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Spatial variation of bacterial and fungal communities of estuarine seagrass leaf microbiomes

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ABSTRACT: The health of seagrass plants, and thereby the ecosystems they form, is linked to their associated microbial communities. However, the role of the microbiome in holobiont function and health remains poorly understood for most seagrass species and environmental pressures, and there is, therefore, a need to better understand the drivers behind the formation of and external influences on the seagrass microbiome. Using a core microbiome framework, we characterised the leaf microbiomes of 6 estuarine seagrass populations after a precipitation event to explore how the microbiomes vary across different sites and salinities over a regional spatial scale. We found that each estuary had distinct core bacterial community structures (beta-diversity), but shared a more similar fungal core community structure. We hypothesise that the differences in the bacterial members of the microbiomes among estuaries are generally the result of each estuary being influenced by unique watersheds and sources of prokaryotes. In contrast, the similarity in the core fungal communities suggests that the eukaryotic components of the microbiomes are likely under selection or result from similar colonisation pathway(s). We also found that the bacterial taxa driving the differences among estuaries were linked to the salinity of the estuary, likely due to (1) the general epibiotic nature of colonisation (i.e. watershed source and exposure) and (2) members or functional groups within the leaf microbiome assisting seagrasses in coping with the extreme salinities. These results are valuable for linking microbiomes to the resilience of seagrasses living within dynamic estuaries experiencing a range of physicochemical pressures.

KEY WORDS: Holobiont · Salinity · *Zostera* · Mycobiome · Estuary · Flood · Intermittently closed-open lakes and lagoons

1. INTRODUCTION

Estuarine ecosystems are transitional environments between terrestrial and marine biomes and play important roles in human socioeconomics and ecosystems services (Barbier et al. 2011). Seagrass meadows are key habitat-forming organisms in estuaries, lagoons, bays and open-coast environments globally (Barbier et al. 2011). Because of their sensitivity to changes in water column characteristics, particularly light availability and eutrophication, seagrass function is tightly linked to ecosystem health (Scanes et al. 2007, Bricker et al. 2008, McKenzie et al. 2012, Macreadie et al. 2014). From a microscale perspective, seagrass function is also tightly linked with their associated microbial communities, or

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microbiomes (reviewed in Seymour et al. 2018, Tarquinio et al. 2019, Trevathan-Tackett et al. 2019). A recent global study showed that the variation in the microbiomes of seagrasses is related to the microbiomes of surrounding water and sediments (Fahimipour et al. 2017), as well as the metabolic processes of the seagrass hosts (Crump et al. 2018). For example, root exudation selects for microbes that can detoxify sulphate and take advantage of the labile carbon sources (Wahbeh & Mahasneh 1984, Cúcio et al. 2016, Ettinger et al. 2017, Martin et al. 2018). For leaf tissues, certain microbes are selected for due to their ability for attachment and biofilm adaptations (Roth-Schulze et al. 2016, Bengtsson et al. 2017), while others can utilise seagrass exudates and may control epiphytic algae populations (Crump et al. 2018).

There is a growing body of work aimed at understanding the drivers behind the formation of and external influences on the seagrass microbiome. For example, environmental conditions, such as season (Wahbeh & Mahasneh 1984), light availability (Mejia et al. 2016), pH (Rotini et al. 2017), water depth (Bengtsson et al. 2017), leaf canopy morphology and salinity (Crump & Koch 2008), are known to influence seagrass microbiomes. For marine microbiomes, the core microbiome concept has been used as a framework to study microbial community patterns and to identify persistent relationships within the microbiome and holobiont, specifically to help tease apart key microbial taxa and functions across space, time and environmental shifts and gradients in marine ecosystems (Hernandez-Agreda et al. 2017). The core microbiome framework can be applied via 2 approaches: (1) the mechanistic approach, which aims to decipher how microbiomes are structured by identifying the core microbiome distribution in local habitats or niches; and (2) the pattern approach, which aims to decipher how biotic and abiotic factors influence the microbiome by examining how environmental gradients formulate their structure at different spatial scales (Hernandez-Agreda et al. 2017).

In seagrasses, the concept of the core microbiome framework has only been applied relatively recently. Core members of the seagrass microbiome have been identified across species (Ugarelli et al. 2019) and within the root compartment (Fahimipour et al. 2017), as well as across micro-niches (within host) and regional spatial scales (Hurtado-McCormick et al. 2019). Hurtado-McCormick et al. (2019) showed that bacterial members of seagrass core microbiomes were specific to the tissue type, but consistent across different estuaries and lagoons. The fungal microbiome members, conversely, had a more generalist relationship with the host, whereby no core microbiome was identified over micro- and regional scales (Hurtado-McCormick et al. 2019). In order to further develop the seagrass core microbiome concept, we used the pattern-based approach and defined the core microbiome in terms of persistence of the association with the leaf (Hernandez-Agreda et al. 2017). The aim is to assess how microbiome members may be contributing to holobiont or ecosystem function across different salinities and over a regional spatial scale (Hernandez-Agreda et al. 2017, Ramsby et al. 2018).

The estuaries along the western coastline of Victoria in southeastern Australia are dynamic systems fed by independent catchment networks dominated by agricultural land use. The estuaries also experience heavy rains during winter, which result in large, significant seasonal shifts in salinity (Mondon et al. 2003). Some of the estuaries are also influenced by sandbar closures, restricting the tidal flows and leading to localised flooding during the winter rains (Mondon et al. 2003). The seagrass *Zostera muelleri* is found at the mouth of most of these estuaries (Mondon et al. 2003), suggesting that these seagrass populations are acclimated to the dynamic salinity conditions the estuaries experience throughout the year. In fact, the *Zostera* genus is commonly found in lower salinities compared to other seagrass species, and low-salinity conditions may serve as an important environmental trigger for seed germination (Sherman et al. 2018). There is growing evidence that salinity shifts and salinity gradients strongly influence leaf-associated microbial communities and their functions (including photosynthesis and metabolism; Crump & Koch 2008, Fraser et al. 2018), and therefore the seagrass–microbiome–salinity relationship could provide useful information on environmental health in dynamic estuaries.

In this study, we took advantage of seasonal rain events to investigate how low-salinity extremes affect leaf microbial communities *in situ*. By using previous annual monitoring data, the 6 estuaries that contain *Z. muelleri* along the western coastline of Victoria were classified into broad *a priori* land-use impact categories and identified for general level of flooding based on winter salinities and time spent as a closed estuary. In using this approach, we could assume that the presence of *Z. muelleri* populations within the regularly flooded estuaries are likely to be acclimated to the seasonal flooding and/or watershed inputs. While seagrass leaf microbial community composition is likely to be highly variable among estuaries due to different watershed sources and

environmental conditions, we hypothesise that there is a persistent or core microbiome, defined as being present in >80% of the samples (Ramsby et al. 2018), that is linked to seasonal flooding events. Characterisation of the variable and core components of the leaf-associated microbial communities of estuarine seagrass would provide a step toward identifying microbial taxa essential or important to the seagrass host and perhaps within estuaries in general.

2. MATERIALS AND METHODS

2.1. Site description and sampling design

Seagrass leaf microbiomes were sampled once during the Australian winter (6–8 July 2016) in 6 estuaries along a 130 km stretch of the western Victoria coastline of Australia: Surrey River, Fitzroy River, Yambuk Inlet, Moyne River, Curdies River and Sherbrook River (from west to east; Fig. 1, Table 1). Each estuary contained a *Zostera muelleri* Irmisch ex Ascherson, 1867 seagrass meadow near the mouth of the estuary. Each estuary also receives inputs from distinct watersheds, ranging from 35 to 1435 km^2 (Mondon et al. 2003). They are hydrologically dynamic and influenced by seasonal winter flooding and spring/summer tidal salt wedges, and most of the sites are classified as intermittently closed-open lakes and lagoons (ICOLLs; Mondon et al. 2003). Four of the estuaries were intermittently closed at the time of sampling, i.e. with a sand bar blocking the tidal influence, except Moyne River (permanently open) and Sherbrook River (tidally stratified) (Mondon et al. 2003). Two weeks prior to sampling, heavy rains were experienced at all sites from Surrey River (Port Fairy, 41 mm) to Sherbrook River (ca. Port Campbell, 74 mm) (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/a084p059_supp.pdf). As a result of the seasonal winter flooding and closed estuaries, the salinities measured at the Surrey River, Fitzroy River and Curdies River sites were fresh or brackish (<5 psu; Table 1). Yambuk Inlet was also flooded but had mesohaline (5–18 psu) salinities. Sherbrook River was open and tidally influenced at the time of sampling (polyhaline defined at 18–30 psu), while Moyne River was the only site that experienced full marine salinities (>30 psu; Table 1). Additionally, the estuaries have historically had varying degrees of human impact and watershed land use, primarily agricultural development (Mondon et al. 2003). The conditions of the estuaries were previously assessed by the Department of Primary Industries (DEPI 2010) and within the OzCoasts Estuary Search (ozcoasts.gov.au/search_data/estuary_search.jsp). The conditions were based on multiple factors, including hydrology, land use, water quality and aquatic life. Based on the degree of modification in these 2 reports (Table 1), the estuaries were *a priori* classified as relatively 'unimpacted' (Surrey, Yambuk, Sherbrook) or 'impacted' (Fitzroy, Curdies, Moyne). Salinity and temperature were measured at the time of sampling using the HQ40D Portable Multi-Meter (Hach Company; Table 1) and were the basis of the salinity classifications and descriptions (Table 1).

Individual seagrass plants were sampled along a transect parallel to the shore, approximately 5–10 m apart (n = 5) to avoid sampling from the same seagrass clone. Sampling locations were primarily in the intertidal regions, although at Surrey River, Yambuk Lagoon and Curdies River the flooding levels from recent winter rains were still above the leaf canopy. The second and third oldest leaves were sampled so as to avoid sampling heavily epiphytised older leaves. The leaves were rinsed with 0.22 µm filtered local water and stored in ~1 ml of nucleic acid preservative (to pH 5.2 following Malmstrom 2015), so as to capture both firmly attached epiphytes and endophytes. The samples were refrigerated at 4°C while in the field (<3 d), then stored frozen at −20°C until processed.

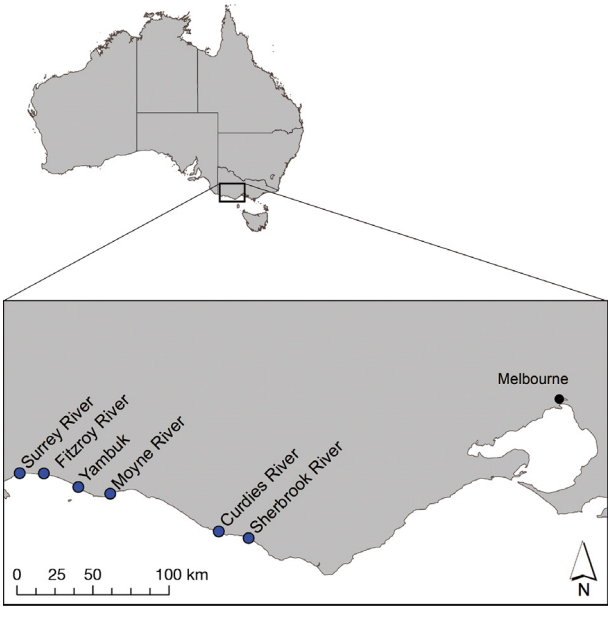


Fig. 1. Estuary mouths where seagrass *Zostera muelleri* leaf microbiomes were sampled in southeastern Australia (upper map: Australia)

Table 1. Estuaries sampled for leaf microbiomes in this study. Estuaries were chosen based on the presence of seagrass *Zostera muelleri*. Salinity classification and description were based on data taken at the time of sampling. Estuary classification was based on previous assessments. n.d.: no data available

Site	Latitude (° S)	Longitude (° E)	Sampling details (6–8 Jul 2016)				Estuary classification	
			Salinity (psu)	Water temp. (°C)	Salinity classification[a]	Salinity description[a]	DEPI condition[b]	OzCoast condition[c]
Surrey River	38.258281	141.705084	0.8	10	Oligohaline	Fresh/brackish	Moderate	Largely unmodified
Fitzroy River	38.259672	141.847366	2.5	11.7	Oligohaline	Fresh/brackish	n.d.	Modified
Yambuk Inlet	38.340362	142.051469	10.6	10.9	Mesohaline	Estuarine	Moderate	Largely unmodified
Moyne River	38.380451	142.240387	38.7	12.8	Euhaline	Marine	Moderate	Modified
Curdies River	38.603381	142.883518	4.4	10.6	Oligohaline	Fresh/brackish	Very poor	Modified
Sherbrook River	38.642901	143.059055	18	12.3	Meso-polyhaline	Estuarine	n.d.	Largely unmodified

[a]Oertli (1964), Tagliapietra et al. (2009); [b]DEPI (2010); [c]www.ozcoasts.gov.au/search_data/estuary_search.jsp

2.2. DNA extraction and sequencing

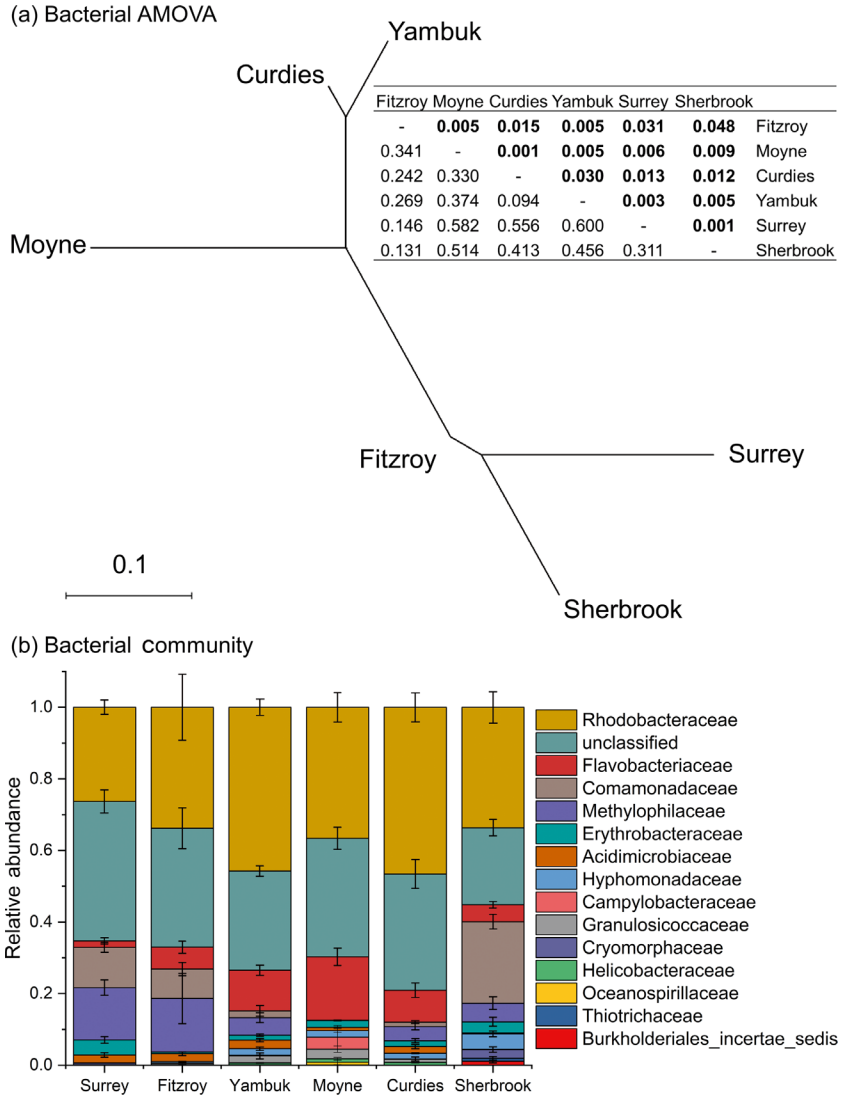
Genomic DNA was extracted from whole seagrass leaves (site means ranged from 15 to 30 mg fresh weight) using the PowerSoil DNA Isolation Kit (MoBio Technologies). The sites had a wide range of leaf lengths (3–70 cm). In order to best normalise input and still represent the whole leaf microbiome, 2–3 leaves of the small-morphology sites (Curdies, Fitzroy, Yambuk, Moyne) and 1 leaf sub-sampled at top, middle and bottom for large-morphology sites (Surrey, Sherbrook) were included in the bead-beating step. Bead-beating was performed using the Vortex Genie 2 adaptor on high for 15 min. Extracted DNA (site means from 5.5 to 10.8 ng μl^{-1}) was amplified using the V3–V4 variable regions of the 16S rRNA gene (341F: 5′-CCT AYG GGR BGC ASC AG-3′; 806R: 5′-GGA CTA CNN GGG TAT CTA AT-3′) (Martin et al. 2018) and the eukaryotic internal transcribed spacer ITS1 region (forward: 5′-CTT GGT CAT TTA GAG GAA GTA A-3′; reverse: 5′-GCT GCG TTC TTC ATC GAT GC-3′) (Bissett et al. 2016) prior to sequencing using the Illumina MiSeq platform at the Australian Genome Research Facility (www.agrf.org.au).

2.3. Bioinformatic and statistical analyses

The complete analysis pipeline and Python scripts are available at https://github.com/theo-allnutt-bioinformatics/Trevathan-Tackett_2018/. The sequencing data were analysed using the UPARSE pipeline (Edgar 2013) of the Usearch package (v.8.1.1861_i86linux64). A total of 5796366 16S rRNA gene reads and 6752922 ITS1 reads were produced, with 65% and 68% of reads remaining, respectively, after merging with the Usearch -fastq_mergepairs and -fastq_filter commands. Merged reads were filtered at maximuum error numbers (E_max) = 1, minimum length = 170 bp. Following quality control filtering, 72% of 16S and 92% of ITS reads remained. A UPARSE taxonomic assignment at 90% identity was performed followed by an operational taxonomic unit (OTU) clustering using a radius of 3% for both 16S and ITS reads (Edgar 2013). 16S taxonomic assignment used the Greengenes database v.13_8 (DeSantis et al. 2006) and ITS used the UNITE database v.7 (Kõljalg et al. 2013). The use of the Greengenes database has been recently criticised since it has not been updated since 2013 (Balvočiūte & Huson 2017). To compare the databases, we also assigned the 16S taxonomy with the Silva v.123 database (Quast et al. 2012). A BLAST search was performed for any ITS reads that were unknown in order to identify them as fungal or non-fungal. Raw reads are available at the European Nucleotide Archive (accession number PRJEB36104).

Initial inspection revealed some 16S rRNA gene reads of plant or algal origin. A BLAST search of the chloroplast OTUs revealed that the most abundant OTU_1 was most likely from the seagrass, and was found across all samples at high proportions (Table S1 in the Supplement). The other assigned taxonomies indicated the presence of epiphytic microalgae from the *Bacillariophyta* genus (Table S1). Extremely rare OTUs were filtered to include only those with 0.1% or greater total abundance and to exclude OTUs that only occurred in 1 sample (these were invariably also <0.1% abundance). To calculate alpha diversity, post-filtered OTU abundances were rarefied to the lowest total among all samples (30079 for each amplicon dataset; Fig. S2 in the Supplement) and alpha diversity measures compared using QIIME v.1.8 (Caporaso et al. 2010). Beta diversity was analysed by first aligning OTUs′ representative

sequences with MUSCLE v.3.8.31 (Edgar 2004), followed by maximum likelihood tree constructions using FastTree v.2.1.9 (Price et al. 2010), normalisation using cumulative sum scaling (Paulson et al. 2013) and calculation of weighted UniFrac (Lozupone & Knight 2005) distances using QIIME. The beta diversity using the UniFrac distance matrix was then analysed by analysis of molecular variance (AMOVA) and pairwise PhiPT tests across sites (Excoffier et al. 1992, Peakall & Smouse 2006). Differential OTU abundances among samples were tested using ANOVA with the metadata categories: site, impacted/unimpacted status, and salinity description. The OTUs were further filtered to identify the core microbiome, which was defined as being present in >80% of the samples (≥1 count in >24 samples; Ramsby et al. 2018). Variable OTUs were considered to be present in >10% of the samples (or >3 samples; Ramsby et al. 2018). Multidimensional scaling plots of the core and variable communities were constructed using relative abundances of the OTUs after square-root transformation and calculation of a Bray-Curtis resemblance matrix (Jeffries et al. 2016). A network analysis was performed to identify any correlations among 16S and ITS OTUs. The relative abundance data for the 100 most abundant 16S and ITS OTUs were cumulative sum squaring normalised (QIIME v.1.9.1) prior to Pearson's R correlation. Significant correlations ($p < 0.05$ false discovery rate using the Benjamini-Hochberg method) of equal to or stronger than ±0.75 were plotted using Cytoscape v.3.7.1.



	Fitzroy	Moyne	Curdies	Yambuk	Surrey	Sherbrook	
Fitzroy	-	**0.005**	**0.015**	**0.005**	**0.031**	**0.048**	Fitzroy
Moyne	0.341	-	**0.001**	**0.005**	**0.006**	**0.009**	Moyne
Curdies	0.242	0.330	-	**0.030**	**0.013**	**0.012**	Curdies
Yambuk	0.269	0.374	0.094	-	**0.003**	**0.005**	Yambuk
Surrey	0.146	0.582	0.556	0.600	-	**0.001**	Surrey
Sherbrook	0.131	0.514	0.413	0.456	0.311	-	Sherbrook

Fig. 2. Site-level variance across the seagrass leaf-associated bacterial communities. (a) Neighbour-joining tree based on genetic distance produced from analysis of molecular variance (AMOVA) pairwise PhiPT values. PhiPT values are below diagonal, and the probability based on 999 permutations is shown above diagonal (significant values in **bold**). (b) Family-level taxonomic diversity across sites, in order of west to east location along the coastline. Relative abundances represent mean proportions ± SEM (n = 5)

3. RESULTS AND DISCUSSION

3.1. Seagrass leaf-associated bacterial communities

Alpha diversity (OTU richness and Shannon index) was statistically similar across sites and estuary classification for the bacterial members of the microbiome, with the exception of a higher Shannon index at Yambuk compared to Curdies (Table S2 in the Supplement). Additionally, the marine site, Moyne, showed a significantly higher OTU richness than fresh/brackish sites (Table S2), supporting previous observations that the more stable ends of a salinity gradient tend to have higher alpha diversity than mixing zones (Campbell & Kirchman 2013). We detected a strong beta-diversity separation of the bacterial leaf microbiomes across sites, suggesting specific site-level influences on the leaf microbiomes (Fig. 2a). However, there was no significant effect of the estuary classification (unimpacted vs. impacted) on

beta diversity (0% variance in the AMOVA). The AMOVA pairwise PhiPT tests indicated that the bacterial communities were significantly different across all sites, with greater similarities shared among Fitzroy/Surrey/Sherbrook and Yambuk/Curdies/Moyne ($p \leq 0.048$; Fig. 2a). The taxonomic classifications generally matched between the Greengenes and Silva databases, with 31% of the OTUs not changing and 65% of the OTUs matching but having a better resolution for the Silva classifications (e.g. 25% improved by 1 taxonomic level; Tables S3–S5 in the Supplement). The classified bacterial OTUs were dominated by the *Alphaproteobacteria*, with at least 25% of the total community across all sites from the *Rhodobacteraceae* family (Fig. 2b). The *Flavobacteriaceae* family was most common at Yambuk, Moyne and Curdies (on average 9–18% relative abundance). These groups are common to seagrass leaf surfaces and coastal surfaces in general, likely due to an ability to be opportunistic and persist in rapidly changing environments (Mejia et al. 2016). In contrast, the other 3 sites — Surrey, Fitzroy and Sherbrook — had high relative abundances of the *Betaproteobacteria* (~23–28%), predominantly from members of the *Comamonadaceae* and *Methylophilaceae* families (Fig. 2b). While these taxonomic groups are also common to estuaries, they have been linked to seagrasses and other aquatic plants in brackish conditions (Crump & Koch 2008, Mejia et al. 2016). We also noted the order *Sphingomonadales* occurring at high relative abundances, most notably at Surrey River (OTUs 20, 495, 29 and 33; Table S3). This taxonomic group contains putative hydrocarbon degraders, in particular, members from the *Erythrobacteraceae* family (Kertesz et al. 2019) and *Porphyrobacter* genus (OTU_20; Hiraishi et al. 2002). While the Surrey River site was classified as an unimpacted site, localised hydrocarbon run-off from a nearby holiday park could be linked to these OTUs, suggesting a potential for these taxa to be sensitive to hydrocarbons in the environment.

We found that salinity at the time of sampling seemed to drive, at least in part, the differences within the bacterial component of the microbial communities. A pairwise ANOVA indicated that the greatest separation in bacterial communities occurred at Moyne, the only fully marine site (Tables S3 & S4). This separation was driven by high relative abundances of a diverse range of taxa, including *Flavobacteraceae*, *Verrucomicrobiae* and the *Gammaproteobacteria* (i.e. *Arenicella*, *Marinicella* and *Neptunomonas* genera and *Leucothrix* and *Cocleimonas* genera with *Thiotrichaceae*) OTUs. The latter *Thiotrichaceae* family are putative sulphate-oxidisers, with the *Leucothrix* genus a known filamentous macroalgal epiphyte (Brock 1966). In contrast, *Methylophilaceae* (OTU_5) was explicitly absent from Moyne but present in high abundances at the other sites. Other taxa that were significantly more common to the estuarine and brackish sites included *Comamonadaceae*, *Sphingomonadales*, *Cytophagaceae* and *Cellulophaga*. While the predominant taxonomic groups at Moyne are not strictly marine, they are commonly or preferentially found in marine seawater, sediments and *Zostera* seagrass leaves (Freitas et al. 2012, Campbell & Kirchman 2013, Liu et al. 2015, Bengtsson et al. 2017). *Methylophilaceae* and *Comamonadaceae*, conversely, are sensitive to environmental conditions, including salinity, and are associated with seagrass leaves, as well as water and sediments in salinities below 15 psu (Crump & Koch 2008, Campbell & Kirchman 2013, Bengtsson et al. 2017). The *Comamonadaceae* is a metabolically and functionally diverse family (Willems 2014), so it is difficult to infer their function within the leaf microbiome. One OTU_7 belonged to the *Hydrogenophaga* genus, putative hydrogen-oxidisers found in aquatic and soil environments (Yoon et al. 2008). Preference of *Hydrogenophaga* for low salinities has been shown to be environment-driven, suggesting the seagrass surface is providing a niche habitat under favourable hydrogen-rich conditions (Aragno & Schlegel 1981, Stratil et al. 2014). The *Methylophilaceae* family including the *Methylotenera* genus (OTU_489), however, consists of obligate or facultative methylotrophs that are important in the cycling of singe-carbon compounds in the environment, e.g. methanol, methylamines and dichloromethane (Doronina et al. 2014). In estuaries and marine environments, methylamines can be produced by bacteria (Yang et al. 1994), as well as emergent coastal plants (e.g. mangroves, saltmarshes; King 1988). In seagrasses, methylamines, such as betaine, are one of several groups of organic osmolytes that the plants use to cope with salinity changes (Touchette 2007, Exadactylos 2015). The breakdown and release of methlyamines in the lower-salinity sites may be providing seagrass-mediated resources for the methylotrophs.

The core bacterial microbiome, defined as the OTUs present in >80% of samples, consisted of 51 OTUs, or 33% of the OTUs (Fig. S3a in the Supplement). The core community consisted of OTUs with both low (0.1%) and high (up to 8%) relative abundances (Fig. S3a). The *Alphaproteobacteria* dominated the core community (40% of all sequences, and

26–90% relative abundance in any given sample; Table S5), which consisted primarily of *Rhodobacteraceae* OTUs (33% of all sequences). There was also a high representation of the *Betaproteobacteria* (15% of all sequences), namely the *Hydrogenophaga* OTU_7 and the *Methylophilaceae* OTU_5 (6% of all sequences each; Table S5). These patterns in the *Proteobacteria* were consistent across sites, although each site had distinct clustering of their core communities (Fig. 3a,c). Moyne, Yambuk and Curdies sites had similar proportions of classes, although this was not explained by salinity description or estuary condition. Similar to the overall community, salinity at the time of sampling did have some influence on core community clustering of the leaf surface (Fig. 3c), driven by the differences in relative abundances of *Methylophilaceae* OTU_5 in estuarine and fresh/brackish sites and *Loktanella rosea* OTU_4 (*Rhodobacteraceae* family) in the marine site. These results suggest that either the surrounding water column is the primary source of colonisation (Fahimipour et al. 2017), or the bacterial taxa themselves are responding directly or indirectly to the different salinity conditions (e.g. osmolyte consumption hypothesis).

The variable bacterial community, or the OTUs present in >10% of samples, was characterised by a higher number and variation of taxonomic groups, including a greater proportion of *Flavobacteriia* and unknown classes compared to the core community (Fig. 3b). Unlike the core communities, the variable communities did not cluster as closely by site or salinity (Fig. 3c,d), particularly between the Curdies and Yambuk sites (Fig. 3d). Further investigations into the functional attributes of the bacterial microbiomes would provide insight into additional colonisation and selection pressures within these estuaries. For example, the core communities, although different across each site, could be providing similar, specific functions to the seagrass host (e.g. nitrogen fixation and cycling or osmolyte regulation; Dupont et al. 2014, Seymour et al. 2018), which would further suggest that functional redundancy (Burke et al. 2011, Roth-Schulze et al. 2016) is important to leaf-associated microbiomes living in dynamic estuaries.

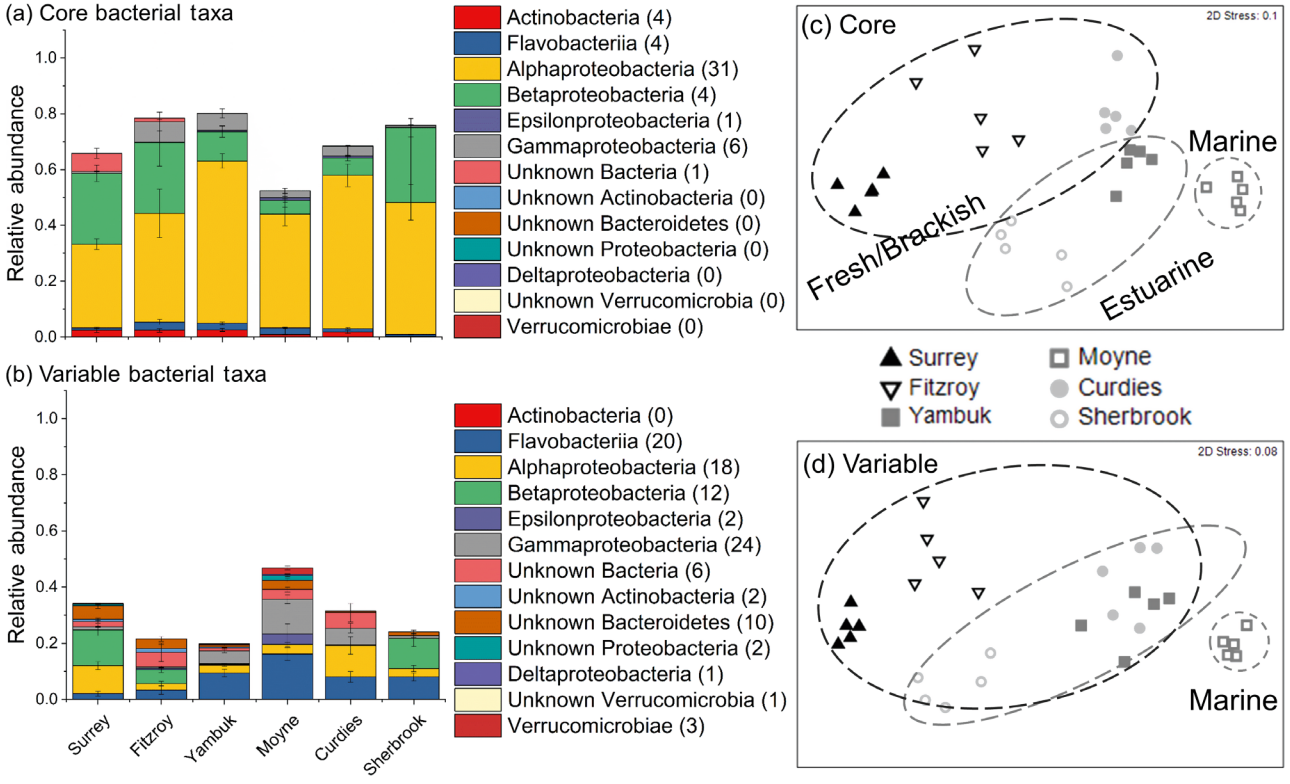


Fig. 3. (a,c) Core and (b,d) variable bacterial taxonomic communities associated with seagrass leaves. (a,b) Proportions were calculated across OTUs from all samples and averaged across sites. Values after the taxonomic name represent the number of OTUs within the class. (c,d) Multidimensional scaling plots were constructed using relative abundances after square-root transformation and calculation of a Bray-Curtis resemblance matrix. Salinity description from Table 1 are marine (Moyne), estuarine (Sherbrook, Yambuk) and freshwater/brackish (Fitzroy, Curdies, Surrey). Circles are drawn to help visualise the salinity descriptions. Relative abundances represent mean proportions ± SEM (n = 5)

3.2. Seagrass leaf-associated fungal communities

Alpha diversity was not statistically different across sites for the fungal members of the microbiome. The fungal communities in estuarine and fresh/brackish sites had higher Shannon index compared to the marine site, while sites with the unimpacted estuary classification had a higher OTU richness than the impacted sites (Table S2). The taxonomic resolution of the fungal communities was poor, with on average one-third of the class-level OTUs unclassified across all sites and two-thirds unclassified at Moyne (Fig. 4). This is likely because the UNITE database is biased toward terrestrial fungi and is under-represented for aquatic fungi (Reich & Labes 2017). However, BLAST searches of the unknown OTUs important across sites, salinity and core community comparisons (Table S6 in the Supplement) indicated they were fungal, except for OTU_133, which was returned as a brown aquatic microalga.

The low taxonomic resolution hindered our ability to make inferences about the ecology or providence of the fungal groups, but comparisons with previous literature did provide some insight into fungal colonisation of seagrass leaves. Similar to the bacterial communities, the fungal communities were generally significantly different across sites (i.e. beta diversity; Fig. 4a; Table S7 in the Supplement). Fungal phyla consisted primarily of Ascomycota, including the classes Sordariomycetes, Leotiomycetes and Dothideomycetes (Fig. 4b). These results align with the seagrass leaf-associated fungal endophytes identified from India, USA and Thailand, which were predominantly within the Sordariomycetes and Dothideomycetes classes (Newell 1981, Devarajan & Suryanarayanan 2002, Sakayaroj et al. 2010, Mata & Cebrián 2013, Venkatachalam et al. 2015). The AMOVA tests indicated sites grouped differently than the bacterial communities (Fig. 4a). The fungal community at Surrey was most similar to both Sherbrook and Moyne (Fig. 4a). Basidiomycota, a phylum less common in marine ecosystems (Jones et al. 2015), was relatively site-specific, with the greatest prevalence at Surrey, Fitzroy and Sherbrook (11%, 4% and 3% relative abundances, respectively; Fig. 4). Yambuk was more similar to Fitzroy (Fig. 4a), likely driven by the unresolved Chytridiomycota OTUs found in each of the Fitzroy samples (1–17% relative abundance of phyla), and upwards of 25–40% rela-

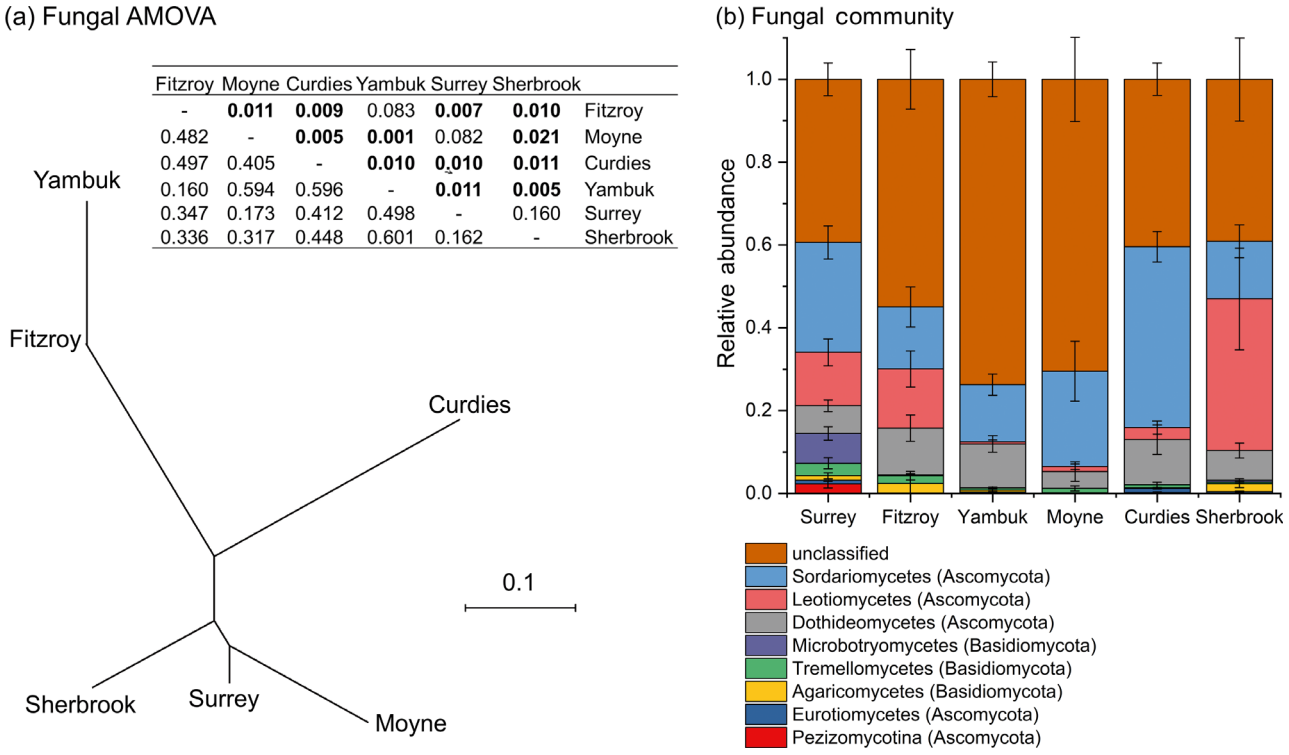


Fig. 4. Site-level variance across the seagrass leaf-associated fungal communities. (a) Neighbour-joining tree based on genetic distance produced from analysis of molecular variance (AMOVA) pairwise PhiPT values. PhiPT values are below diagonal, and the probability based on 999 permutations is shown above diagonal (significant values in **bold**). (b) Class-level fungal diversity across sites in order of west to east location along the coastline. Relative abundances represent mean proportions ± SEM (n = 5)

tive abundance in Yambuk samples. Chytridiomycota typically act as parasites and saprobes in aquatic (freshwater) environments (Gleason et al. 2008). While one species has been shown to be a parasite of a brown seaweed (Gleason et al. 2013) and another OTU is a seagrass root endophyte (Vohník et al. 2017), the frequency and ecological role of this phylum on a seagrass host is unclear. The estuarine modification classification (i.e. impacted vs. unimpacted) was not a significant driver for fungal community beta diversity, and in contrast to the bacterial members of the leaf microbiomes, the fungal communities were not strongly influenced by the salinity at the time of sampling. However, in a pairwise ANOVA test, the marine site (Moyne) had significantly higher abundances of Dothideomycetes (OTU_75), a Chytridiomycota (OTU_96), as well as 2 unknown Ascomycota and 2 unknown OTUs (Table S8 in the Supplement). A BLAST search identified the unknown OTUs as a fungus (OTU_3) and the brown microalga *Ectocarpus* sp. (OTU_133; Table S6).

Defining the core and variable fungal OTUs provided further insight into the site drivers of the fungal microbial communities. In contrast to the bacterial core members, the core fungal microbiome consisted of very few OTUs (8) that generally had high relative abundances (0.8–10.7 % of all sequences; Fig. S3b in the Supplement). The core community was dominated by 3 Sordariomycetes OTUs, including a Hypocreales OTU (17 % of all sequences; Table S9 in the Supplement, Fig. 5a,c). A Pleosporales (class Dothideomycetes) OTU was also found at all sites (Table S9, Fig. 5a,c). Both Hypocreales and Pleosporales orders are common endophytes for marine and estuarine seagrass leaves around the world (Newell 1981, Devarajan & Suryanarayanan 2002, Sakayaroj et al. 2010, Mata & Cebrián 2013, Venkatachalam et al. 2015). The core fungal community was not as distinct among sites as the core bacterial community (Fig. 5c), possibly because of the small number of OTUs belonging to the former. The Yambuk core community clustered tightly by itself, likely because

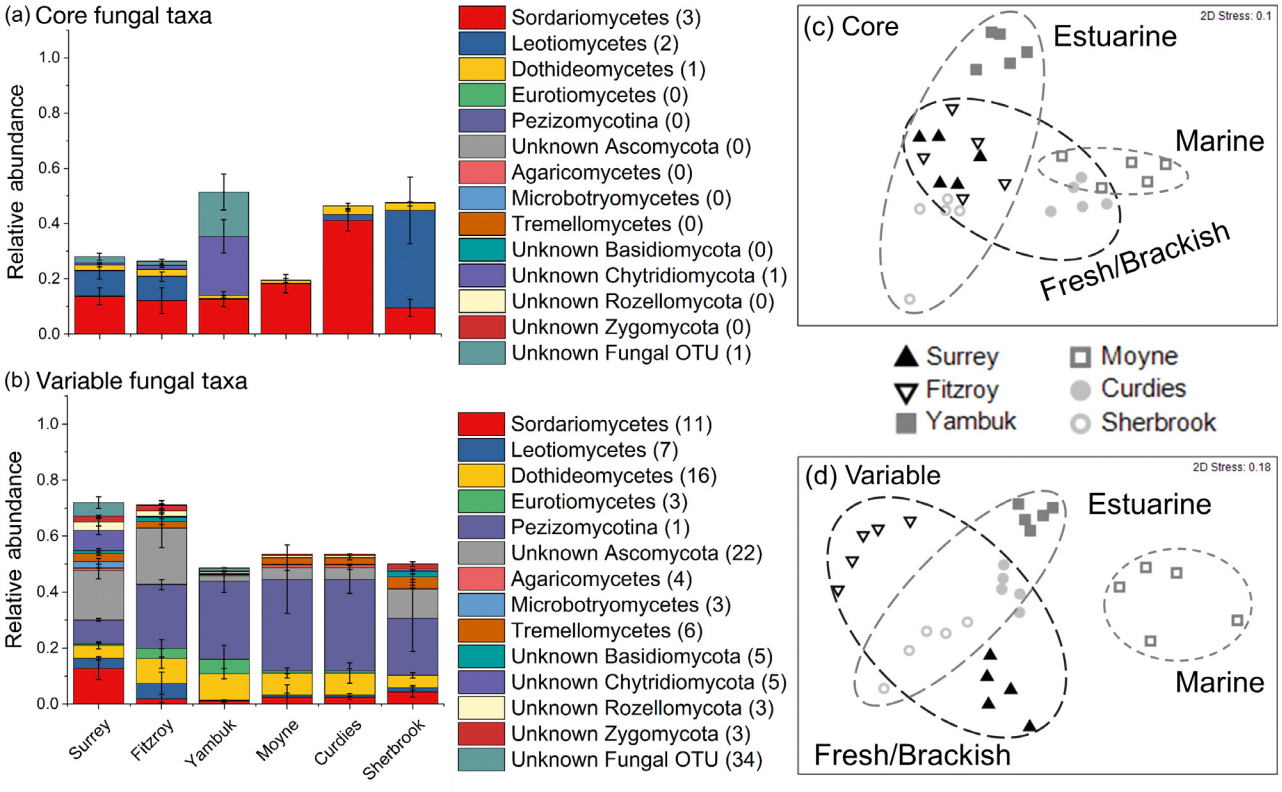


Fig. 5. (a,c) Core class-level and (b,d) variable fungal taxonomic communities associated with seagrass leaves. (a,b) Proportions were calculated across OTUs from all samples and averaged across sites. Values after the taxonomic name represent the number of OTUs within the class. (c,d) Multidimensional scaling plots were constructed relative abundances after square-root transformation and Bray-Curtis resemblance matrix. Salinity description from Table 1 are marine (Moyne), estuarine (Sherbrook, Yambuk) and freshwater/brackish (Fitzroy, Curdies, Surrey). Circles are drawn to help visualise the salinity descriptions. Relative abundances represent mean proportions ± SEM (n = 5)

of the high relative abundance of the Chytridiomycota OTU_6 (Fig. 5c, Table S9). Moyne and Curdies clustered closely because they shared on average >80% high relative abundance of Hypocreales (Sordariomycetes OTU_1). In contrast, the variable fungal community members consisted of more OTUs of relatively lower abundance (Fig. S3b). Of these, roughly one-third were classified to phylum, while one-third were unclassified. Despite the taxonomic unknowns, there was a high degree of site-level clustering for the variable fungal community, due to a higher amount of taxonomic groups of various relative proportions compared to the core group (Fig. 5b,d). These results suggest that each estuary is providing a relatively unique source of transient fungal endophytes, but that pressures from the seagrass or other microbiome members are selecting for a relatively narrow range of endophytes that make up the core community.

3.3. Bacterial–fungal correlations

The correlation network of bacterial and fungal microbiome members resulted in 3 network clusters (Pearson's R > ±0.75; Fig. 6) that were overall linked to presence/absence at specific sites. First, there were correlations between 2 fungal taxa (Dothideomycetes OTU_75 and unknown OTU_3) and 10 bacterial OTUs that were found in high relative abundances or strictly at the marine Moyne site. These 2 fungal OTUs also had negative correlations with the *Methylophilaceae* OTU_5 and *Flavobacterium ponti* OTU_44, both absent at Moyne. This network highlights the selective effect that marine salinities have on fungal members of the seagrass leaf microbiome (Shearer et al. 2007). The other 2 networks highlight the differences between Surrey and Sherbrook and the other sites. The Leotiomycetes OTU_2 and Hypocreales OTU_1 are both core fungal members, so were present in high abundances at all or most of the sites (Table S7). However, most of the positive and negative correlations in this network are to bacterial OTUs in high abundances or absent at Surrey and Sherbrook sites (Fig. 6). In particular, the Leotiomycetes OTU had only 1 positive correlation with a *Comamonadaceae* OTU_1486. The only quantified similarities between these 2 sites were the leaf lengths (>40 cm). This network could suggest that the water depth or hydrology allowing for longer leaves or the long leaf length itself could be providing additional niches for bacterial–fungal interactions not seen at the other sites. Furthermore, the third network highlighted OTUs that were in low or high relative abundances at Surrey River only (Fig. 6). Given that the Surrey site was the most flooded site, these strong correlations between fungal and bacterial OTUs could be linked to the nearly fresh salinity (0.8 psu). For example, the yeast Sporobolomyces (OTU_38) is rare in estuarine and marine environments (Ahearn et al. 1968).

3.4. Environmental influence on seagrass leaf microbiomes

The aim of the present study was to characterise the variable and consistent (core) components of the microbiomes associated with seagrass leaves living in dynamic estuarine systems. Our results show that there is a large component of seagrass leaf microbiomes that are highly variable and likely influenced by estuarine-specific conditions and/or sources. This suggests that broad classifications of estuaries based on historical modifications of the estuaries are not likely to be representative of the highly dynamic nature of the estuaries, both temporal and spatially. Rather, exploring additional microbiomes (e.g. roots, sediments; Sun et al. 2013, Mejia et al. 2016, Fahimipour et al. 2017), as well as a manipulative mesocosm or time-series approach to capture monthly or seasonal shifts in the microbiome could tease apart environmental pressures and better reflect the temporal and spatial scale at which the estuary condition parameters are reported (Mondon et al. 2003). The strong site-level differences of the seagrass leaf-associated microbiomes found in this study shows that there is an avenue toward identifying key microbiome members within the seagrass holobiont. However, understanding the functions of the microbiome in relation to its environment and host need to be resolved to fully understand its role in the estuary, for example the degree of functional redundancy across the unique estuarine microbial communities. A combination of laboratory and fieldwork using next-generation sequencing (NGS) techniques (Seymour et al. 2018), as well as molecular and physiological responses of host stress (Macreadie et al. 2014) are needed for understanding the seagrass holobiont (see other examples in Weigel & Erwin 2017, Pratte & Richardson 2018).

The responses in site-specificity were strongest in the core bacterial and variable fungal community signatures, suggesting different drivers of colonisation and generalist versus specialist functions between the prokaryotic and eukaryotic members of the sea-

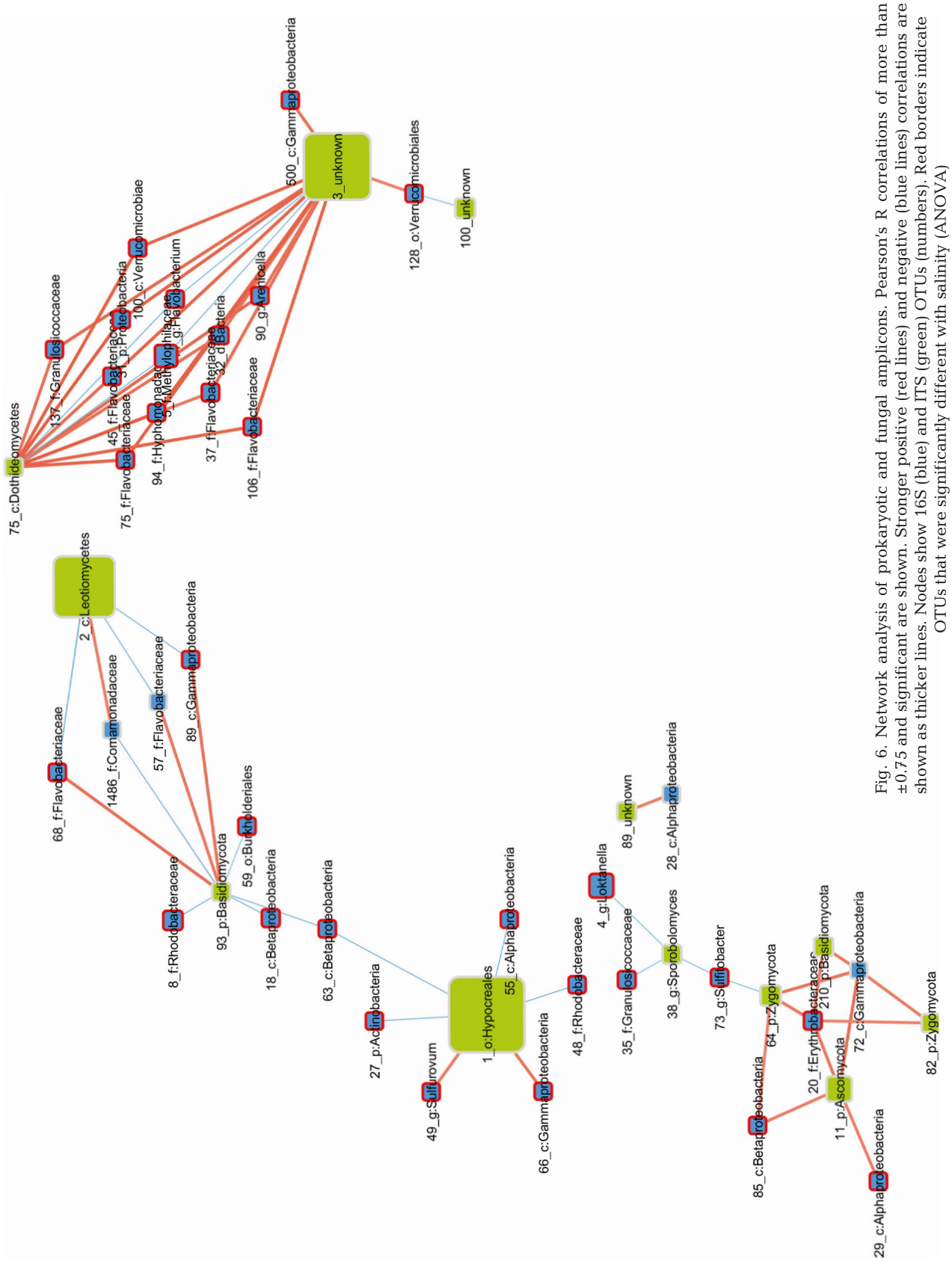


Fig. 6. Network analysis of prokaryotic and fungal amplicons. Pearson's R correlations of more than ±0.75 and significant are shown. Stronger positive (red lines) and negative (blue lines) correlations are shown as thicker lines. Nodes show 16S (blue) and ITS (green) OTUs (numbers). Red borders indicate OTUs that were significantly different with salinity (ANOVA)

grass leaf microbiome. Bacterial communities in seagrass leaf microbiomes are strongly influenced by the members present in the surrounding seawater (Fahimipour et al. 2017) and this influence is likely driving the bacterial community structure in this study. Perhaps taxa sensitive to pollutants could be useful indicators of environmental condition or as bioremediators (e.g. *Sphingomonadales*; Festa et al. 2016). Leaf bacteria are also dependent on seagrass leaf exudates or waste products (Wang et al. 2014), so changes in the seagrass' physiology and metabolism under hyposalinity conditions could also influence the resources and structure of the bacterial community, e.g. the hydrogen-, single carbon- and sulphide-oxidising taxonomic groups *Hydrogenophaga*, *Methylophilaceae* and *Thiotrichaceae*, respectively. In contrast, the core fungal members were generally independent of the site. We hypothesise that this independence is due to one or a combination of factors. First, the endophytic nature of fungi could provide protection within the cell wall (Saikkonen et al. 1998), and thus established core members are less likely to fluctuate with frequent changes in environmental conditions. The variable fungal community, by contrast, may reflect loosely established colonies or transient propagules (Newell 1981), or a generalist approach as suggested for other *Z. muelleri* mycobiomes (Hurtado-McCormick et al. 2019). Second, fungal endophytes typically have a low colonisation frequency on seagrass leaves (Devarajan & Suryanarayanan 2002, Venkatachalam et al. 2015). The low success rate on living tissue could be due to competition, selection or inhibition by the other members of the microbiome or the seagrass itself (Suetrong et al. 2009, Sakayaroj et al. 2010, Wahl et al. 2012, Venkatachalam et al. 2015). Lastly, marine fungi have saprobic roles and are decomposers of seagrass detritus (Cuomo et al. 1987, Sathe & Raghukumar 1991). Since we sampled younger leaves, it is possible that only a few fungal taxa in the core microbiome have a functional role on a healthy, living seagrass blade, and some of the fungi we captured in the microbiome are simply colonising throughout the life of the leaf until it can take advantage of the nutrients released during senescence (Newell 1981).

Across the sites, salinity, particularly at the extremes, was a secondary influence on the seagrass leaf microbiome composition and was a key factor in the bacteria–fungi correlations. The influence of salinity on entire community structure was mostly limited to the bacterial component of the microbiomes. This suggests that the fungal communities could be more protected from salinity shifts at the estuarine sites and that there is a strong selection of the core fungal endophytes under marine conditions leading to unique OTUs at Moyne (Table S8) and strong correlations between marine bacteria and fungi (Fig. 6). Salinity has been known to influence bacterial microbial communities in several ecosystems, including coral reefs, pelagic bacterioplankton and estuaries (Herlemann et al. 2011, Campbell & Kirchman 2013, Röthig et al. 2016, Gołębiewski et al. 2017). Gołębiewski et al. (2017) posited that while marine and freshwater bacterial communities are typically distinct and have limited ability to acclimatise to varying salinities, freshwater-marine transitions, such as the ICOLLs in this study, provide a unique environment where co-existence is possible. The bacterial taxa in this study have been found to tolerate mixing zones and freshwater conditions (i.e. *Hyphomonadaceae*) or to belong to families that contain both marine and freshwater members (i.e. *Sphingomonadaceae*, *Rhodobacteraceae*) (Gołębiewski et al. 2017). Furthermore, studies tracking the microbiome shifts along salinity gradients within a single estuary system have shown similar shifts from *Burkholderiales* (*Comamonadaceae*-) and *Methylophilaceae*-dominated communities in upstream, low-salinity regions to higher relative abundances in the *Gammaproteobacteria* and *Rhodobacteraceae* groups in marine salinities (Campbell & Kirchman 2013, Dupont et al. 2014, Liu et al. 2015). These same taxa were also found in the core microbiome across the sites, which raises the question of whether we detected a post-flooding legacy effect or a specific selection or protection of certain members of the microbiome that are helping cope with or taking advantage of resources during semi-regular influx of freshwater/run-off. Additionally, the links between bacteria and fungi at the salinity bookend sites (fresh i.e. Surrey and marine i.e. Moyne) could suggest that some transient fungal taxa or transient bacteria–fungal interactions could be important to the seagrass holobiont in these dynamic estuaries.

Studies have shown that microbiomes of benthic coastal or marine organisms provide a beneficial role in protecting or alleviating environmental stress on the host, i.e. the probiotic hypothesis (Reshef et al. 2006). For example, long-term (~1 mo) changes in salinity was shown to restructure the members of the coral bacterial microbiome (Röthig et al. 2016). In a filamentous brown alga, the bacterial microbiome was essential for survival from seawater to freshwater (Dittami et al. 2016). In seagrasses, members of the microbiome are shown to benefit the seagrass resilience and fitness (Tarquinio et al. 2019). For

salinity stress, *Zostera* seagrass species have been repeatedly shown to be able to tolerate hyposaline environments for relatively long periods of time before showing negative morphological or physiological effects (Fernández-Torquemada & Sánchez-Lizaso 2011, Collier et al. 2014). Low-salinity environments are also hypothesised to aid in the clearing of potential seagrass pathogens (Trevathan-Tackett et al. 2018). While the link between seagrass physiological acclimation and their microbiome under environmental stress is unresolved, potential indicator taxa, such as *Methylophilaceae*, could assist in cycling single-carbon and nitrogen compounds (methlyamines, methanol) or provide alternate metabolic pathways for ion transport in meso- or oligohaline estuaries (Touchette 2007, Crump & Koch 2008, Dupont et al. 2014, Taubert et al. 2017). Identification of potential indicator taxa in coastal and estuarine microbiome research has been recently suggested as a tool to assess the state of host and ecosystem health and pollution (Sun et al. 2013, O'Brien et al. 2016, Birrer et al. 2017, Glasl et al. 2017, Filippini et al. 2019, Santini et al. 2019), as well as ecosystem restoration (Santini et al. 2019). In order to progress this growing research field, Glasl et al. (2017) outlined key baseline questions needed to implement microbiota indicators in practice, including developing baselines for healthy ecosystems and the response of the microbial community and holobiont resilience to environmental change.

In summary, we detected a strong site-differentiation in both bacterial and fungal members of the microbiomes, driven in part by environmental conditions that were also linked to correlations between bacterial and fungal OTUs. The seemingly differential responses to site and salinity of the core and variable members of both fungal and prokaryotic components of the microbiomes suggests different selection pressures and colonisation routes that could be useful for linking microbiomes to the spatiotemporal and environmental changes within the estuary. To progress our understanding of how environmental drivers influence the resilience, health and function of the seagrass holobiont, we want to highlight these future research directions:

- There is a need for better taxonomic resolution of NGS databases to better identify baseline structure and functioning of the eukaryotic members of the microbiomes.
- Comparative studies on how microbiomes associated across multiple seagrass tissues under controlled environmental conditions would provide different indicators of stress and health of both the microbiome and holobiont, including resilience under environmental change.
- Integrative studies that link the microbiome with seagrass health and environmental stressors at multiple spatial and temporal scales would allow us to integrate 'healthy' and 'disturbed' microbiomes into the existing estuary health assessment framework.

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