°C for 45 s, 50°C for 60 s and 72°C for 90 s, and a final extension at 72°C for 10 min. Negative extraction and PCR controls were included to identify any potential contamination issues. Replicate PCR was not performed (Smith & Peay 2014, Marotz et al. 2019).

PCR products were visualised on a 1% Tris-borate-EDTA (TBE) buffer agarose gel. Cleaning and normalisation of PCR products were performed in SequalPrep normalisation plates (Invitrogen) and sequenced on the Illumina MiSeq platform (600 cycles, V3 chemistry, 300 bp paired end reads) with a 30% PhiX spike (Macrogen Korea).

2.2. Bioinformatics and data processing

Sequences were demultiplexed by Macrogen, and Cutadapt (Martin 2011) was used to remove barcodes and adaptors. Reads were filtered based on quality scores and trimmed using the DADA2 package version 1.14.1 (Callahan et al. 2016) in R version

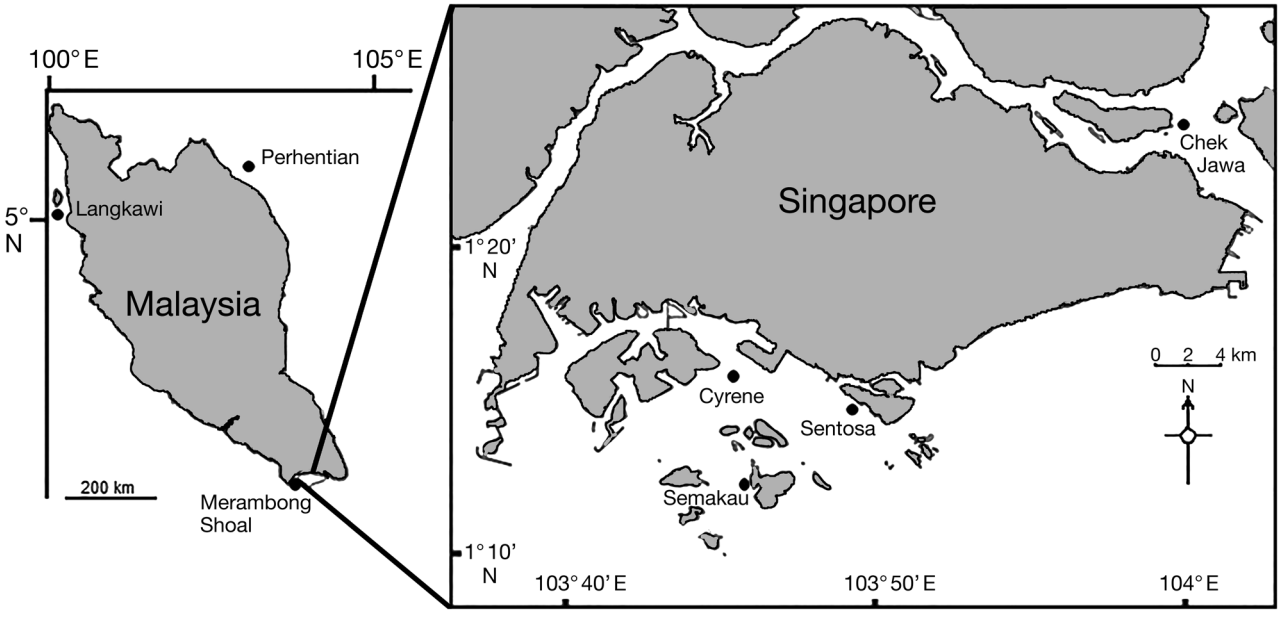

Fig. 1. Location of the 7 sampling sites throughout Singapore and Peninsular Malaysia

3.6.2 (R Core Team 2017). Unless otherwise indicated, all DADA2 processing was performed using the default parameters. Forward and reverse reads were truncated at 250 and 200 bp, respectively. Both forward and reverse reads were filtered to remove any reads with a max EE (expected error) of 2, and reads were additionally truncated at the end of a good quality sequence with the parameter truncQ = 2 (see https://benjjneb.github.io/dada2/ for a detailed explanation of filtering parameters).

The DADA2 algorithm was used to estimate error rates from all quality-filtered reads; forward and reverse reads were then merged to infer amplicon sequence variants (ASVs). De novo detection was used to find and remove chimeras. Sequenced extraction negatives were used to identify potential contaminants using the prevalence method in the decontam R package (Davis et al. 2018). ASVs were assigned taxonomy with the RDP classifier (Cole et al. 2007) against a training set based on the Silva v138 16S database (Quast et al. 2013).

Any ASVs assigned to mitochondrial or chloroplast genomes and those not present in at least 5% of samples were removed (Cao et al. 2021). Rarefaction curves were produced using the rarecurve() function implemented in the vegan R package version 2.5.6. Raw sequence counts were then converted to relative abundance (Becker et al. 2020). The Shannon diversity for each sample was calculated, and non-metric multi-dimensional scaling (NMDS) was performed on the Bray-Curtis dissimilarity matrix of samples using the phyloseq R package version 1.30.0 (McMurdie & Holmes 2013). NMDS plots were generated for each part individually (leaf, rhizome, root, sediment), and then all samples combined, and bar plots of relative abundance were made using ggplot2 version 3.3.1 (Wickham 2011).

To test for differences in community structure between locations and sampled parts, we performed permutational multivariate ANOVA with 999 permutations using the adonis() function of the vegan R package version 2.5.6. To test for homogeneity of dispersion, a beta-dispersion test was performed with 999 permutations using the betadisper function in the vegan R package. A Mantel test (Spearman correlation) and multiple regression on distance matrices (Bray-Curtis) were performed using the ecodist R package to test for distance–decay similarity with 9999 permutations. Indicator species were identified for each part and each site using the indicspecies R package version 1.7.9 with 999 permutations using default indicator values and an alpha of 0.05 (De Cáceres et al. 2012). Mann-Whitney *U*-tests were used to determine whether significant differences in diversity exist between locations and part sampled. All sequences associated with the present study have been deposited at the National Center for Biotechnology Information under BioProject ID: PRJNA649078.

3. RESULTS

In total, 280 samples from 7 sampling sites had their bacterial communities profiled; see Table S1 in Supplement 1 at www.int-res.com/articles/suppl/a087p139_supp1.xlsx for details of sequencing depth and the number of reads used in downstream analysis after quality filtering steps had been performed. Rarefaction curves show that sequencing depth was sufficient to recover all bacterial diversity, and ASV richness was exhausted in every single sample (Fig. S1 in Supplement 2 at www.int-res.com/articles/suppl/a087p139_supp2.pdf). After quality filtering and removing low prevalence sequences (<5%), a total of 1888 unique ASVs were identified.

ASVs represented 44 phyla, comprising 169 families with members of *Proteobacteria*, *Firmicutes* and *Desulfobacterota* phyla dominating all sample types (Figs. 2 & S2–S5). Shannon diversity between sampling locations was similar, with the highest diversity found at Merambong Shoal (Fig. S6), although this higher diversity was not significant ($p > 0.05$). Sediment samples had the highest diversity and were significantly different from all other samples ($p < 0.01$). Leaves, roots and rhizomes were not significantly different from one another ($p > 0.05$) (Fig. S7). Heatmaps showed that phylum *Bacteroidota* tended to be found in higher abundance in roots in comparison to other parts, while *Cyanobacteria* were more common in living seagrass parts but less abundant in sediment samples, and the *Proteobacteria* were abundant throughout all parts and in the sediment (Fig. S8), with the orders *Oceanospirillalesm, Rhizobiales, Vibrionales* and *Alteromonadales* also found in all samples. Root tissues had a higher abundance of bacteria from the genus *Halomonas*, and *Pseudoalteromonas* were abundant in all parts other than leaves (Figs. S9 & S10).

Indicator species analysis showed that a number of bacterial taxa could be used as indicators at each location with different parts of the seagrass plant showing significant associations with particular bacterial genera; see Tables 1 & 2 for the top 5 indicator species in each part and location and Tables S2 & S3 for a complete list. *Vibrio* was the most commonly encountered genus, found at 6 of the 7 sampling

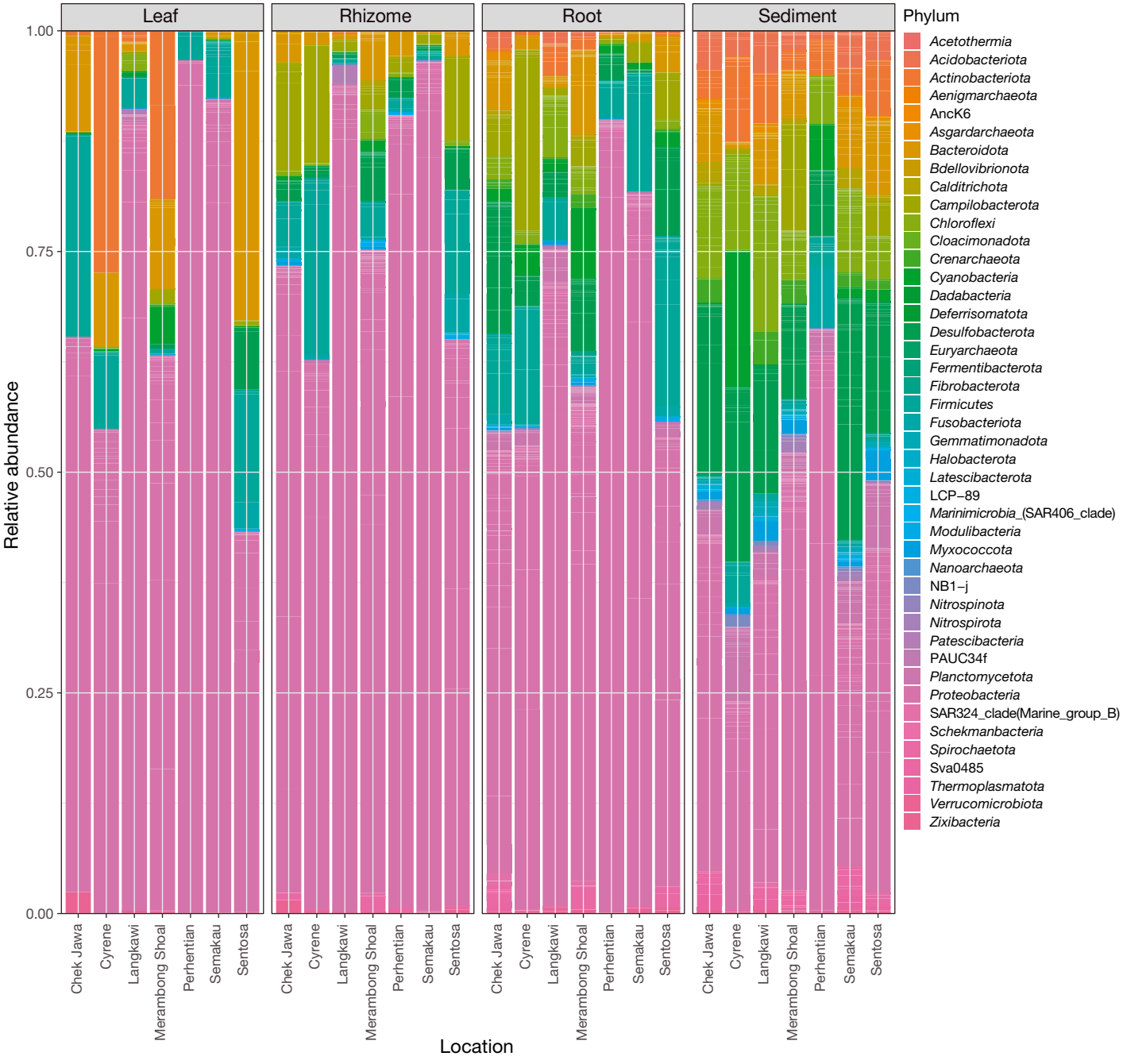

Fig. 2. Stacked bar plots of relative bacterial abundance. All samples of a specific type from one location combined

locations (Table 2). Roots, rhizomes and sediment samples tend to associate with bacteria that play roles in nitrogen fixation, the cycling of other nutrients such as carbon, and the degradation of complex organic compounds, or those that can alleviate the sulphide stresses associated with water-logged sediment (e.g. *Thiodiazotropha, Tistlia, Thalassotalea, Oceanospirillales* and *Blastopirellula*) (Díaz-Cárdenas et al. 2010, Jensen et al. 2010, Petersen et al. 2016, Kim et al. 2020) (Table 1).

NMDS plots indicate that bacterial communities can be differentiated by location and part sampled (Fig. 3), with sediment samples showing particularly well-defined clusters (Fig. 3). Permutational multivariate analysis of variance (PERMANOVA) shows significant differences in bacterial community be-

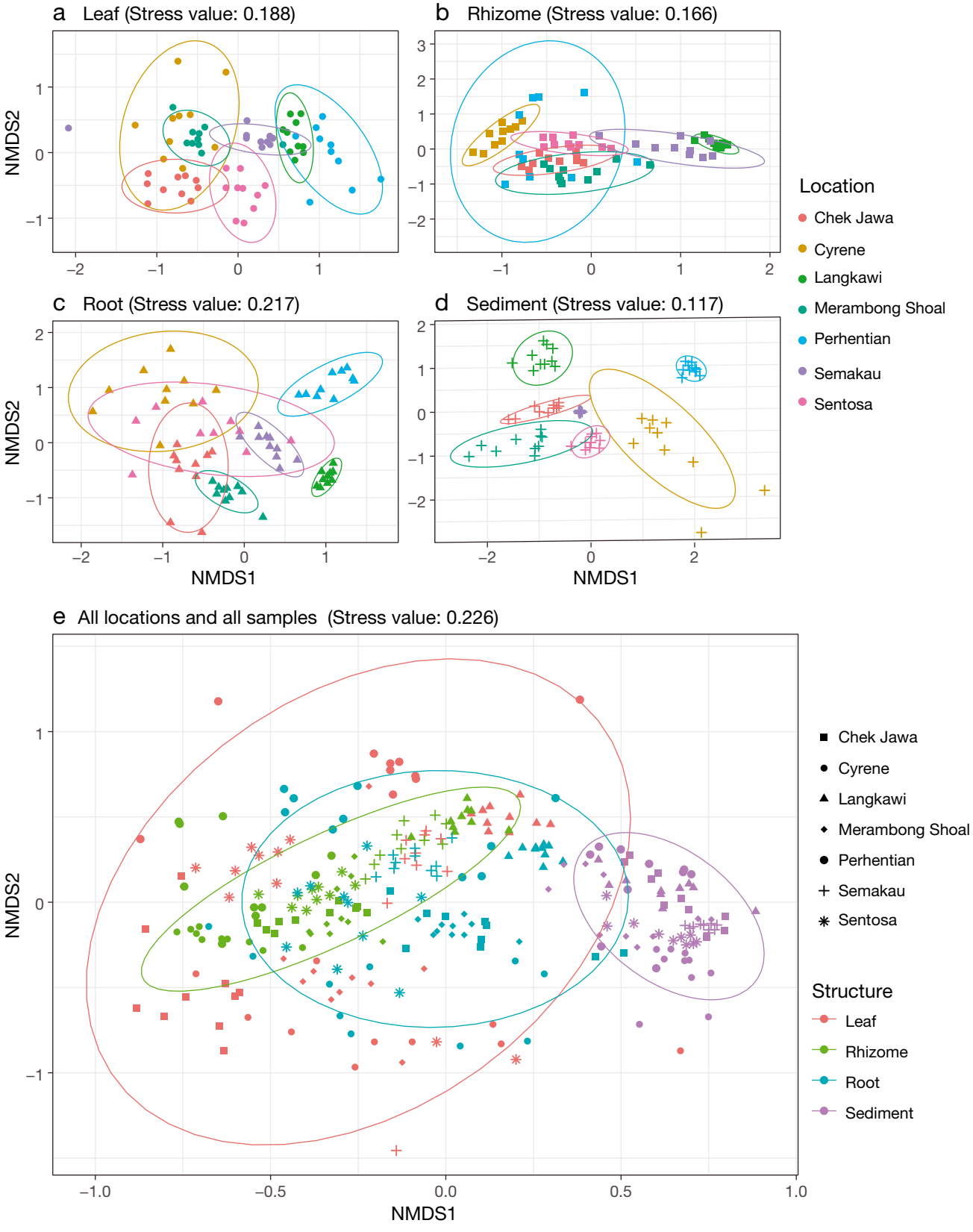

Fig. 3. Non-metric multidimensional scaling (NMDS) of bacterial communities based on Bray-Curtis dissimilarity

Table 1. Bacterial genera of the top 5 amplicon sequence variants (ASVs) that were significantly associated with each plant structure of *Halophila ovalis*. See Table S2 in Supplement 1 for a complete list of significant associations

Structure	Genus of associated ASV	No. of corresponding ASVs
Leaf	*Ilumatobacter*	1624
	Agaribacterium	4937
	Ruegeria	7059
	Arenicella	964
	Hyphomonas	989
Rhizome	*Methylotenera*	27 467
	Candidatus_Thiodiazotropha	17 359
	Candidatus_Thiodiazotropha	36 170
	Methylotenera	6877
	Hypnocyclicus	6184
Root	*Epulopiscium*	11 712
	Thalassotalea	3491
	Reinekea	81 816
	Romboutsia	1327
	Dasania	33 451
Sediment	*Defluviicoccus*	1690
	Thiogranum	20 171
	Blastopirellula	4088
	Sva0081_sediment_group	6304
	Tistlia	1547

tween locations and between plant parts (both $p = 0.001$; Table S4); location and sampled part had a significant interaction ($p = 0.001$; Table S4). Beta-dispersion tests indicated significant differences in dispersion ($p < 0.001$); however, the balanced design of our work means our PERMANOVA results and interpretations are robust and unaffected (Anderson & Walsh 2013). We found a significant distance–decay of community similarity, with sites in close proximity tending to have more similar bacterial communities than those further away. Sediment samples showed the strongest distance–decay relationship, and the weakest relationship of any one part was found in the leaf samples. When all samples were combined, a weak but significant distance–decay relationship was observed (Table 3).

4. DISCUSSION

Our work here, like other work performed in the region (Pootakham et al. 2017, Lee et al. 2019, 2020, Wainwright et al. 2019a,b, 2020b, Tan et al. 2020, Oh et al. 2021), shows that bacterial communities associated with marine taxa differ strongly over comparatively small spatial scales, even when the distance

Table 2. Bacterial genera of the top 5 amplicon sequence variants (ASVs) that were significantly associated with the seagrass *Halophila ovalis* at each location. See Table S3 in Supplement 1 for a complete list of significant associations

Location	Genus of associated ASV	No. of corresponding ASVs
Chek Jawa	*Thiohalophilus*	2511
	Muriicola	1034
	Labrenzia	21 331
	Desulfatiglans	983
	Vibrio	10 721
Cyrene	*Stenotrophomonas*	3673
	Vallitalea	5418
	Clostridium	759
	Cyanobacterium	4298
	Vibrio	10 090
Langkawi	*Vibrio*	46 534
	Marinomonas	23 764
	Exiguobacterium	25 065
	Roseibacillus	1438
	Halarcobacter	11 712
Merambong Shoal	*Vibrio*	79 591
	Thalassotalea	9455
	Pseudoalteromonas	14 054
	Thalassotalea	1326
	Photobacterium	33 451
Perhentian	*Halomonas*	29 854
	Unidentified (CI75cm.2.12)	457
	Unidentified (CI75cm.2.12)	5082
	Vibrio	1842
	Aestuariibacter	404
Semakau	*Turicibacter*	1711
	Vibrio	8500
	Romboutsia	758
	Photobacterium	519
	Epulopiscium	4899
Sentosa	*Dokdonia*	1199
	Tenacibaculum	8221
	Muricauda	2738
	Psychrosphaera	1173
	Altererythrobacter	6139

Table 3. Mantel test and multiple regression on distance matrices (MRM) results for all structure combined and each individual structure

	Mantel R statistic	Mantel significance	MRM R^2	MRM significance
All	0.09	<0.001	0.02	<0.001
Leaf	0.34	<0.001	0.04	<0.001
Rhizome	0.37	<0.001	0.11	<0.001
Root	0.40	<0.001	0.10	<0.001
Sediment	0.45	<0.001	0.22	<0.001

between sampling sites is less than 10 km. This is contrary to the generally accepted notion that microbes show weak biogeographic patterns, an apparent consequence of their high abundance, longevity, high dispersal ability, high degree of ecological redundancy and ability to rapidly acquire the traits needed for survival in new habitats through horizontal gene transfer (Finlay 2002, Horner-Devine et al. 2004, Meyer et al. 2018).

As others have suggested (Horner-Devine et al. 2004, Tisthammer et al. 2016, Martin et al. 2020), it is likely that environmental differences are contributing to the structuring of bacterial communities associated with *Halophila ovalis* observed here. Similar ideas regarding differences in habitat type have been invoked to explain the high diversity of marine taxa found on coral reefs throughout Southeast Asia (Palumbi 1994, Benzie 1999, Bowen et al. 2013, 2020, Wainwright et al. 2018a, 2020a). While not explicitly tested for here, it is not unreasonable to suggest that the differences we observe in bacterial communities at different sampling sites are a consequence of environmental heterogeneity. For example, an environmental cline has been identified as one moves in a south-to-north direction through the Malay Peninsula, and a gradual increase in salinity and dissolved oxygen with increasing latitude is observed in the Strait of Malacca (Muhaimin et al. 2011). Peninsular Malaysia is split in half by the Titiwangsa mountain range. The strata on either side of this range is different in composition; coastal western Malaysia is predominantly Permian in origin, while coastal eastern Malaysia is predominantly Carboniferous strata (Hutchison 2014). Additionally, the more than 2000 m elevation Titiwangsa mountain range has the potential to severely restrict, if not completely curtail dispersal between coastal regions. Dispersal limitations, the different physical characteristics of water masses, and the differences in substrate chemistry created by strata of different origins all have the potential to structure bacterial communities. Correspondingly, clinal effects over similar distances, and differences in substrate pH, organic and inorganic carbon content have all been shown to significantly influence microbial community composition (Horner-Devine et al. 2004, Goldmann et al. 2016, Vincenot et al. 2017, Tan et al. 2020). Further limiting dispersal, water currents on the western side of Peninsular Malaysia flow in a northerly direction all year round (Rizal et al. 2010). This likely severely limits dispersal in a southerly direction. It is plausible that these differences are responsible for, or at least contribute to, the differences we see in bacterial communities associated with *H. ovalis* throughout Singapore and Peninsular Malaysia. This idea is further supported by the results of our Mantel test and multiple regression analyses, both suggesting a positive pattern of distance–decay of similarity.

Consistent with other research, we found that soil and sediment samples contain the highest diversity of microbes when compared to living plant parts (Serna-Chavez et al. 2013, Fierer 2017, Rabbani et al. 2021). Plants are able to exert a degree of control over the bacterial communities they associate with, and act somewhat like a filter, limiting or regulating their associated communities (Goldmann et al. 2016, Jones et al. 2019, Wang et al. 2020). This filtering cannot happen in the sediment, and correspondingly we see the highest bacterial diversity in these samples. The idea of filtering by living parts is given more credibility by our NMDS plots: living parts, while showing clustering, tend to exhibit a higher degree of overlap in bacterial community composition. This suggests that each part, irrespective of sampling location, requires a reasonably similar bacterial community to function optimally and promote host health. Whereas, sediment samples form more distinct clusters, indicating more unique bacterial communities; this uniqueness in the absence of filtering is likely shaped by the different environmental conditions at each sampling location. The significant pattern of distance–decay seen in the bacterial communities further supports this idea, with the strongest relationship observed in the sediment samples.

All seagrass parts and sediment samples contained members from the order *Oceanospirillales,* a common marine bacteria that is able to excrete hydrolytic enzymes to degrade complex organic compounds and aid in carbon fixation (Jensen et al. 2010). Also found throughout the *H. ovalis* microbiome are the *Rhizobiales*; members of this order include a number of nitrogen-fixing microbes that are frequently observed in symbiotic relationships with plants (Fischer et al. 2012, Martin et al. 2018). Like other work, we found the genera *Halomonas* and *Pseudoalteromonas* associated with seagrass samples. *Halomonas* and *Pseudoalteromonas* have both previously been identified as putative pathogens, *Halomonas* of humans and *Pseudoalteromonas* of invertebrates (Liu et al. 2018). Additionally, *Pseudoalteromonas* have been isolated from seagrass sediments (Lee et al. 2016). Similarly, we found this genus in our sediment samples and belowground seagrass parts. Members of this genus have been associated with the facilitation of marine plant growth and promoting

stress tolerance in plants (Lee et al. 2016). Finding bacteria that may promote stress tolerance could be an adaptation to the increasingly anthropogenically stressed seagrass habitats of Southeast Asia; however, additional work is required to determine if this is the case.

Analysis of indicator species by plant part or associated sediment sample revealed a number of taxa significantly associated with each other in this study; however, none of the bacteria we found associated with leaves have previously been identified as indicative of stressed or healthy seagrass meadows in other studies. Members of the genus *Methylotenera* have been identified as indicator species found in belowground seagrass parts (Crump et al. 2018), and we found the same genera associated with rhizomes in this study. *Methylotenera* and other methylotrophs play a key role in the metabolism of organic C_1 compounds (e.g. methane and methanol) (Chistoserdova et al. 2009). All angiosperms produce methanol during cell-wall synthesis, and there is evidence that methanol can support diverse populations of bacterial methylotrophs which can enhance the growth of photosynthetic C_3 plants (Fall & Benson 1996), thus accounting for our finding of this taxon in belowground parts. *Methylotenera* species have also been identified as indicator species associated with healthy seagrass beds in Western Australia (Martin et al. 2020); however, the same study suggests that the sulphide-oxidising genera *Thiodiazotropha* can be indicative of stressed seagrass. Like the *Methylotenera,* we found *Thiodiazotropha* significantly associated with belowground parts. These inconsistencies suggest that caution is needed when using indicator species identified outside the region of interest, and it is possible that a unique set of indicator species needs to be identified and developed for each region and each host species. The genera *Hypnocyclicus* and *Reinekea* were both identified as indicators in belowground parts. *Hypnocyclicus* is a marker of wastewater input and eutrophication (Kopprio et al. 2021), and *Reinekea* is a genus that has been implicated in the degradation of organic matter following phytoplankton blooms and has previously been associated with seagrasses (Teeling et al. 2012, Hassenrück et al. 2015). Finding *Hypnocyclicus* and *Reinekea* identified as indicator taxa within our samples could be indicative of the pressures associated with increasing coastal urbanisation in Southeast Asia. We also found the genus *Tistlia* in our sediment samples, a known nitrogen fixer that is associated with higher seagrass cover (Alsaffar et al. 2020). *Tistlia* is thought to form symbiotic relationships with seagrasses by supplying a source of fixed nitrogen that can be used by growing seagrass (Alsaffar et al. 2020). Similar to our rhizome samples, we found the sulphur-cycling bacterial genera *Thiogranum* and Sva0081 (*Desulfobacteraceae*) associated with sediment samples. Sulphur cycling has been linked to healthy and productive seagrass ecosystems throughout the world (Martin et al. 2019), where the mineralisation of organic matter in the sediment into biologically available forms is tightly coupled to microbial sulphate reduction (Jørgensen 1982, Holmer et al. 2001). Six of the 7 sampling sites showed significant associations with the genus *Vibrio*. Multiple studies identify *Vibrio* spp. as probable bacterial pathogens in seagrass beds, and these species tend to be more abundant in areas that are subjected to greater disturbance (Liu et al. 2018, Tarquinio et al. 2019). The presence of *Vibrio* spp. in the majority of our sampling sites is in general agreement with the declining seagrass cover and increasing anthropogenic disturbances the region is experiencing (Fortes et al. 2018).

The identification of bacterial indicator species, along with the continued monitoring of seagrass beds for change in bacterial communities, could allow the development of proactive seagrass management strategies. For example, the discovery of *Vibrio* spp. in a seagrass meadow could be indicative of increasing stress. If this happens, it may be possible to isolate the cause of the stressor (e.g. sediment inputs from building, coastal development or forestry) and implement mitigation strategies to reduce the stress (i.e. sediment curtains to prevent sediment runoff). It may also be possible to seed areas with beneficial microbes (e.g. the genus *Tistlia* that aids in nitrogen fixation). However, much more work is required in controlled settings before this should be attempted. Indeed, the complex nature of natural ecosystems necessitates a detailed understanding of the processes and unintended consequences that microbial seeding could have on ecosystem functioning. Nevertheless, seeding areas with bacteria that promote nitrogen fixation and those that are associated with higher seagrass cover could help curtail future seagrass losses (Unsworth et al. 2019).

Work examining the seagrass microbiome is still in its infancy in Southeast Asia, but work such as this, which seeks to characterise bacterial communities, is important, and it allows us to build on these foundations and develop more explicit hypothesis-driven research that aims to facilitate a better understanding of the seagrass ecosystem. Ultimately, we

hope this will assist in the design and implementation of more effective conservation and restoration schemes.

Acknowledgements. All samples from Singapore were collected under permit number NP/RP16-156 issued by the National Parks Board of Singapore. Collections from Malaysia were made under permit JTLM 630-7Jld.9(9). The authors declare no conflict of interest. This study was funded by the National Research Foundation, Prime Minister's Office, Singapore under its Marine Science R&D Programme (MSRDP-P03) and the Mandai Nature Fund. Student support was provided by the Summer Research Programme, administered by the Yale-NUS College Centre for International & Professional Experience (CIPE) and co-funded by the Yale-NUS Dean of Faculty Office. The funders played no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

LITERATURE CITED

Alsaffar Z, Pearman JK, Cúrdia J, Ellis J and others (2020) The role of seagrass vegetation and local environmental conditions in shaping benthic bacterial and macroinvertebrate communities in a tropical coastal lagoon. Sci Rep 10:13550

Anderson MJ, Walsh DCI (2013) PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: What null hypothesis are you testing? Ecol Monogr 83:557–574

Archer SDJ, Lee KC, Caruso T, King-Miaow K and others (2020) Air mass source determines airborne microbial diversity at the ocean–atmosphere interface of the Great Barrier Reef marine ecosystem. ISME J 14:871–876

Bar-On YM, Phillips R, Milo R (2018) The biomass distribution on Earth. Proc Natl Acad Sci USA 115:6506–6511

Becker CC, Weber L, Suca JJ, Llopiz JK, Mooney TA, Apprill A (2020) Microbial and nutrient dynamics in mangrove, reef, and seagrass waters over tidal and diurnal time scales. Aquat Microb Ecol 85:101–119

Benzie JAH (1999) Genetic structure of coral reef organisms: ghosts of dispersal past. Integr Comp Biol 39:131–145

Bowen BW, Rocha LA, Toonen RJ, Karl SA (2013) The origins of tropical marine biodiversity. Trends Ecol Evol 28: 359–366

Bowen BW, Forsman ZH, Whitney JL, Faucci A and others (2020) Species radiations in the sea: What the flock? J Hered 111:70–83

Burgess MG, Gaines SD (2018) The scale of life and its lessons for humanity. Proc Natl Acad Sci USA 115: 6328–6330

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP (2016) DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods 13: 581–583

Cao Q, Sun X, Rajesh K, Chalasani N and others (2021) Effects of rare microbiome taxa filtering on statistical analysis. Front Microbiol 11:607325

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D and others (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci USA 108:4516–4522

Chistoserdova L, Kalyuzhnaya MG, Lidstrom ME (2009) The expanding world of methylotrophic metabolism. Annu Rev Microbiol 63:477–499

Cole JR, Chai B, Farris RJ, Wang Q and others (2007) The ribosomal database project (RDP-II): introducing myRDP space and quality controlled public data. Nucleic Acids Res 35:D169–D172

Conte C, Rotini A, Manfra L, D'Andrea MM, Winters G, Migliore L (2021) The seagrass holobiont: what we know and what we still need to disclose for its possible use as an ecological indicator. Water 13:406

Crump BC, Wojahn JM, Tomas F, Mueller RS (2018) Metatranscriptomics and amplicon sequencing reveal mutualisms in seagrass microbiomes. Front Microbiol 9:388

Cullen-Unsworth L, Unsworth R (2013) Seagrass meadows, ecosystem services, and sustainability. Environment 55: 14–28

Davis NM, Proctor DM, Holmes SP, Relman DA, Callahan BJ (2018) Simple statistical identification and removal of contaminant sequences in marker-gene and metagenomics data. Microbiome 6:226

De Cáceres MD, Legendre P, Moretti M (2010) Improving indicator species analysis by combining groups of sites. Oikos 119:1674–1684

De Cáceres MD, Legendre P, Wiser SK, Brotons L (2012) Using species combinations in indicator value analyses. Methods Ecol Evol 3:973–982

Díaz-Cárdenas C, Patel BKC, Baena SY (2010) *Tistlia consotensis* gen. nov., sp. nov., an aerobic, chemoheterotrophic, free-living, nitrogen-fixing alphaproteobacterium, isolated from a Colombian saline spring. Int J Syst Evol Microbiol 60:1437–1443

Egan CP, Koko JH, Muir CD, Zahn G, Swift SOI, Amend AS, Hynson NA (2021) Restoration of the mycobiome of the endangered Hawaiian mint *Phyllostegia kaalaensis* increases its resistance to a common powdery mildew. Fungal Ecol 52:101070

Fall R, Benson AA (1996) Leaf methanol—the simplest natural product from plants. Trends Plant Sci 1:296–301

Fierer N (2017) Embracing the unknown: disentangling the complexities of the soil microbiome. Nat Rev Microbiol 15:579–590

Finlay BJ (2002) Global dispersal of free-living microbial eukaryote species. Science 296:1061–1063

Fischer D, Pfitzner B, Schmid M, Simões-Araújo JL and others (2012) Molecular characterisation of the diazotrophic bacterial community in uninoculated and inoculated field-grown sugarcane (*Saccharum* sp.). Plant Soil 356:83–99

Fortes MD, Ooi JLS, Tan YM, Prathep A, Bujang JS, Yaakub SM (2018) Seagrass in Southeast Asia: a review of status and knowledge gaps, and a road map for conservation. Bot Mar 61:269–288

Fourqurean JW, Duarte CM, Kennedy H, Marbà N and others (2012) Seagrass ecosystems as a globally significant carbon stock. Nat Geosci 5:505–509

Goldmann K, Schröter K, Pena R, Schöning I and others (2016) Divergent habitat filtering of root and soil fungal communities in temperate beech forests. Sci Rep 6:31439

Harborne AR, Mumby PJ, Micheli F, Perry CT, Dahlgren CP, Holmes KE, Brumbaugh DR (2006) The functional value of Caribbean coral reef, seagrass and mangrove habitats to ecosystem processes. Adv Mar Biol 50:57–189

Hassenrück C, Hofmann LC, Bischof K, Ramette A (2015) Seagrass biofilm communities at a naturally CO_2-rich vent. Environ Microbiol Rep 7:516–525

Hernandez-Agreda A, Leggat W, Ainsworth TD (2018) A comparative analysis of microbial DNA preparation methods for use with massive and branching coral growth forms. Front Microbiol 9:2146

Holmer M, Andersen FØ, Nielsen SL, Boschker HTS (2001) The importance of mineralization based on sulfate reduction for nutrient regeneration in tropical seagrass sediments. Aquat Bot 71:1–17

Horner-Devine MC, Lage M, Hughes JB, Bohannan BJM (2004) A taxa–area relationship for bacteria. Nature 432: 750–753

Hurtado-McCormick V, Kahlke T, Petrou K, Jeffries T, Ralph PJ, Seymour JR (2019) Regional and microenvironmental scale characterization of the *Zostera muelleri* seagrass microbiome. Front Microbiol 10:1011

Hutchison CS (2014) Tectonic evolution of Southeast Asia. Bull Geol Soc Malaysia 60:1–18

Jensen S, Duperron S, Birkeland NK, Hovland M (2010) Intracellular *Oceanospirillales* bacteria inhabit gills of *Acesta* bivalves. FEMS Microbiol Ecol 74:523–533

Jones P, Garcia BJ, Furches A, Tuskan GA, Jacobson D (2019) Plant host-associated mechanisms for microbial selection. Front Plant Sci 10:862

Jørgensen BB (1982) Mineralization of organic matter in the sea bed—the role of sulphate reduction. Nature 296: 643–645

Kannan RRR, Arumugam R, Anantharaman P (2010) Antibacterial potential of three seagrasses against human pathogens. Asian Pac J Trop Med 3:890–893

Kendrick GA, Fourqurean JW, Fraser MW, Heithaus MR, Jackson G, Friedman K, Hallac D (2012) Science behind management of Shark Bay and Florida Bay, two P-limited subtropical systems with different climatology and human pressures. Mar Freshw Res 63:941

Kennedy H, Beggins J, Duarte CM, Fourqurean JW, Holmer M, Marbà N, Middelburg JJ (2010) Seagrass sediments as a global carbon sink: isotopic constraints. Global Biogeochem Cycles 24:GB4026

Kim M, Cha IT, Lee KE, Lee EY, Park SJ (2020) Genomics reveals the metabolic potential and functions in the redistribution of dissolved organic matter in marine environments of the enus *Thalassotalea*. Microorganisms 8:1412

Kopprio GA, Cuong LH, Luyen ND, Duc TM, Ha TH, Huong LM, Gärdes A (2021) Carrageenophyte-attached and planktonic bacterial communities in two distinct bays of Vietnam: eutrophication indicators and insights on ice-ice disease. Ecol Indic 121:107067

Lamb JB, van de Water JAJM, Bourne DG, Altier C and others (2017) Seagrass ecosystems reduce exposure to bacterial pathogens of humans, fishes, and invertebrates. Science 355:731–733

Lee RD, Jospin G, Lang JM, Eisen JA, Coil DA (2016) Draft genome sequences of two *Pseudoalteromonas porphyrae* strains isolated from seagrass sediment. Genome Announc 4:e00092–16

Lee NLY, Huang D, Quek ZBR, Lee JN, Wainwright BJ (2019) Mangrove-associated fungal communities are differentiated by geographic location and host structure. Front Microbiol 10:2456

Lee NLY, Huang D, Quek ZBR, Lee JN, Wainwright BJ (2020) Distinct fungal communities associated with different organs of the mangrove *Sonneratia alba* in the Malay Peninsula. IMA Fungus 11:17

Liu S, Jiang Z, Deng Y, Wu Y and others (2018) Effects of nutrient loading on sediment bacterial and pathogen communities within seagrass meadows. Microbiology-Open 7:e00600

Marotz C, Sharma A, Humphrey G, Gottel N and others (2019) Triplicate PCR reactions for 16S rRNA gene amplicon sequencing are unnecessary. Biotechniques 67:29–32

Martin BC, Gleeson D, Statton J, Siebers AR, Grierson P, Ryan MH, Kendrick GA (2018) Low light availability alters root exudation and reduces putative beneficial microorganisms in seagrass roots. Front Microbiol 8:2667

Martin BC, Bougoure J, Ryan MH, Bennett WW and others (2019) Oxygen loss from seagrass roots coincides with colonisation of sulphide-oxidising cable bacteria and reduces sulphide stress. ISME J 13:707–719

Martin BC, Alarcon MS, Gleeson D, Middleton JA and others (2020) Root microbiomes as indicators of seagrass health. FEMS Microbiol Ecol 96:fiz201

Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.journal 17: 10–12

McMurdie PJ, Holmes S (2013) Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLOS ONE 8:e61217

Meyer KM, Memiaghe H, Korte L, Kenfack D, Alonso A, Bohannan BJM (2018) Why do microbes exhibit weak biogeographic patterns? ISME J 12:1404–1413

Muhaimin AA, Zaiton Ibr Z, Aizat Isma S (2011) Water mass characteristics in the Strait of Malacca using Ocean Data View. Res J Environ Sci 5:49–58

O'Callaghan M (2016) Microbial inoculation of seed for improved crop performance: issues and opportunities. Appl Microbiol Biotechnol 100:5729–5746

Oh RM, Bollati E, Maithani P, Huang D, Wainwright BJ (2021) The microbiome of the reef macroalga *Sargassum ilicifolium* in Singapore. Microorganisms 9:898

Palumbi SR (1994) Genetic divergence, reproductive isolation, and marine speciation. Annu Rev Ecol Syst 25:547–572

Peixoto RS, Rosado PM, Leite DC de A, Rosado AS, Bourne DG (2017) Beneficial microorganisms for corals (BMC): proposed mechanisms for coral health and resilience. Front Microbiol 8:341

Peixoto RS, Sweet M, Bourne DG (2019) Customized medicine for corals. Front Mar Sci 6:1–5

Petersen JM, Kemper A, Gruber-Vodicka H, Cardini U and others (2016) Chemosynthetic symbionts of marine invertebrate animals are capable of nitrogen fixation. Nat Microbiol 2:16195

Pootakham W, Mhuantong W, Yoocha T, Putchim L and others (2017) High resolution profiling of coral-associated bacterial communities using full-length 16S rRNA sequence data from PacBio SMRT sequencing system. Sci Rep 7:2774

Quast C, Pruesse E, Yilmaz P, Gerken J and others (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41:D590–D596

R Core Team (2017) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. www.r-project.org

Rabbani G, Yan BC, Lee NLY, Ooi JLS, Lee JN, Huang D, Wainwright BJ (2021) Spatial and structural factors shape seagrass-associated bacterial communities in Singapore and Peninsular Malaysia. Front Mar Sci, https://doi.org/10.3389/fmars.2021.659180

Rizal S, Setiawan I, Iskandar T, Ilhamsyah Y, Musman M, Mulyadi AW (2010) Currents simulation in the Malacca Straits by using three-dimensional numerical model. Sains Malaysiana 39:519–524

Rosado PM, Leite DCA, Duarte GAS, Chaloub RM and others (2019) Marine probiotics: increasing coral resistance to bleaching through microbiome manipulation. ISME J 13:921–936

Salinas C, Duarte CM, Lavery PS, Masque P and others (2020) Seagrass losses since mid-20th century fuelled CO_2 emissions from soil carbon stocks. Global Change Biol 26:4772–4784

Schile LM, Kauffman JB, Crooks S, Fourqurean JW, Glavan J, Megonigal JP (2017) Limits on carbon sequestration in arid blue carbon ecosystems. Ecol Appl 27:859–874

Serna-Chavez HM, Fierer N, van Bodegom PM (2013) Global drivers and patterns of microbial abundance in soil. Glob Ecol Biogeogr 22:1162–1172

Short F, Carruthers T, Dennison W, Waycott M (2007) Global seagrass distribution and diversity: a bioregional model. J Exp Mar Biol Ecol 350:3–20

Smith DP, Peay KG (2014) Sequence depth, not PCR replication, improves ecological inference from next generation DNA sequencing. PLOS ONE 9:e90234

Sogin ML, Morrison HG, Huber JA, Welch DM and others (2006) Microbial diversity in the deep sea and the underexplored 'rare biosphere.'. Proc Natl Acad Sci USA 103: 12115–12120

Tan YTR, Wainwright BJ, Afiq-Rosli L, Ip YCA and others (2020) Endosymbiont diversity and community structure in *Porites lutea* from Southeast Asia are driven by a suite of environmental variables. Symbiosis 80:269–277

Tarquinio F, Hyndes GA, Laverock B, Koenders A, Säwström C (2019) The seagrass holobiont: understanding seagrass-bacteria interactions and their role in seagrass ecosystem functioning. FEMS Microbiol Lett 366:fnz057

Teeling H, Fuchs BM, Becher D, Klockow C and others (2012) Substrate-controlled succession of marine bacterioplankton populations induced by a phytoplankton bloom. Science 336:608–611

Tisthammer KH, Cobian GM, Amend AS (2016) Global biogeography of marine fungi is shaped by the environment. Fungal Ecol 19:39–46

Todd PA, Ong X, Chou LM (2010) Impacts of pollution on marine life in Southeast Asia. Biodivers Conserv 19: 1063–1082

Trevathan-Tackett SM, Sherman CDH, Huggett MJ, Campbell AH and others (2019) A horizon scan of priorities for coastal marine microbiome research. Nat Ecol Evol 3: 1509–1520

Unsworth RKF, Bertelli CM, Cullen-Unsworth LC, Esteban N and others (2019) Sowing the seeds of seagrass recovery using hessian bags. Front Ecol Evol 7:311

Vandenkoornhuyse P, Quaiser A, Duhamel M, Van AL, Dufresne A (2015) The importance of the microbiome of the plant holobiont. New Phytol 206:1196–1206

Vincenot L, Popa F, Laso F, Donges K and others (2017) Out of Asia: biogeography of fungal populations reveals Asian origin of diversification of the *Laccaria amethystina* complex, and two new species of violet *Laccaria*. Fungal Biol 121:939–955

Wainwright BJ, Arlyza IS, Karl SA (2018a) Population genetics of the collector urchin, *Tripneustes gratilla*, in the Indonesian archipelago. PSZNI: Mar Ecol 39: e12530

Wainwright BJ, Zahn GL, Arlyza IS, Amend AS (2018b) Seagrass-associated fungal communities follow Wallace's line, but host genotype does not structure fungal community. J Biogeogr 45:762–770

Wainwright BJ, Afiq-Rosli L, Zahn GL, Huang D (2019a) Characterisation of coral-associated bacterial communities in an urbanised marine environment shows strong divergence over small geographic scales. Coral Reefs 38: 1097–1106

Wainwright BJ, Zahn GL, Zushi J, Lee NLY, Ooi JLS, Lee JN, Huang D (2019b) Seagrass-associated fungal communities show distance decay of similarity that has implications for seagrass management and restoration. Ecol Evol 9:11288–11297

Wainwright BJ, Arlyza IS, Karl SA (2020a) Population genetics of the banded coral shrimp, *Stenopus hispidus* (Olivier, 1811), in the Indonesian archipelago. J Exp Mar Biol Ecol 525:151325

Wainwright BJ, Zahn GL, Afiq-Rosli L, Tanzil JTI, Huang D (2020b) Host age is not a consistent predictor of microbial diversity in the coral *Porites lutea*. Sci Rep 10:14376

Wang L, Tomas F, Mueller RS (2020) Nutrient enrichment increases size of *Zostera marina* shoots and enriches for sulfur and nitrogen cycling bacteria in root-associated microbiomes. FEMS Microbiol Ecol 96:fiaa129

Waycott M, Duarte CM, Carruthers TJB, Orth RJ and others (2009) Accelerating loss of seagrasses across the globe threatens coastal ecosystems. Proc Natl Acad Sci USA 106:12377–12381

Whitman WB, Coleman DC, Wiebe WJ (1998) Prokaryotes: the unseen majority. Proc Natl Acad Sci USA 95: 6578–6583

Wickham H (2011) Ggplot2. Wiley Interdiscip Rev Comput Stat 3:180–185

Yaakub SM, McKenzie LJ, Erftemeijer PLA, Bouma T, Todd PA (2014) Courage under fire: seagrass persistence adjacent to a highly urbanised city–state. Mar Pollut Bull 83: 417–424

Yaakub SM, Ooi JLS, Buapet P, Unsworth RKF (2018) Seagrass research in Southeast Asia. Bot Mar 61:177–179

Zahn G, Amend AS (2017) Foliar microbiome transplants confer disease resistance in a critically-endangered plant. PeerJ 5:e4020

Editorial responsibility: Eva Lindström, Uppsala, Sweden
Reviewed by: V. Hurtado-McCormick, M. Vass

Submitted: December 14, 2020
Accepted: July 27, 2021
Proofs received from author(s): September 21, 2021